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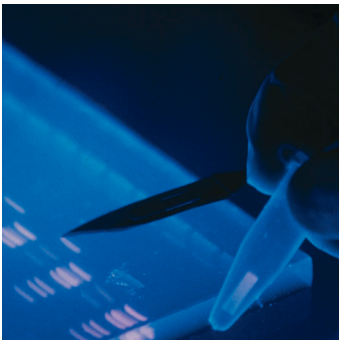
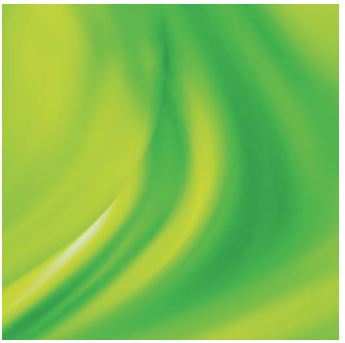
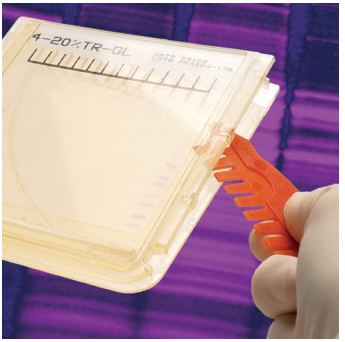



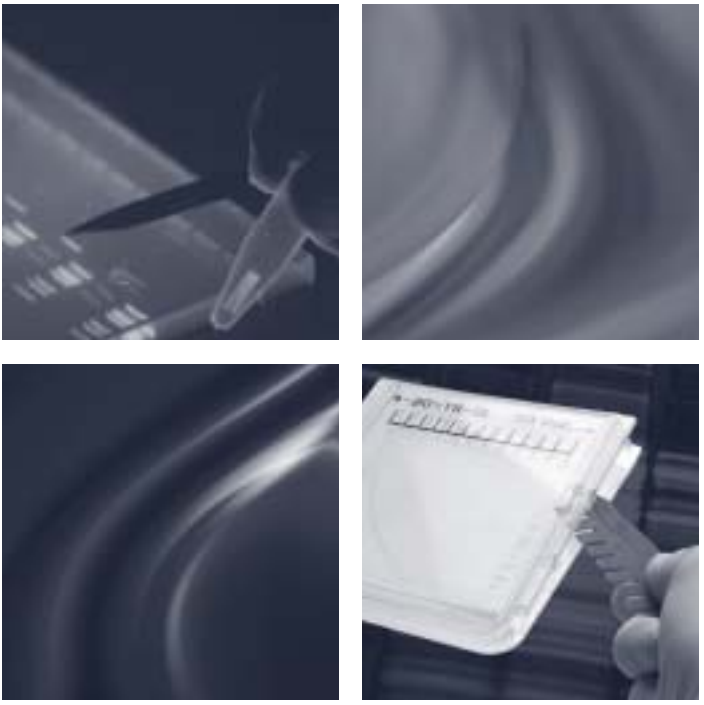


A Handbook for Gel Electrophoresis

CAMBREX

Innovation. Experience. Performance.

THE sourcebook



A Handbook for Gel Electrophoresis

CAMBREX

Innovation. Experience. Performance.

Preface

Genomics. Functional Genomics. Proteomics. Metabolomics. Molecular Biology. Biochemistry. Biology.

The names used to define areas of research in the life sciences are continually changing, but many of the basic questions researchers ask remain the same. Thus, many of the techniques they use remain the techniques that have been applied for many years. Techniques such as separation of nucleic acids by agarose gel electrophoresis and separation of proteins by polyacrylamide gel electrophoresis.

Marine Colloids. FMC BioProducts. BMA. BioWhittaker Molecular Applications. Cambrex.

Our name has also changed over the years. But our high quality products and our commitment to our customers have not. For over 35 years we have supplied the research community with the best agarose products in the world and enabled nucleic acid separation, detection, modification and recovery. Those years of experience led to the development of the original *The Sourcebook* – "everything you could possibly want to know about agarose."

This new edition of *The Sourcebook* expands coverage to include information about protein separation and detection. You can still find the information and techniques necessary to get the maximum performance from our SeaKem®, NuSieve® and MetaPhor® Agarose products for DNA electrophoresis, including the latest information on staining. In addition, you will find the same comprehensive information and techniques for RNA electrophoresis, as well as for separation of nucleic acids in polyacrylamide gels. We've expanded the section on special applications of agarose, especially in cell culture. Plus, new to this edition of *The Sourcebook*, are sections covering the separation of proteins by polyacrylamide slab gels, IEF gels, and agarose gels. In each case, thorough information is provided on preparation, loading, running and detection. In keeping with our original Sourcebook, the information is presented in easy to access tables and charts.

This manual can also be used at the bench as you perform your experiments. The step-by-step protocols are easy to follow and we include recipes for all the buffers used in this manual. We hope this becomes one of your preferred resources for all your electrophoretic separation needs.

We look forward to and welcome your comments. Please contact us with your comments at: **biotechserv@cambrex.com**

THE sourcebook

A Handbook for Gel Electrophoresis

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Chapter I

Preparation of Agarose Gels

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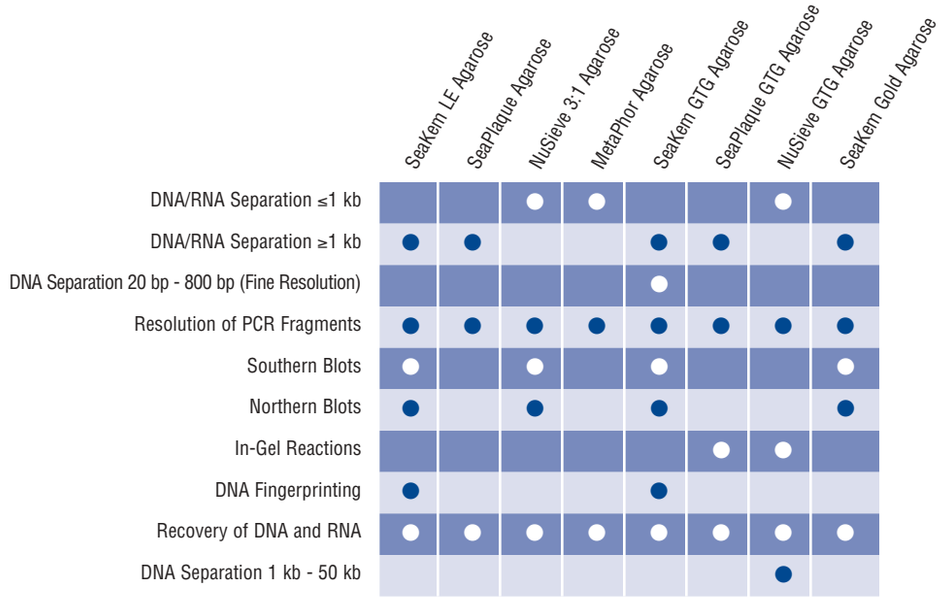
Selecting the Appropriate Agarose

Introduction

In this section we present information in two different formats, an Agarose Selection Guide and an Application Matrix to help you choose the appropriate agarose for your application.

	Fragments ≤1 kb	Fragments ≥1 kb	Fragments from 1 kb to 50 kb
Standard Analytical	NuSieve® 3:1 Agarose	SeaKem® LE Agarose	SeaKem Gold Agarose
Fine Resolution	MetaPhor® Agarose	—	—
Blotting	NuSieve 3:1 Agarose	SeaKem LE Agarose	SeaKem Gold Agarose
Preparative (In-Gel)	*Low Melting* NuSieve GTG® Agarose	*Low Melting* SeaPlaque® GTG Agarose	*Low Melting* SeaPlaque GTG Agarose
Preparative (Recovery)	*Low Melting* NuSieve GTG Agarose *Standard Melting* SeaKem GTG Agarose	*Low Melting* SeaPlaque GTG Agarose *Standard Melting* SeaKem GTG Agarose	*Low Melting* SeaPlaque GTG Agarose *Standard Melting* SeaKem Gold Agarose



How to Select the Appropriate Agarose

Nucleic acid applications take advantage of the specific properties of different agaroses and derivatized agaroses. Gelling/melting temperatures, electroendosmosis and gel strength are especially important in choosing the right agarose for your application.

The appropriate choice of an agarose depends on the size of the DNA to be analyzed and any subsequent manipulations required.

Genetic Technology Grade (GTG) agaroses

Our Genetic Technology Grade™ (GTG®) agarose products are specially prepared for demanding molecular biology applications for nucleic acids, including PCR† amplified products. Cambrex's GTG quality tests go beyond standard assays such as, DNase and RNase testing, to include enzymatic performance measurements. Our additional testing provides a more realistic index of overall product quality and reliability. You no longer need to screen agarose lots to find those that yield biologically active DNA.

The following agaroses are GTG certified:

- SeaKem® GTG® Agarose
- SeaPlaque® GTG Agarose (low melting temperature agarose)
- NuSieve® GTG Agarose (low melting temperature agarose)
- SeaKem Gold Agarose

Cambrex performs the following tests on GTG certified agaroses:

- DNA binding
- DNase and RNase activity
- DNA resolution
- Gel background-gel exhibits low background fluorescence after ethidium bromide staining
- In-gel cloning
- In-gel restriction digestion
- In-gel DNA labeling

Molecular Biology grade agaroses

Molecular Biology grade agaroses are generally suitable for analytical separation of DNA.

The following agaroses are considered molecular biology grade agaroses:

- MetaPhor® Agarose
- SeaKem® LE Agarose
- NuSieve® 3:1 Agarose
- SeaPlaque® Agarose (low melting temperature agarose)

Cambrex screens our molecular biology grade agaroses for the following parameters:

- DNA binding
- DNase and RNase activity
- Gel background staining

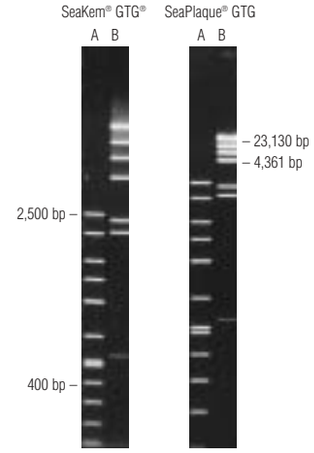
Precast agarose gels

Cambrex offers an extensive line of ready-to-use gels for nucleic acid separation using our SeaKem and NuSieve Agarose. Reliant® and Latitude® Precast Agarose Gels for DNA and RNA electrophoresis are designed to help increase efficiency, provide flexibility and maximize sample throughput. Our precast gels are offered in a wide variety of sizes, formats, concentrations and well configurations.

- **Reliant Gel System –** Easy-to-use precast minigels that provide results in 30 minutes or less. Reliant Precast Gels fit most minigel chambers and all medium to large electrophoresis systems.
- **Latitude Precast Agarose Gels –** Easy-to-use precast midigels, prestained with ethidium bromide. Latitude Precast Gels fit most medium to large electrophoresis systems.
- **Latitude HT Precast Agarose Gels –** Designed for high throughput screening applications. Latitude HT Precast Gels fit most large electrophoresis systems.
- **Reliant and Latitude HS Gel System –** Precast gels containing GelStar® PC Stain designed to increase detection sensitivity and decrease electrophoresis times without compromising resolution quality.
- **Latitude and Reliant Precast RNA Gels –** Ready-to-use RNase-free agarose gels suitable for a variety of applications including Northern blotting and analysis of RNA transcripts.

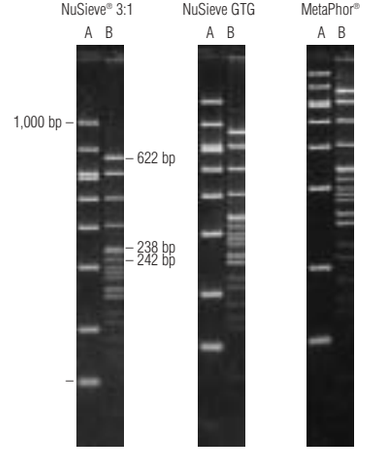
DNA resolution

The photographs below show the different resolution properties of Cambrex Agaroses.



Resolution Performance of Cambrex Agarose for DNA >1 kb

Resolution performance of Cambrex Agaroses for DNA >1 kb. Separation of DNA markers in 1% SeaKem® GTG® and SeaPlaque® GTG Agarose gels in 1X TBE buffer (Prepared from AccuGENE® 10X TBE Buffer).
Lane A: Cambrex's 50 - 2,500 bp DNA Marker (~0.25 ng/band)
Lane B: New England BioLab's Hind III digest of lambda DNA (0.125 mg/lane)
20 cm long gels were run at 6 V/cm for ~ 2.5 hrs. Gels were post stained using Cambrex's 1X GelStar® Nucleic Acid Gel Stain for 30 minutes. No destain.



Resolution Performance of Cambrex Agaroses for DNA <1 kb

Separation of DNA markers in 3% NuSieve® 3:1, NuSieve GTG® and MetaPhor® Agarose gels in 1X TBE (Prepared from AccuGENE® 10X TBE Buffer).
Lane A: Cambrex's 50 - 1,000 bp DNA Marker (~25 ng/band)
Lane B: New England BioLab's Msp I digest of pBR322 (0.125 mg/lane)
20 cm long gels were run at 6 V/cm for 2 hrs. & 20 mins. Gels were post stained using Cambrex's 1X GelStar® Nucleic Acid Gel Stain for 30 minutes. No destain.

Buffers for Electrophoresis

Introduction

During electrophoresis, water is electrolyzed, which generates protons at the anode, and hydroxyl ions at the cathode. The cathodal end of the electrophoresis chamber then becomes basic and the anodal end acidic. The use of a buffering system is therefore required when charged molecules are electrophoresed through a separation medium. The two buffers commonly used for DNA electrophoresis are Tris-acetate with EDTA (TAE; 40 mM Tris-acetate, 1 mM EDTA) and Tris-borate with EDTA (TBE; 89 mM Tris-borate, 2 mM EDTA). Because the pH of these buffers is neutral, the phosphate backbone of DNA has a net negative charge and migrates towards the anode.

Properties of TAE and TBE buffer systems

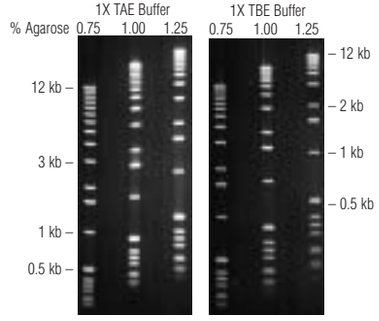
Despite the apparent similarity of TAE and TBE buffers, each has different properties which make it best suited for different applications (see table below).

Buffer	Suggested Uses and Comments
TAE	Use when DNA is to be recovered. Use for electrophoresis of large (>12 kb) DNA. **Properties:** Low buffering capacity – recirculation may be necessary for extended electrophoretic times (>6 hours).
TBE	Use for electrophoresis of small (<1 kb) DNA. Increased resolution of small (<1 kb) DNA. **Properties:** Decreased DNA mobility. High buffering capacity – no recirculation required for extended run times.

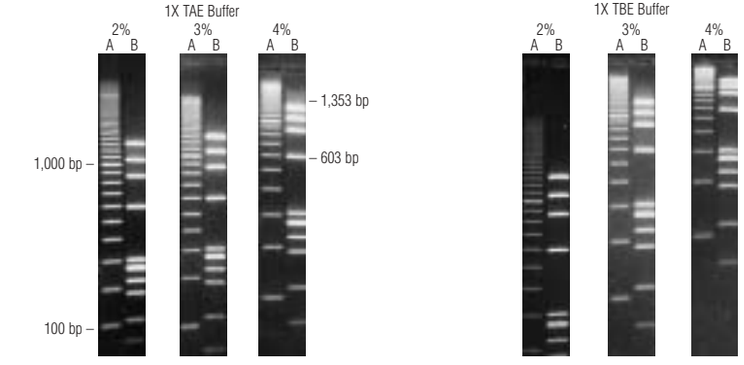
DNA electrophoresis in TAE or TBE buffer

When DNA will not be recovered, either 1X TAE or TBE (1X or 0.5X) buffer is suitable for use when the DNA is less than 12 kb to 15 kb. For larger DNA, the best buffer to use for electrophoresis is TAE in combination with a low field strength (1-2 V/cm). During these extended electrophoretic runs, larger apparent gel porosity, lower EEO and low field strength decrease the tendency of large DNA to smear.

TBE buffer is preferred for separation of small DNA (<1 kb) when DNA recovery is not required. TBE buffer's interaction with agarose results in a smaller apparent pore size. The tighter gel reduces the broadening of DNA bands due to dispersion and diffusion.



Separation of DNA markers in 0.75% to 1.25% SeaKem® GTG® Agarose gels in 1X TAE and TBE buffers. 1 kb DNA ladder (GIBCO-BRL, Life Technologies); 1 µg/lane. The gels were cast in a 25.5 cm framing gel of 1% SeaKem GTG Agarose in a submarine chamber and run under 5 mm of buffer overlay at 5 V/cm for 3 hours, 30 minutes (TBE buffer), 3 hours (TAE buffer).



Separation of DNA fragments in 2% to 4% NuSieve® 3:1 Agarose gel in 1X TAE and TBE buffers.
Lane A: Cambrex's 100 bp Extended Range DNA Ladder (4 - 7.5 ng/band)
Lane B: Life Technologies' *Hae* III digest of ϕX174 (0.25 ng/band)
20 cm long gels were run at 6 V/cm for 2.5 hrs. in 1X TBE (Prepared from AccuGENE® 10X TBE Buffer).
Gels were post stained using Cambrex's 1X GelStar® Nucleic Acid Gel Stain for 30 minutes. No destain.

Buffer depth

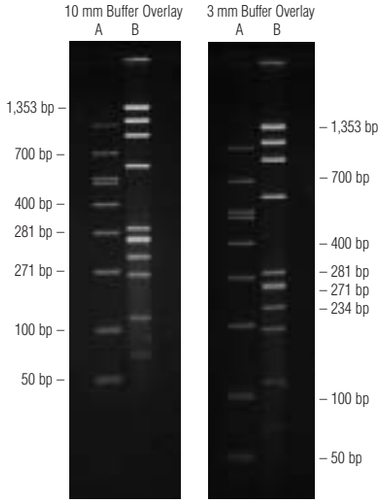
Whichever buffer is used, the depth over the gel in a horizontal electrophoretic system should be 3 mm -5 mm. Excessive buffer depth will decrease DNA mobility, promote band distortion and can cause excessive heating within the system. Less buffer and the gel may dry out during electrophoresis.

The photograph below depicts the effect of buffer depth on DNA electrophoresis. The DNA mobility in the gel with a 10 mm buffer overlay is slower than the gel with a 3 mm buffer overlay. In the *Hae* III digested ϕX174 marker, the 281/271 bands are starting to resolve in the gel with the 3 mm buffer overlay whereas in the gel with the 10 mm buffer overlay they are not.

Buffer depletion

The rate of buffer depletion is influenced by the buffer used and its buffering capacity. Evidence of buffer depletion is gel melting, smearing of DNA and/or overheating. A 0.5X TBE buffer has greater buffering capacity than a 1X TAE buffer at the pH used because the pK_a of borate is closer to the initial buffer pH than that of acetate. Standard-sized electrophoresis chambers (15 cm x 30 cm) with a 1.5 L to 2 L capacity will tolerate 40- to 50-Watt hours before buffer depletion, and buffer depletion will not occur in mini-electrophoresis chambers for 10- to 13-Watt hours. Consult the electrophoresis chamber manufacturer for specific values.

Effects of buffer depletion and development of a pH gradient can be reduced by recirculating the buffer. This is usually necessary only when electrophoresis is done for extended times or the electrophoresis buffer has a low buffering capacity.



Separation of DNA markers in a 3% NuSieve® 3:1 Agarose gel prepared and run in 1X TBE buffer. Lane A: DNA marker 50 bp - 1,000 bp DNA Marker (Cambrex); 0.4 µg/lane.
Lane B: *Hae* III digested ϕX174 DNA (New England Biolabs); 0.5 µg/lane. The gels were run at 5 V/cm until the bromophenol blue tracking dye was 1 cm from the bottom of each gel.

Buffer preparation

50X TAE (TRIS-ACETATE) STOCK	5X TBE (TRIS-BORATE) STOCK
(1X=40 mM Tris base, 40 mM acetic acid, 1 mM EDTA)	(1X=89 mM Tris base, 89 mM boric acid, 2 mM EDTA) (0.5X=45 mM Tris-borate, 1 mM EDTA)
242.0 g Tris base	54.0 g Tris base
57.1 ml Glacial acetic acid	27.5 g Boric acid
18.61 g $Na_2EDTA \bullet 2H_2O$	3.72 g $Na_2EDTA \bullet 2H_2O$
To 1 liter with distilled water	To 1 liter with distilled water

Alternatively, Cambrex offers AccuGENE® TBE Buffer in 5X and 10X stock solutions and AccuGENE TAE Buffer in 10X and 50X stock solutions.

Other buffering systems

Tris-phosphate buffer (TPE) may also be used for DNA electrophoresis. Like TBE buffer, TPE has a high buffering capacity, and will not interfere with DNA recovery procedures. However, TPE can not be used when recovered DNA will be used in a phosphate-sensitive reaction.

10X Tris-phosphate stock (TPE)

(1X=90 mM Tris base, 90 mM phosphoric acid, 2 mM EDTA)
108.0 g Tris base
15.5 ml 85% Phosphoric acid
7.44 g $Na_2EDTA \bullet 2H_2O$
To 1 liter with distilled water

Alkaline electrophoresis buffer is used for the analysis of single-stranded DNA. This is discussed in more detail in Chapter IX.

Urea in agarose gels

Urea can be included in agarose gels as an alternative to alkaline gel electrophoresis for the analysis of single-stranded DNA or as an alternative denaturant to formaldehyde or glyoxal for RNA electrophoresis.

The presence of urea inhibits agarose gelation by interfering with hydrogen bonding of the agarose polysaccharide chains. This can be circumvented by careful gel preparation and a refrigeration cure time, generally overnight. The resulting gel will be more fragile than a regular agarose gel. For this reason, it is best not to blot an agarose-urea gel.

Agarose-urea gels (4 M - 6 M urea) can be prepared by first dissolving the agarose in a reduced volume of water or buffer, cooling to 50°C, then adding urea while stirring. The dissolution of either crystalline, ultrapure urea or a concentrated solution of ultrapure urea is an endothermic process. Although the solution will cool noticeably and the viscosity will increase, the solution will not gel. Once the urea has dissolved, adjust to the desired total weight/volume with distilled water or buffer, cast the gel and refrigerate overnight. Alternatively, some researchers prefer to "quick chill" gels by initially placing them in a freezer for 15 - 30 minutes. The urea prevents the formation of ice crystals. If urea crystals form, allow the gel to stand at room temperature before use until the crystals disappear (usually a few minutes).

If a satisfactory gel cannot be obtained by the above procedures, cast an agarose gel in the normal manner, then equilibrate in a urea solution prepared from a buffer or other solute.

Avoid exposing urea to high temperatures. Urea solutions decompose to ammonia and biuret with excessive heat. Cyanate formation of other gel components may also occur.

Suggested Agarose Concentrations

Introduction

The optimal gel concentration to use depends on the size of the DNA fragments to be resolved.

CAMBREX PRODUCT	SIZE RANGE (BASE PAIRS)	FINAL AGAROSE CONCENTRATION % (W/V) 1X TAE BUFFER	1X TBE BUFFER
NuSieve® 3:1 Agarose*	500 - 1,000	3.0	2.0
	100 - 500	4.0	3.0
	10 - 100	6.0	5.0
NuSieve GTG® Agarose*	500 - 1,000	2.5	2.0
	150 - 700	3.0	2.5
	100 - 450	3.5	3.0
	70 - 300	4.0	3.5
	10 - 100	4.5	4.0
	8 - 50	5.0	4.5
MetaPhor® Agarose*	150 - 800	2.0	1.8
	100 - 600	3.0	2.0
	50 - 250	4.0	3.0
	20 - 130	5.0	4.0
	<80	—	5.0
SeaPlaque® and SeaPlaque GTG Agarose	500 - 25,000	0.75	0.70
	300 - 20,000	1.00	0.85
	200 - 12,000	1.25	1.00
	150 - 6,000	1.50	1.25
	100 - 3,000	1.75	1.50
	50 - 2,000	2.00	1.75
SeaKem® GTG and SeaKem LE Agarose	1,000 - 23,000	0.60	0.50
	800 - 10,000	0.80	0.70
	400 - 8,000	1.00	0.85
	300 - 7,000	1.20	1.00
	200 - 4,000	1.50	1.25
	100 - 3,000	2.00	1.75
SeaKem Gold Agarose	5,000 - 50,000	0.3	TBE buffer is not
	1,000 - 20,000	0.5	recommended for
	800 - 10,000	0.8	the separation of
	400 - 8,000	1.0	DNA >12,000 bp.

*We do not recommend preparing NuSieve 3:1, NuSieve GTG and MetaPhor Agarose gels at less than 2% because these gels are more difficult to handle.

Dissolving Agarose

Introduction

Agarose undergoes a series of steps when it is dissolved; dispersion, hydration and melting/dissolution.

Dispersion

Dispersion simply refers to the separation of the particles by the buffer without clumping. Clumping occurs when the agarose starts to dissolve before it is completely dispersed, coating itself with a gelatinous layer which inhibits the penetration of water and keeps the powder inside from dispersing. Dissolution then becomes a long process.

Hydration

Hydration is the surrounding of agarose particles by a solution (e.g., water or running buffer). Problems are sometimes encountered with hydration when using a microwave oven to dissolve agarose. In part, this occurs because hydration is time dependent and microwave ovens bring the temperature up rapidly. The problem is exacerbated by the fact that the agarose is not being agitated to help dilute the highly concentrated solution around each particle and dissolution is slowed.

Melting and dissolution

The final stage in dissolving the agarose is the melting and dissolution. Melting can be done in either a microwave oven or on a hot plate. As the particles hydrate, they become small, highly concentrated gels. Since the melting temperature of a standard agarose gel is about 93°C, merely heating a mixture to 90°C will not completely dissolve agarose. Even low melting temperature agaroses should be boiled to ensure that all the agarose is fully dissolved.

Microwave instructions for agarose preparation for gel concentrations of ≤2% w/v

1. Choose a beaker that is 2 - 4 times the volume of the solution.
2. Add room temperature 1X or 0.5X buffer and a stir bar to the beaker.
3. Sprinkle in the premeasured agarose powder while the solution is rapidly stirred.
4. Remove the stir bar if not Teflon® coated.
5. Weigh the beaker and solution before heating.
6. Cover the beaker with plastic wrap.
7. Pierce a small hole in the plastic wrap for ventilation.
8. Heat the beaker in the microwave oven on HIGH power until bubbles appear.
   CAUTION: Any microwaved solution may become superheated and foam over when agitated.
9. Remove the beaker from the microwave oven.
10. GENTLY swirl the beaker to resuspend any settled powder and gel pieces.
11. Reheat the beaker on HIGH power until the solution comes to a boil.
12. Hold at boiling point for 1 minute or until all of the particles are dissolved.
13. Remove the beaker from the microwave oven.
14. GENTLY swirl the beaker to mix the agarose solution thoroughly.
15. After dissolution, add sufficient hot distilled water to obtain the initial weight.
16. Mix thoroughly.
17. Cool the solution to 60°C prior to casting.

Materials

- Microwave oven or hot plate
- Beaker that is 2 - 4 times the volume of the solution
- Teflon-coated magnetic stir bar
- Magnetic stir plate
- Plastic wrap

Reagents

- Distilled water
- 1X TAE, 1X TBE or 0.5X TBE electrophoresis buffer
- Agarose powder

Caution

Always wear eye protection, and guard yourself and others against scalding solutions.

Microwave instructions for agarose preparation for gel concentrations >2% w/v

1. Choose a beaker that is 2 - 4 times the volume of the solution.
2. Add room temperature or chilled buffer (for MetaPhor® and NuSieve® GTG® Agarose) and a stir bar to the beaker.
3. Sprinkle in the premeasured agarose powder while the solution is rapidly stirred to prevent the formation of clumps.
4. Remove the stir bar if not Teflon® coated.
5. Soak the agarose in the buffer for 15 minutes before heating. This reduces the tendency of the agarose solution to foam during heating.
6. Weigh the beaker and solution before heating.
7. Cover the beaker with plastic wrap.
8. Pierce a small hole in the plastic wrap for ventilation. For agarose concentrations >4%, the following additional steps will further help prevent the agarose solution from foaming during melting/dissolution:
   - **8A.** Heat the beaker in the microwave oven on MEDIUM power for 1 minute.
   - **8B.** Remove the solution from the microwave.
   - **8C.** Allow the solution to sit on the bench for 15 minutes.
9. Heat the beaker in the microwave oven on MEDIUM power for 2 minutes.
   CAUTION: Any microwaved solution may become superheated and foam over when agitated.
10. Remove the beaker from the microwave oven.
11. GENTLY swirl to resuspend any settled powder and gel pieces.
12. Reheat the beaker on HIGH power for 1 - 2 minutes or until the solution comes to a boil.
13. Hold at the boiling point for 1 minute or until all of the particles are dissolved.
14. Remove the beaker from the microwave oven.
15. GENTLY swirl to mix the agarose solution thoroughly.
16. After dissolution, add sufficient hot distilled water to obtain the initial weight.
17. Mix thoroughly.
18. Cool the solution to 60°C prior to gel casting.

Hot plate instructions for preparing agarose

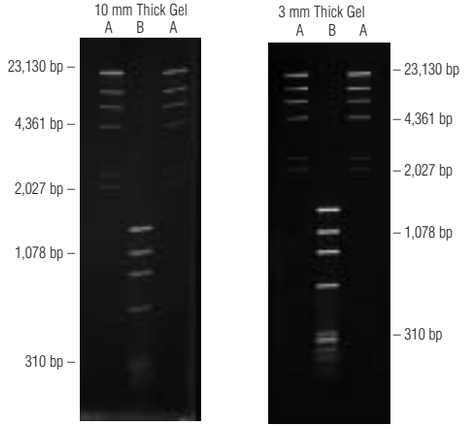
1. Choose a beaker that is 2 - 4 times the volume of the solution.
2. Add room temperature or chilled buffer (for MetaPhor® or NuSieve® GTG® Agarose) and a stir bar to the beaker.
3. Sprinkle in the premeasured agarose powder while the solution is rapidly stirred to prevent the formation of clumps.
4. Weigh the beaker and solution before heating.
5. Cover the beaker with plastic wrap.
6. Pierce a small hole in the plastic wrap for ventilation.
7. Bring the solution to a boil while stirring.
8. Maintain gentle boiling until the agarose is dissolved (approximately 5 -10 minutes).
9. Add sufficient hot distilled water to obtain the initial weight.
10. Mix thoroughly.
11. Cool the solution to 60°C prior to casting.

Casting Agarose Gels

Introduction

For optimal resolution, cast horizontal gels 3 mm - 4 mm thick (see figure below). The volume of gel solution needed can be estimated by measuring the surface area of the casting chamber, then multiplying by the gel thickness. Thinner gels can be cast on GelBond® Film and/or in a vertical electrophoresis apparatus.

The photographs below depict the effect of gel depth on DNA electrophoresis. Gel thickness has a profound effect on the resolution of smaller fragments. The smaller DNA fragments in the 10 mm thick gel are fuzzy, whereas in the 3 mm thick gel the resolution is sharp throughout the gel. There is also a higher background staining in gels thicker than 5 mm.



Separation of DNA markers in 1% SeaKem® GTG® Agarose gels prepared and run in 1X TBE buffer.
Lane A: *Hin*d III digest of lambda DNA (Boehringer Mannheim); 0.1 µg/lane.
Lane B: *Hae* III digested ϕX174 DNA (New England Biolabs); 0.5 µg/lane.
20 cm long gels were run at 6 V/cm for 2 hours 15 minutes (10 mm thick gel) and 2 hours 10 minutes (3 mm thick gel).

Horizontal gel casting instructions

1. Allow the agarose solution to cool to 60°C.

2. While the agarose solution is cooling:

2A. Assemble the gel casting tray.

2B. Level the casting tray prior to pouring the agarose solution.

2C. Check the comb(s)* teeth for residual dried agarose. Dried agarose can be removed by scrubbing the comb teeth with a lint-free tissue soaked in hot distilled water.

2D. Allow a small space (approximately 0.5 mm - 1 mm) between the bottom of the comb teeth and the casting tray.

3. Pour the agarose solution into the gel tray.

4. Replace the comb(s).

5. Allow the agarose to gel at room temperature for 30 minutes.

6. Low melting temperature agaroses and MetaPhor® Agarose require an additional 30 minutes of gelling at 4°C to obtain the best gel handling. The additional cooling step is essential for obtaining fine resolution in MetaPhor Agarose.

7. Once the gel is set, flood with running buffer.

8. Slowly remove the comb.

9. Place the gel casting tray into the electrophoresis chamber.

10. Fill the chamber with running buffer until the buffer reaches 3 mm - 5 mm over the surface of the gel.

11. Gently flush the wells out with electrophoresis buffer using a Pasteur pipette to remove loose gel fragments prior to loading the samples.

12. Load DNA and electrophorese.

*The thickness of the comb in the direction of the electric field can affect the resolution. A thin comb (≤1 mm) will result in sharper DNA bands. With a thicker comb, more volume can be added to the well but the separated DNA bands may be broader.

Materials
- Horizontal electrophoresis apparatus
- Combs
- Pasteur pipette

Reagents
- Agarose solution
- Electrophoresis buffer

Materials

- Vertical electrophoresis apparatus
- Combs and side spacers
- Whatman 3MM chromatography paper
- Clamps
- Silicone tubing or electrical tape
- Two 60 ml syringes with 16-gauge needles
- Heat gun or 55°C oven
- Scalpel or razor blade

Reagents

- Agarose solution
- Electrophoresis buffer

Vertical gel casting instructions

Follow the steps below to cast a vertical agarose gel. This protocol is divided into the following segments:

- Cassette assembly
- Casting the gel
- Cassette sealing
- Preparing for electrophoresis

Cassette assembly

Unlike polyacrylamide gels, agarose gels do not adhere to glass plates and may slide out during electrophoresis. To prevent this from happening, frosted glass plates or plastic plates can be used.

Follow the steps below to assemble the glass plates.

1. Use clean glass plates. Clean with soap and water, rinse with distilled water and dry.

2. Wipe the plates with ethanol and a lint-free tissue.

3. Place two side spacers on the back plate. Follow the steps below if using glass plates.

3A. For 1 mm thick standard size gels, cut a strip of Whatman® 3mm chromatography paper (1 mm thick and 5 - 10 mm wide) long enough to fit between the two spacers.*

3B. Wet with running buffer.

3C. Place at the bottom of the back plate in contact with the spacers on each side (see Figure 1, page 25).

4. Put on the front plate.

5. Clamp the glass plates together.

6. Use the manufacturer's casting apparatus or seal the cassette against leaks with silicone tubing or tape.

*Alternatively, use GelBond® Film as a support for the gel, which obviates the need for the use of Whatman 3MM chromatography paper to hold the gel in the cassette during electrophoresis (see Chapter IX). GelBond Film is put into the casting cassette and the gel attaches to the film during the gelling process. After electrophoresis, the gel may be dried down on the GelBond Film and kept as a permanent record. However, GelBond Film blocks UV light below 300 nm and exhibits background fluorescence. To overcome these problems, gels cast on GelBond Film may be photographed inverted (gel side down) on the UV light box. Background fluorescence can be screened out by using red, orange (Wratten® #22 or #25 gelatin filter) and UV filters (Wratten #2B gelatin filter).

Cassette sealing

Follow one of the methods below to seal the cassette prior to casting the gel.

Silicone tubing method

1. Use silicone tubing which is the same diameter as the spacer thickness.

2. Cut a piece long enough to extend along the bottom and up both sides of the cassette.

3. Place the tubing across the bottom of the back plate below the blotting paper strip (see Figure 2).

4. Place the top plate over the bottom plate.

5. Clamp the glass plates together at the bottom.

6. Run the tubing up either side of the plates and finish clamping the plates together (see Figure 3).

Tape method

1. Place the top plate over the bottom plate.

2. Tape the sides of the cassette with separate pieces of tape.

3. Tape the bottom of the cassette with a separate piece of tape. This way the tape on the bottom can be removed for electrophoresis without disturbing the tape at the sides of the gel (see Figure 4).

4. Clamp the plates together.

Casting a vertical agarose gel

1. Prepare agarose solution as described previously.

2. Pre-warm the assembled cassette and a 60 ml syringe for 15 minutes by placing in a 55°C oven or by using a heat gun.

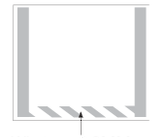
3. Cool the dissolved agarose to 60°C.

4. Pour into a pre-warmed 60 ml syringe fitted with a 16-gauge needle.

5. Wedge the needle tip between the plates in the upper corner of the cassette with the needle opening directed toward the back plate (see Figure 5).

Continued on page 26

FIGURE 1.



Whatman® 3MM chromatography paper

FIGURE 2.

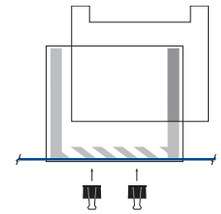


FIGURE 3.

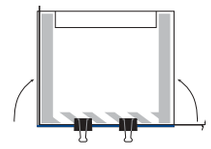


FIGURE 4.

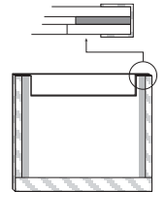


FIGURE 5.

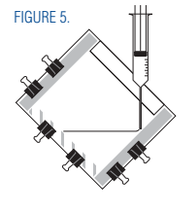
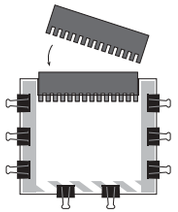


FIGURE 6.



6. Inject the agarose solution at a moderate, steady rate. Keep a constant flow to prevent air bubble formation (see Fig. 5, page 25).
7. Angle the cassette while pouring so the agarose solution flows down one side spacer, across the bottom and up the other side.
8. Fill until the agarose solution goes just above the glass plates.
9. Insert one end of the comb, then slowly insert the rest of the comb until the teeth are at an even depth.
   Insert the comb into the agarose to the minimal depth necessary to accommodate your samples (see Figure 6).
10. Place extra clamps on the side of the glass plates to hold the comb in place.
11. Cool the gel at room temperature for 15 minutes.
12. Place the gel at 4°C for 20 minutes.
13. Remove the clamps at the top of the gel.
14. Remove any excess agarose with a scalpel or razor blade.
15. Squirt running buffer in the spaces between the comb and the gel.
16. Slowly and gently lift the comb straight up. Allow air or buffer to enter the well area to release the vacuum which forms between the agarose and the comb.
17. The wells can be further cleaned by flushing with running buffer.
18. The gel can be stored overnight in a humidity chamber or in a sealed bag with a buffer-dampened paper towel.

Preparing for electrophoresis

1. Remove the silicone tubing or tape at the bottom of the cassette

   NOTE: If you have placed Whatman® 3MM chromatography paper between the plates at the bottom, it is not necessary to remove it as it will not interfere with electrophoresis.

2. Place the cassette into the chamber at an angle to minimize the number of bubbles which can collect in the well area.
3. Rinse out well area with a syringe.

   NOTE: Since agarose does not adhere well to glass, leave as many clips in place as possible. For some electrophoresis chambers, it is helpful to seal the spacers at the top of the gel with molten agarose.

Chapter II

Loading and Running DNA in Agarose Gels

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DNA Loading

Introduction

The amount of DNA to load per well is variable. Most important are the quantities of DNA in the bands of interest.

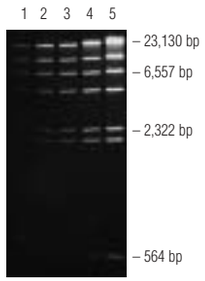
Optimal DNA loading amount

The amount of DNA that may be loaded on a gel depends on several factors:

- Well volume
- Fragment size: The capacity of the gel drops sharply as the fragment size increases, especially over a few kilobases
- Distribution of fragment sizes
- Voltage gradient: Higher voltage gradients are better suited to DNA fragments ≤1 kb, lower voltages are better suited to fragments >1 kb
- Detection Method: The least amount of dsDNA in a single band that can be reliably detected with ethidium bromide is approximately 10 ng, with GelStar® Nucleic Acid Gel Stain is approximately 20 pg and with SYBR® Green I Stain is 60 pg.

Overloaded DNA results in trailing and smearing, a problem that will become more severe as the size of the DNA increases.

The photograph below shows the effect of overloaded and underloaded DNA on an agarose gel. Where samples are loaded in excess (0.5 µg/lane), you can see band broadening and smearing of the larger molecular weight fragments. Where samples are underloaded, you lose the small molecular weight fragments. The optimum loading level for the marker used in the photograph is 0.1 µg/lane.



Separation of DNA markers in a 1% SeaKem® GTG® Agarose gel prepared and run in 1X TBE buffer. *Hin*d III digested lambda DNA (Boehringer Mannheim) was loaded from left to right at 0.025, 0.05, 0.1, 0.2 and 0.5 µg/lane. 20 cm long gels were run at 6 V/cm for 2 hours, 5 minutes.

The optimal amount of DNA to load in the well may be calculated by the fraction of the total DNA which is in the band of interest, represented by the following:

$$\frac{\text{Fragment of interest (kbp)}}{\text{Size of DNA sample (kbp)}} \text{ X 100 = \% DNA in band of interest}$$

NOTE: The most DNA compatible with a clean sharp band is approximately 100 ng.

For example:

The size of your DNA sample is 48.5 kbp and when run on the gel 8 fragments are separated. Your fragment of interest is 2.3 kbp.

Calculation:

$$\frac{2.3 \text{ kbp}}{48.5 \text{ kbp}} \text{ X 100 = 4.7\% DNA in fragment of interest}$$

If you load 1 µg of DNA, then 4.7% of the 1 µg of loaded sample will appear in your fragment of interest (47 ng).

Loading Buffers

Introduction

Gel loading buffers serve three purposes in DNA electrophoresis:

- Increase the density of the sample: This ensures that the DNA will drop evenly into the well
- Add color to the sample: Simplifies loading
- Add mobility dyes: The dyes migrate in an electric field towards the anode at predictable rates. This enables one to monitor the electrophoretic process

Loading buffers

At least five loading buffers are commonly used for agarose gel electrophoresis. They are prepared as six-fold concentrated solutions. If needed, 10X solutions of each buffer can also be prepared. Alkaline loading buffer is used when performing alkaline gel electrophoresis (see Chapter IX).

Loading Buffer	6X Recipe	Storage Temperature
Sucrose Based	40% (w/v) Sucrose 0.25% Bromophenol Blue 0.25% Xylene cyanol FF	4°C
Glycerol Based	30% Glycerol in distilled water 0.25% Bromophenol Blue 0.25% Xylene cyanol FF	4°C
Ficoll® Based	15% Ficoll® (type 400) polymer in distilled water 0.25% Bromophenol Blue 0.25% Xylene cyanol FF	room temperature
Alkaline	300 mN NaOH 6 mM EDTA 18% Ficoll (type 400) polymer in distilled water 0.15% Bromocresol Green 0.25% Xylene cyanol FF	4°C

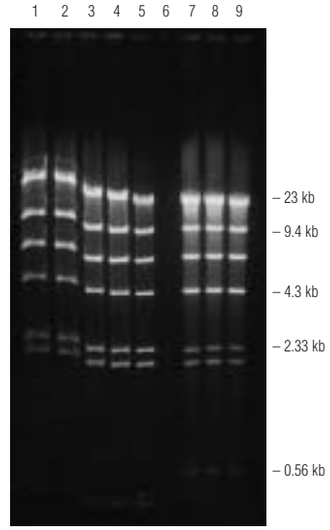
Ficoll® based loading buffers

To increase the sharpness of DNA bands, use Ficoll® (type 400) polymer as a sinking agent instead of glycerol. The use of the lower molecular weight glycerol in the loading buffer allows DNA to stream up the sides of the well before electrophoresis has begun and can result in a U-shaped band. In TBE gels, glycerol also interacts with borate which can alter the local pH.

Sample preparation

Loading buffer that is too high in ionic strength causes bands to be fuzzy and migrate through the gel at unpredictable rates. Ideally, the DNA sample should be resuspended in the same solution as the running buffer. If this is not possible, use a sample buffer with a lower ionic strength than the running buffer.

The photograph below shows the effect of high salt concentrations in loading buffers on DNA resolution.



Separation of *Hin*d III digested lambda DNA (GIBCO-BRL, Life Technologies) marker (5 µg/lane) in a 1% SeaKem® GTG® Agarose gel prepared and run in 1X TAE buffer. 20 cm long gels were run at 6 V/cm for 2 hours. The sample buffer was mixed with varying amounts of NaCl to obtain different final salt concentrations.
Lane 1: 4 M NaCl, **Lane 2**: 3 M NaCl, **Lane 3**: 1 M NaCl, **Lane 4**: 0.5 M NaCl, **Lane 5**: 0.25 M NaCl and **Lanes 7 - 9**: No addition of NaCl.

Dye Mobility Table

Introduction

The following table is a migration table of double-stranded DNA in relation to bromophenol blue (BPB) and xylene cyanol (XC) in Cambrex Agarose and Reliant®, Latitude® and Latitude HT Precast Agarose Gels.

NuSieve® 3:1 Agarose

1X TAE BUFFER XC	1X TAE BUFFER BPB	% AGAROSE	1X TBE BUFFER XC	1X TBE BUFFER BPB
950	130	2.50	700	70
650	80	3.00	500	40
350	40	4.00	250	20
200	30	5.00	140	8
120	20	6.00	90	4

NuSieve GTG® Agarose

1X TAE BUFFER XC	1X TAE BUFFER BPB	% AGAROSE	1X TBE BUFFER XC	1X TBE BUFFER BPB
750	175	2.50	460	75
400	120	3.00	210	35
115	<20	4.00	150	<20
100	<20	5.00	80	<20
85	<20	6.00	50	<20

SeaKem® GTG and SeaKem LE Agarose

1X TAE BUFFER XC	1X TAE BUFFER BPB	% AGAROSE	1X TBE BUFFER XC	1X TBE BUFFER BPB
24,800	2,900	0.30	19,400	2,850
11,000	1,650	0.50	12,000	1,350
10,200	1,000	0.75	9,200	720
6,100	500	1.00	4,100	400
3,560	370	1.25	2,500	260
2,800	300	1.50	1,800	200
1,800	200	1.75	1,100	110
1,300	150	2.00	850	70

SeaKem Gold Agarose

1X TAE BUFFER XC	1X TAE BUFFER BPB	% AGAROSE	1X TBE BUFFER XC	1X TBE BUFFER BPB
24,800	3,550	0.30	19,000	2,550
12,200	2,050	0.50	9,200	1,500
9,200	1,050	0.75	7,100	800
6,100	760	1.00	4,000	500
4,100	600	1.25	2,550	350
2,600	400	1.50	1,900	250
2,000	330	1.75	1,400	180
1,500	250	2.00	1,000	100

SeaPlaque® and SeaPlaque GTG Agarose

1X TAE BUFFER XC	1X TAE BUFFER BPB	% AGAROSE	1X TBE BUFFER XC	1X TBE BUFFER BPB
11,700	1,020	0.50	6,100	400
4,000	500	0.75	2,850	280
2,300	350	1.00	1,700	180
1,500	200	1.25	1,000	100
1,000	150	1.50	700	70
700	100	1.75	500	50
550	60	2.00	400	30
320	30	2.50	250	10

MetaPhor® Agarose

1X TAE BUFFER XC	1X TAE BUFFER BPB	% AGAROSE	1X TBE BUFFER XC	1X TBE BUFFER BPB
480	70	2.00	310	40
200	40	3.00	140	35
120	35	4.00	85	30
85	30	5.00	60	15

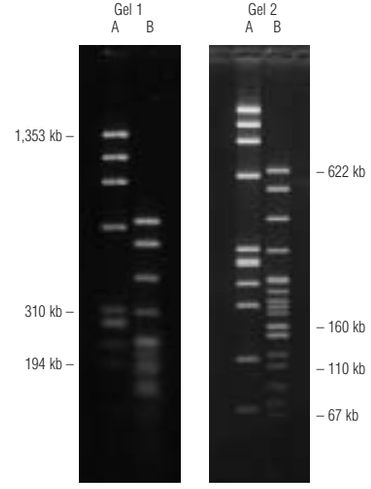
Optimal Voltage and Electrophoretic Times

Optimal voltage

The distance used to determine voltage gradients is the distance between the electrodes, not the gel length. If the voltage is too high, band streaking, especially for DNA ≥12 kb - 15 kb, may result. When the voltage is too low, the mobility of small (≤1 kb) DNA is reduced and band broadening will occur due to dispersion and diffusion.

The photographs below show the effect of voltage on small DNA. The small fragments on the gel run at 1 V/cm show severe band broadening and fuzziness. The gel run under 5 V/cm has sharp bands both in the small fragments and the larger fragments. Buffer also plays a role in band sharpness.

The photographs on the next page show the effect of voltage and buffer on large DNA. When large DNA is subjected to very high voltage, smearing occurs.



Separation of DNA markers in 3% NuSieve® 3:1 Agarose gels. Lane A: *Hae* III digested ϕX174 DNA (New England Biolabs); 0.5 µg/lane. Lane B: *Msp* I digested pBR322 DNA (New England Biolabs); 0.5 µg/lane. Gel 1: 21 cm long gel run at 1 V/cm in 1X TAE buffer (recirculating) for 16 hours. Gel 2: 20 cm long gel run at 5 V/cm in 1X TBE buffer for 2 hours, 10 minutes.

Voltage table

The table below provides a quick reference for optimal voltage for DNA electrophoresis.

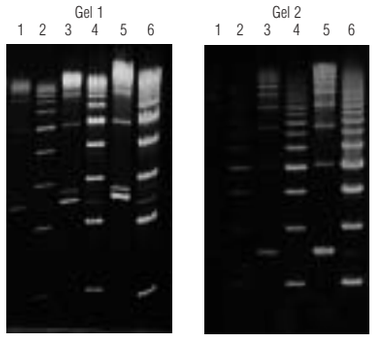
Recommended Voltages and Buffers Related to DNA Size and Application

SIZE	VOLTAGE	BUFFER	
		RECOVERY	ANALYTICAL
≤1 kb	5 V/cm	TAE	TBE
1 kb to 12 kb	4 - 10 V/cm	TAE	TAE/TBE
>12 kb	1 - 2 V/cm	TAE	TAE

NOTE: MetaPhor® Agarose can also be run at very high voltages to achieve 1% - 2% resolution. See "Fast Running Protocols for High Resolution in MetaPhor Agarose Gels," this chapter.

Optimal electrophoretic time

The gel should be run until the band of interest has migrated 40% - 60% down the length of the gel (see the Dye Mobility Table). Band broadening resulting from dispersion and diffusion results in a decrease in resolution in the lower third of the gel. Resolution may also be decreased in smaller gels, since longer electrophoretic runs result in greater separation between two fragments.



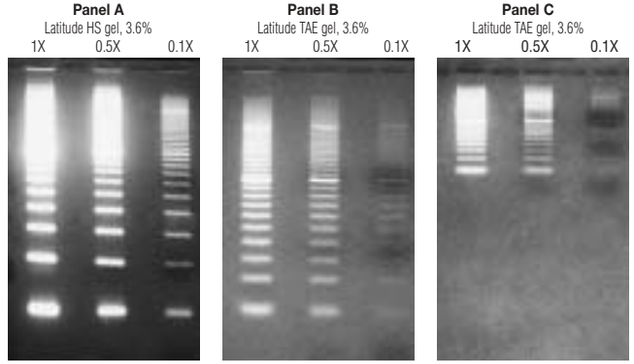
Separation of Gensura's 5 kb and 2 kb DNA ladders in 0.5% SeaKem® LE Agarose gels. Odd numbered lanes are 5 kb ladders; even numbered lanes are 2 kb ladders. Lane 1: 5 ng/band; Lane 2: 5 ng/band; Lane 3: 20 ng/band; Lane 4: 20 ng/band; Lane 5: 60 ng/band; Lane 6: 60 ng/band. Gel 1: 20 cm long gel run at 8 V/cm in 1X TBE buffer without recirculation. Gel 2: 21 cm long gel run at 1 V/cm in 1X TAE buffer with recirculation.

Fast Electrophoresis Protocol for Reliant® and Latitude® High Sensitivity Precast Agarose Gels

Introduction

This protocol describes how to achieve DNA detection sensitivity 5 - 10 times greater than the sensitivity of ethidium bromide and decrease electrophoresis time by using Cambrex's HS Precast Agarose Gels. Reliant and Latitude HS Precast Agarose Gels contain GelStar® PC Gel Stain, which can detect as little as 20 pg of dsDNA, and provide sharp band visualization with low background. These gels also use HS Electrophoresis Buffer, a high sensitivity buffer formulation that allows increased voltage and faster runs while optimizing separation performance. TAE and TBE buffers may be used as running buffers, however, HS Electrophoresis Buffer is recommended for speed and optimal resolution.

The photographs below demonstrate the detection sensitivity in Latitude HS Precast Agarose Gels compared to standard Latitude Precast Agarose Gels.



Separation of 20 bp DNA Ladder (Cambrex) on 3.6% NuSieve® Hi-Res Latitude® Precast Agarose Gels in 1X HS or TAE buffer. Gel 1: Latitude HS Gel containing GelStar PC Stain, run at 325 volts for 30 minutes in HS buffer. Gel 2: Latitude Gel containing ethidium bromide run at 150 volts for 70 minutes in 1X TAE buffer. Gel 3: Latitude Gel containing ethidium bromide run at 150 volts for 30 minutes in 1X TAE buffer.

Chamber Compatibility and Gel Size

PRECAST GEL	GEL DIMENSIONS	TRAY DIMENSIONS*	COMPATIBLE CHAMBERS
Reliant® Gels	6.0 x 9.5 cm	6.8 x 10.2 cm	Most minigel chambers All medium and large chambers.
Latitude® Gels	24 x 14 cm	25 x 15 cm	OWL Centipede™ or Millipede™ Horizontal Systems Bio-Rad® Sub-Cell® Models 96 and 192 systems FisherBiotech® Wide-Format Horizontal System FB-SB-2318

**Reliant and Latitude HS Precast Gels are supplied in trays. We recommend that both gels be run in the tray with the TruBand™ Anchor for optimal performance*

Tips for Running Reliant® and Latitude® HS Precast Agarose Gels

Materials
- Electrophoresis chamber
- Power supply
- Scissors
- TruBand Anchor
- Reliant UV Transparent Tray (optional)

Reagents
- Reliant or Latitude HS Precast Gel
- 1X HS Buffer 1X TAE or 1X TBE buffer
- Tri-Dye Loading Buffer

Caution
GelStar® PC Gel Stain should be handled with care. Gloves should be worn when handling the gels. Avoid skin and eye exposure to UV light.

- Latitude HS Precast Gels must be used with the TruBand™ Anchor to achieve optimal results.
- Do not flood buffer over the top surface of the anchor.
- UV transparent, open-ended trays are available for use with the Reliant HS Precast Gels.
- Use of Tri-Dye™ Loading Buffer is recommended with the High Sensitivity System. Bromophenol blue will not migrate in HS gels and remains in the well.
- Use Clare Chemical's Dark Reader™ transilluminator to visualize or photograph the gels without removing them from the tray.
- For optimal sensitivity and band resolution use 1X HS Buffer.
- For optimal detection sensitivity with Polaroid® photography use the GelStar® Gel Stain Photographic Filter.
- GelStar PC Stain is compatible with most CCD and video imaging systems. Due to variations in the filters for these systems you may need to purchase a new filter. Contact your systems manufacturer and using the excitation and emission information listed they can guide you to an appropriate filter. **The excitation and emission maxima of GelStar PC Gel Stain are 493 nm and 527 nm respectively.**

Follow the steps below for running Reliant and Latitude HS Precast Gels

1. Remove the gel from the Latitude packaging or remove the lid from the Reliant packaging.

2. Place the tray directly on the chamber platform.

3. Cover the gel with 1X electrophoresis buffer to a depth of 2 mm - 3 mm over the gel surface.

4. If using the Reliant® HS Precast Gels proceed to step 5. Follow the steps below if using Latitude® HS Precast Agarose Gels:

4A. Place the Cambrex TruBand™ Anchor over the tray so the white gaskets fit snugly on the flanges on the short ends of the tray.

4B. Position the anchor so both the top and middle well tiers are exposed.

4C. Add electrophoresis buffer until the level is even with the top of the TruBand Anchor. Do not flood buffer over the surface of the anchor.

5. For Reliant HS Precast Gels, add electrophoresis buffer to a depth of 5 mm over the tray flange.

6. Prepare samples using Tri-Dye™ Loading Buffer. Tri-Dye Loading Buffer contains Orange G, Cresol Red and Xylene Cyanol. See dye mobility table below.

Migration of marker dyes in Reliant and Latitude HS Precast Gel

AGAROSE CONCENTRATION	ORANGE G (YELLOW)	CRESOL RED (RED)	XYLENE CYANOL (BLUE)
1%	800 - 900 bp	1,750 bp	12,000 bp
2%	75 bp	375 bp	1500 bp
3.6%	40 bp	100 bp	350 bp

** Gels were run in 1X HS Buffer.*

7. Load up to 12 µl - 15 µl of DNA per well.

8. If using the TruBand Anchor, cover the wells completely and center over the gel.

9. Run the gels using the following guidelines:

GEL TYPE	ELECTROPHORESIS BUFFER	VOLTAGE
Latitude HS Gels	1X HS Buffer	18 V/cm
Latitude HS Gels	1X TAE or 1X TBE Buffer	10 V/cm
Reliant HS Gels	1X HS, TAE or TBE Buffer	10 V/cm

**V/cm is determined by the total voltage divided by the interelectrode distance in cm.*

10. Remove the gel tray from the electrophoresis chamber.

11. **Carefully** remove the gel from the tray.

12. View with a 300 nm transilluminator, Clare Chemical's Dark Reader™ transilluminator or CCD imaging system.

Fast Running Protocols for High Resolution in MetaPhor® Agarose Gels

Materials

- Horizontal electrophoresis chamber to accommodate a 20 cm long gel or vertical electrophoresis chamber
- Power supply
- Recirculator-chiller water bath

Reagents

- Electrophoresis buffer (TAE or TBE)
- MetaPhor Agarose
- GelStar® or SYBR® Green Nucleic Acid Gel Stains or ethidium bromide solution

Introduction

The protocols in the following section describe how to increase resolution to a 1% size difference with DNA between 100 bp and 500 bp, and decrease your electrophoretic time to 1.5 hours.

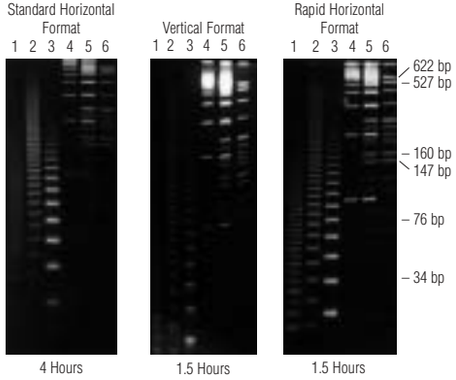
A standard horizontal submarine gel apparatus can be used to achieve resolution which is comparable to polyacrylamide gels at ≤8%. MetaPhor Agarose can also be used to achieve similar resolution in a standard vertical gel electrophoretic system.

The photographs below show the resolution achieved in 4% MetaPhor Agarose gels, using the same markers in three different formats.

Electrophoretic conditions

	VERTICAL FORMAT	RAPID HORIZONTAL FORMAT
Resolution	1% (≤500 bp)	1% (≤500 bp)
Run Time	1 - 1.5 hours	1.5 hours
Gel Concentration	3% - 4%	3% - 4%
Gel Length	16 cm - 26 cm	20 cm
Gel Thickness	1 mm	3 mm
Gel Buffer	1X TAE or 1X TBE	1X TAE or 1X TBE
Running Buffer	1X TAE or 1X TBE	0.5X TAE or 0.5X TBE
Voltage*	17 V/cm	17 V/cm
Temperature	ambient	15°C**

**V/cm is determined by the total voltage divided by the interelectrode distance in cm.*
***Circulate electrophoresis buffer with a recirculator-chiller water bath.*



Lane 1: 4 bp linker ladder; Lane 2: 8 bp linker ladder; Lane 3: 12 bp linker ladder; Lane 4: 600 ng of 100 bp ladder; Lane 5: 400 ng of pBR322/*Msp* I digest + 600 ng of 100 bp ladder; Lane 6: 400 ng of pBR322/*Msp* I digest.

Fast running protocol for horizontal gels

NOTE: This protocol cannot be used with only a peristaltic pump in the cold room; the gel will melt.

1. Prepare a 3% - 4% MetaPhor® Agarose gel in 1X electrophoresis buffer.
2. Cast a 3 mm thick, 20 cm long agarose gel.
3. Allow the gel to solidify at room temperature.
4. Place at 4°C for 30 minutes.
5. Place the gel in the electrophoresis chamber.
6. Use 0.5X TBE or 1X TAE running buffer in the electrophoresis chamber.
7. Add the running buffer to a depth of 3 mm over the surface of the gel.
8. Load 20 ng - 50 ng of DNA. If this cannot be estimated, load varying amounts of the sample. The sharpest bands are seen with small (5 µl - 10 µl) loading volumes.
9. Run the gel at 17 V/cm (interelectrode distance).
10. When the sample has left the well and moved into the gel, begin recirculating the electrophoresis buffer.
11. Chill and circulate the electrophoresis buffer with a recirculator-chiller water bath.
12. Run the gel for approximately 1.5 hours.

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Troubleshooting

The fast running protocol will not work if the buffer is not chilled or recirculated. The photograph below shows the effect of DNA resolution on running the gel in a cold room (4°C), without buffer recirculation.

Fast running protocol for vertical gels

1. Cast a vertical agarose gel in 1X electrophoresis buffer following the Vertical Gel Casting Instructions in Chapter I.
2. Carefully flush the wells with running buffer.
3. Load 20 ng - 50 ng of DNA in the band of interest. If this cannot be estimated, load varying amounts of the sample. The sharpest bands are seen with a small (5 µl - 10 µl) loading volume.
4. Run the gel at 17 V/cm interelectrode distance.
5. Run the gel approximately 1 - 1.5 hours.

Msp I digest of pBR322 (New England Biolabs), 400 ng/lane and Cambrex's 100 bp DNA Ladder, 600 ng/lane run on a 4% MetaPhor® Agarose gel prepared in 1X TAE buffer. The gel was run at 17 V/cm in 0.5X TAE buffer for 90 minutes in the cold room without buffer recirculation.

NOTE: The gel melted and was allowed to resolidify prior to staining.

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Chapter III

Detection of DNA in Agarose Gels

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Detecting DNA with GelStar®, SYBR® Green I or II Nucleic Acid Gel Stains

Materials

- Clear polypropylene container (e.g., Rubbermaid® recycling #5 plastics)
- GelStar Photographic Filter (Wratten® #9 equivalent) or SYBR Green Photographic Filter (Wratten® #15 equivalent)
- Microcentrifuge
- UV transilluminator, Dark Reader™ transilluminator or CCD imaging system

Reagents

- Buffer between pH 7.5 - 8.5 (TAE, TBE or TE)
- GelStar or SYBR Green I or II Stain stock solution

Caution

GelStar and SYBR Green Nucleic Acid Gel Stains should be handled with care and disposed of properly. Gloves should be worn when handling solutions of this dye and stained gels. Avoid skin and eye exposure to UV light.

Introduction

GelStar and SYBR Green Nucleic Acid Gel Stains are highly sensitive fluorescent stains for detecting nucleic acids in agarose gels. Unlike ethidium bromide, these stains fluoresce only upon binding to nucleic acids. Background staining is minimal and destaining of gels is not required. GelStar Stain gives high detection sensitivity for double-stranded or single-stranded DNA and RNA. SYBR Green I Stain exhibits a preferential affinity for double-stranded nucleic acids and SYBR Green II Stain is most sensitive for single-stranded nucleic acids.

Advantages

High sensitivity – 25 - 100 times more sensitive than ethidium bromide.

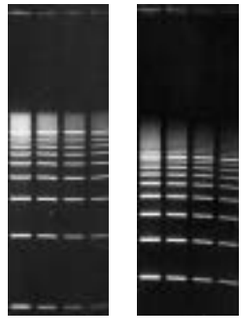
Flexible – Add GelStar Stain directly to the agarose solution or post stain your gel with GelStar or SYBR Green Stains.

Fast – No destaining required.

The table below compares the detection sensitivities of several commonly used staining methods. Samples detected with SYBR Green Stains were post-stained with the dye. Samples detected with ethidium bromide or GelStar Stain were detected by in-gel staining. Detection limits were determined by using DNA samples that were serially diluted and by recording the lowest amount that gave a visible band on photographs. Photographic conditions were varied as needed to use the longest exposure time possible without increasing gel background to an unacceptable level.

Stain and Method	ssDNA	dsDNA
GelStar Stain – in gel	25 pg	20 pg
Ethidium bromide, no destain	1.25 ng	350 pg
Ethidium bromide, destain	350 pg	100 pg
SYBR Green I or II Stain	60 pg	20 - 30 pg

The photographs below demonstrate the detection sensitivity of GelStar® Nucleic Acid Gel Stain when used in-gel or when gels have been post-stained.



Cambrex's 500 bp DNA Ladder was separated on 1% SeaKem® GTG® Agarose gels 20 cm long, 4 mm thick, run in 1X TBE buffer (Prepared from Cambrex's AccuGENE® 10X TBE Buffer) at 6 V/cm for 3 hours. GelStar® Stain was diluted 1:10,000 and added directly to the agarose or the gel was post stained for 30 minutes in a 1:10,000 dilution of GelStar Stain in buffer. Lane 1: 10 ng DNA/band; Lane 2: 5 ng DNA/band; Lane 3: 2.5 ng DNA/band; Lane 4: 1.25 ng DNA/band.

Tips for staining gels with GelStar® or SYBR® Green I Stain

Follow the guidelines below to increase the detection sensitivity of GelStar or SYBR Green Stains.

- New clear polypropylene containers (e.g., Rubbermaid® recycling #5 plastics) should be obtained for use with GelStar and SYBR Green Stains. When stored in the dark in polypropylene containers, the diluted stain can be used for up to 24 hours and will stain 2 to 3 gels with little decrease in sensitivity. The containers should be rinsed with distilled water (do not use detergents) after each use and dedicated to GelStar or SYBR Green Stain use only.
- These stains bind to glass and some non-polypropylene (polystyrene) plastics resulting in reduced or no signal from the nucleic acid.
- A 1X working solution of GelStar or SYBR Green Stain should be prepared just prior to use from the 10,000X stock solution by diluting in a pH 7.5 to 8.5 buffer (e.g., TAE, TBE or TE).
- Agarose gels should be cast no thicker than 4 mm. As gel thickness increases, diffusion of the stain into the gel is decreased, lowering the efficiency of DNA detection.
- Optimal sensitivity for GelStar and SYBR Green Stains is obtained by using the appropriate photographic filters for each stain.
- GelStar Stain: Wratten® or Tiffen® #9 filter

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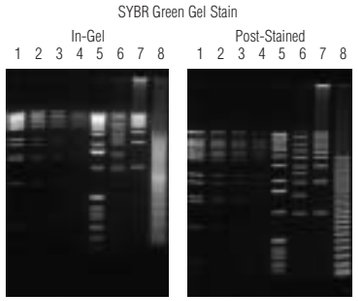
- SYBR® Green Stains: Wratten® or Tiffen® #15 filter
- We do not recommend photographing gels with a 254 nm transilluminator. An outline of the UV light source may appear in photographs. A filter that will allow a 525 nm transmission and exclude infrared light is required.

Procedure for staining DNA with GelStar® or SYBR® Green Stains

For optimal resolution, sharpest bands and lowest background, stain the gel with GelStar or SYBR Green Stain following electrophoresis. Alternatively, GelStar Stain can be included in the agarose gel.

It is not recommended to include SYBR Green Stains in the agarose gel. When the dye is incorporated into the agarose, the gel is more sensitive to DNA overloading, and the electrophoretic separation of DNA may not be identical to that achieved with ethidium bromide.

The photograph below demonstrates the effect of adding SYBR Green I Stain to the agarose.



DNA Markers were separated on 1% SeaKem® Gold Agarose gels 10-cm-long, 3 mm thick, run in 1X TBE buffer for 1 - 1.5 hours. SYBR® Green I Stain was diluted 1:10,000 and added directly to the agarose or the gel was post stained for 30 minutes in a 1:10,000 dilution of SYBR Green Stain in buffer. Lanes 1 - 4: BstE II digest of lambda DNA; 1:2 dilutions with an initial loading of 25 ng/lane. Lane 5: 1 kb DNA ladder (GIBCO-BRL, Life Technologies); 200 ng/lane (SYBR Green I stained gel); 1 µg/lane (Ethidium bromide stained gel). Lane 6: Cambrex's 1-10 kb DNA Ladder; 100 ng/lane. Lane 7: Gensura's 1 kb DNA Ladder; 100 ng/lane. Lane 8: Cambrex's 100 bp DNA Ladder; 100 ng/lane.

Follow the steps below to stain DNA after electrophoresis

1. Remove the concentrated stock solution of GelStar® or SYBR® Green Stain from the freezer and allow the solution to thaw at room temperature.
2. Spin the solution in a microcentrifuge to collect the dye at the bottom of the tube.
3. Dilute the 10,000X concentrate to a 1X working solution (1 ml per 10 ml), in a pH 7.5 - 8.5 buffer, in a clear plastic polypropylene container. Prepare enough staining solution to just cover the top of the gel.
4. Remove the gel from the electrophoresis chamber.
5. Place the gel in staining solution.
6. Gently agitate the gel at room temperature.
7. Stain the gel for 15 - 30 minutes.
   The optimal staining time depends on the thickness of the gel, concentration of the agarose, and the fragment size to be detected. Longer staining times are required as gel thickness and agarose concentration increase.
8. Remove the gel from the staining solution and view with a 300 nm UV transilluminator, CCD camera or Clare Chemical's Dark Reader™ transilluminator.
   GelStar and SYBR Green stained gels do not require destaining. The dyes fluorescence yield is much greater when bound to DNA than when in solution.

Follow this procedure when including GelStar Stain in the agarose gel.

Alternatively, Cambrex offers Reliant® and Latitude® HS Precast Agarose Gels that include GelStar PC Stain.

1. Remove the concentrated stock solution of GelStar Stain from the freezer and allow the solution to thaw.
2. Spin the solution in a microcentrifuge tube.
3. Prepare the agarose solution (See Chapter I).
4. Once the agarose solution has cooled to 70°C, add the stain by diluting the stock 1:10,000 into the gel solution prior to pouring the gel (1 ml per 10 ml).
5. Slowly swirl the solution.
6. Pour the gel into the casting tray (See Chapter I).
7. Load your DNA onto the gel.
8. Run the gel.
9. Remove the gel from the electrophoresis chamber.
10. View with a 300 nm UV transilluminator, CCD camera or Clare Chemical's Dark Reader™ transilluminator.
    GelStar stained gels do not require destaining. The dye's fluorescence yield is much greater when bound to DNA than when in solution.

Staining vertical gels with GelStar® and SYBR® Green stains

Incorporating GelStar and SYBR Green Stains into the gel or prestaining the DNA for use in a vertical format is not recommended. The dye binds to glass or plastic plates and DNA may show little to no signal. Gels should be post-stained as described in the previous section.

Follow this procedure when staining vertical gels with GelStar or SYBR Green Stain

1. Remove the concentrated stock solution of GelStar or SYBR Green Stain from the freezer and allow the solution to thaw at room temperature.
2. Spin the solution in a microcentrifuge to collect the dye at the bottom of the tube.
3. Dilute the 10,000X concentrate to a 1X working solution, in a pH 7.5 - 8.5 buffer, in a clear plastic polypropylene container. Prepare enough staining solution to just cover the top of the gel.
4. Remove the gel from the electrophoresis chamber.
5. Open the cassette and leave the gel in place on one plate.
6. Place the plate, gel side up in a staining container.
7. Gently pour the stain over the surface of the gel.
8. Stain the gel for 5 - 15 minutes.
9. View with a 300 nm UV transilluminator, CCD camera or Clare Chemical's Dark Reader™ transilluminator.
   GelStar or SYBR Green stained gels do not require destaining. The dye's fluorescence yield is much greater when bound to DNA than when in solution.

Visualization by Photography

Gels stained with GelStar and SYBR Green Stains exhibit negligible background fluorescence, allowing long film exposures when detecting small amounts of DNA. Use the appropriate photographic filter for the stain you are using.

The table below provides suggested film types and photographic conditions

Polaroid® Film	f-stop	Exposure time
Type 57 or 667	4.5	0.5 - 2 seconds
Type 55	4.5	15 - 45 seconds

Visualization by image capture system

GelStar® and SYBR® Green Stains are compatible with most CCD and video imaging systems. Due to variations in the filters for these systems you may need to purchase a new filter. Cambrex does not sell filters for this type of camera. Contact your systems manufacturer and using the excitation and emission information listed they can guide you to an appropriate filter.

Stain	Emission (nm)	Excitation (nm)
GelStar Stain	527	493
SYBR Green I Stain	521	494
SYBR Green II Stain	513	497

Application notes

- The fluorescent characteristics of GelStar and SYBR Green Stains make them compatible with argon ion lasers.
- These stains are removed from double-stranded DNA by standard procedures for ethanol precipitation of nucleic acids.
- Gels previously stained with ethidium bromide can subsequently be stained with GelStar or SYBR Green Stain following the standard protocol for post-staining. There will be some decrease in sensitivity when compared to a gel stained with only GelStar or SYBR Green Stain.
- The inclusion of GelStar and SYBR Green Stains in cesium chloride density gradient plasmid preparations is not recommended. The effect of the dye on the buoyant density of DNA is unknown.
- These stains do not appear to interfere with enzymatic reactions.
- We recommend the addition of 0.1% to 0.3% SDS in the prehybridization and hybridization solutions when performing Southern blots on gels stained with these dyes.
- Double-stranded DNA-bound GelStar or SYBR Green Stain fluoresces green under UV transillumination. Gels that contain DNA with single-stranded regions may fluoresce orange rather than green.

Decontamination

Staining solutions should be disposed of by passing through activated charcoal followed by incineration of the charcoal. For absorption on activated charcoal, consult Sambrook, *et al.*, pp. 6.16 - 6.19, (1989). Solutions can also be passed through Schleicher & Schuell®'s S&S® Extractor™ Ethidium Bromide Waste Reduction System, followed by the incineration of the filter.

Detecting DNA with Ethidium Bromide

Materials

- **Staining vessel larger than gel**
- **UV transilluminator**
- **Magnetic stir plate**
- **Magnetic stir bar**

Reagents

- **Ethidium bromide stock solution (10 mg/ml)**
  - 1.0 g Ethidium bromide
  - 100 ml distilled water
  - Stir on magnetic stirrer for several hours
  - Transfer the solution to a dark bottle
  - Store at room temperature
- **Electrophoresis buffer or distilled water**

Caution

Ethidium bromide is a powerful mutagen. Labcoat, safety glasses and gloves should be worn when handling solutions of this dye and stained gels. Also, avoid skin and eye exposure to UV light; see Sambrook *et al.*, p. 6.19 (1989).

Introduction

Ethidium bromide is a fluorescent dye which detects both single- and double-stranded DNA. However, the affinity for single-stranded DNA is relatively low compared to double-stranded DNA. Ethidium bromide contains a planar group which intercalates between the bases of DNA and, when bound to DNA, results in an increase in fluorescence yield. Ethidium bromide-stained DNA is detected by ultraviolet radiation. At 254 nm, UV light is absorbed by the DNA and transmitted to the dye; at 302 nm, and 366 nm, UV light is absorbed by the bound dye itself. In both cases, the energy is re-emitted at 590 nm in the red-orange region of the visible spectrum.

Procedure

For optimal resolution, sharpest bands and lowest background, stain the gel with ethidium bromide following electrophoresis.

Ethidium bromide can also be included in the gel and electrophoresis buffer (0.5 µg/ml) with only a minor loss of resolution. The electrophoretic mobility of DNA will be reduced by approximately 15%.

Follow the steps below to stain DNA after electrophoresis

1. Prepare enough working solution of ethidium bromide. (0.5 - 1 µg/ml of ethidium bromide in distilled water or gel buffer) to cover the surface of the agarose gel.
2. Remove the gel from the electrophoresis chamber.
3. Submerge the gel for 20 minutes in the ethidium bromide solution.
4. Remove the gel from the solution.
5. Submerge the gel for 20 minutes in a new container filled with distilled water.
6. Repeat in fresh distilled water.
7. Gels can be viewed with a hand-held or tabletop UV light. For gel concentrations of 4% or greater, these times may need to be doubled. If after destaining the background is still too high, continue to destain.

Follow the steps below when including ethidium bromide in the agarose gel

1. Prepare agarose solution (see Chapter I).
2. While the agarose solution is cooling, add ethidium bromide to a final concentration of 0.1 to 0.5 µg/ml to the solution.
3. Gently swirl the solution.
4. Pour the gel into the casting tray (see Chapter I).
5. Add ethidium bromide to the running buffer to a final concentration of 0.5 µg/ml.
6. Load and run the gel (see Chapter II).
7. Destain the gel by submerging the gel in distilled water for 20 minutes.
8. Repeat in fresh distilled water.
9. Gels can be viewed with a hand-held or tabletop UV light during or after electrophoresis.

Decontamination of ethidium bromide solutions

Decontamination of ethidium bromide solutions is described in Sambrook, *et al.*, pp. 6.16 - 6.17 (1989).

Solutions can also be passed through Schleicher & Schuell®'s S&S® Extractor™ Ethidium Waste Reduction System, followed by the incineration of the filter.

Detecting DNA with Silver Stain

Materials

- GelBond Film
- Forced hot air oven
- Staining vessels larger than the gel
- Glass plate larger than the gel (must be clean)
- Blotting paper
- Rubber roller or tissue
- Magnetic stir plate
- Magnetic stir bar
- Clamps or clips

Caution

Wear the necessary protective equipment and follow your site's guidelines for disposal of silver solutions.

Introduction

Silver staining of DNA in agarose gels will yield a sensitivity similar to ethidium bromide staining. If it is necessary to detect low levels of DNA (<1 ng), the use of GelStar® or SYBR® Green I Nucleic Acid Gel stain is preferable.

Gel preparation and electrophoresis

For easy handling and to prevent curling during drying, cast the gel on GelBond® Film.

1. Cut GelBond Film to the inner dimension of your casting tray.
2. Spread a few drops of distilled water on the surface of the casting tray.
3. Lay the cut sheet of GelBond Film onto the casting tray with the hydrophilic side up.
4. Cover the film with a sheet of blotting paper and firmly roll with a rubber roller or wipe with tissues to squeeze out any air bubbles and excess fluid from behind the film.
5. Wipe off any excess liquid at the edges.
6. Cast a gel ≤3 mm thick; thin gels are necessary for low background.
7. Electrophorese DNA following your standard protocol.
8. Dry the agarose gel (see Chapter IX).

Silver staining

NOTE: If using Bio-Rad®'s Silver Stain Plus Kit, follow the manufacturer's instructions.

1. Fix the gels for 30 minutes in the Fixative solution.
2. Wash the gel three times in distilled water for 20 minutes.
3. Vigorously stir Solution A on a magnetic stir plate.
4. To prepare the staining solution, add an equal volume of Solution B to Solution A.
5. Place the staining solution in a staining vessel.
6. Place the gel into the staining solution.
7. Allow the gel to stay in the stain until the bands appear.
8. Place the gel in the Stop solution for 5 minutes.
9. Rinse the gel in distilled water.
10. Allow the gel to air dry.

Reagents

- **Distilled water**
- **Bio-Rad Silver Stain Plus Kit OR**
- **Fixative Solution**
  - 500 ml Methanol
  - 120 ml Glacial acetic acid
  - 50.0 g Glycerol
  - Bring volume to 1 liter with distilled water
  - Prepare fresh
- **Solution A**
  - 50.0 g Sodium carbonate
  - Bring volume to 1 liter with distilled water
  - Stable for 2 to 3 weeks at room temperature
- **Solution B**
  - 2.0 g Ammonium nitrate
  - 2.0 g Silver nitrate
  - 10.0 g Tungstosilic acid (Sigma Chemical Co. catalog # T2786)
  - 8.0 ml 37% Formaldehyde
  - Bring volume to 1 liter with distilled water
  - Stable for 1 week when stored in the dark at room temperature.
- **Stop Solution**
  - 1% Glacial acetic acid in distilled water
  - Prepare fresh

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Notes

Chapter IV

Blotting DNA from Agarose Gels

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Tips for Increasing Blotting Efficiency

Recommended agaroses for Southern blotting

When transferring DNA from an agarose gel to a membrane, a standard melting temperature agarose should be used. Standard melting temperature agaroses have more gel strength than low melting temperature agaroses and do not fracture during transfer. The table below is a list of Cambrex Agaroses that are recommended for blotting.

AGAROSE	SIZE RANGE (BP)
SeaKem® LE Agarose	100 - 23,000
NuSieve® 3:1 Agarose	10 - 1,000
SeaKem GTG® Agarose	100 - 23,000
SeaKem Gold Agarose	400 - 50,000

In addition to the agaroses listed above, Cambrex offers Reliant® and Latitude® Precast Agarose Gels that are suitable for Southern blotting.

Tips for agarose gel preparation

- Use a standard melting temperature agarose.
- Avoid casting thick gels (>4 mm) unless absolutely necessary. Thick gels not only require longer electrophoretic times but may interfere with the free transfer of nucleic acids to the hybridization membrane.
- Level your gel tray before casting the gel. This ensures a uniform gel surface. Uneven gel surfaces may produce non-uniform Southern transfers.
- Submerge the gel in electrophoresis buffer as soon as the agarose solution has completely solidified. Submersion immediately after solidification prevents the formation of an impermeable skin on the surface of the gel due to evaporation of water from the gel.

Choosing the appropriate membrane

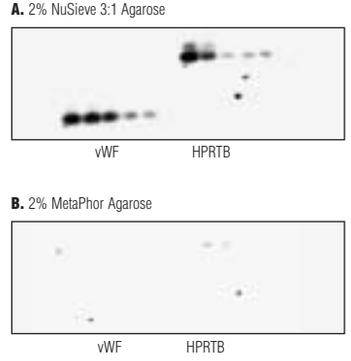
NYLON (CHARGED AND UNCHARGED)	NITROCELLULOSE
High strength	Low strength
Several reprobings	Minimal reprobings
Good binding of DNA <300 bp	Poor binding of DNA <300 bp
UV cross-linking	No UV cross-linking
Higher background	Lower background
Stable if baked	Becomes brittle if baked

Depurination

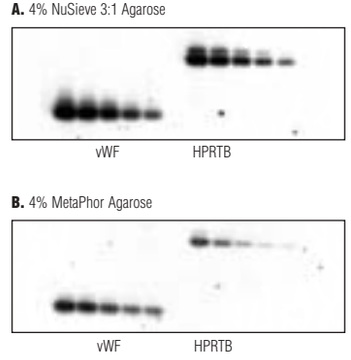
If the DNA fragments to be transferred are larger than 15 kb, treat the gel with 0.25 N HCl for 15 - 30 minutes, then rinse well before alkali treatment. Alternatively, ethidium bromide stained DNA can be photonicked with controlled exposure to UV light. Depurination is neither necessary nor recommended if the DNA fragments to be transferred are smaller than 15 kb.

The figures below (left) compare the DNA transfer from a 2% NuSieve® 3:1 Agarose gel (A) and a 2% MetaPhor® Agarose gel (B) following a 1.5 hour transfer using standard capillary alkaline transfer techniques. All five bands of both the smaller and larger PCR† fragment are visible on the blot from the NuSieve 3:1 Agarose gel, whereas very little product is visible on the blot from the 2% MetaPhor Agarose gel.

The figures below (right) demonstrate the signal from DNA transferred from a 4% NuSieve 3:1 Agarose gel (A) and a 4% MetaPhor (B) Agarose gel, following an overnight alkaline transfer. All five bands from each PCR† product are visible in each gel, but the transfer efficiency is greater with the NuSieve 3:1 Agarose gel than with the MetaPhor Agarose gel.



Lumigraph® film of DNA transferred from a 2% NuSieve® 3:1 Agarose gel (A) and a 2% MetaPhor® Agarose gel (B). Transfer time was 1.5 hours and was performed with a standard alkaline capillary technique. The five bands on the left of each blot are 150 bp PCR† fragments, and the 5 bands to the right are 300 bp PCR† fragments.



Lumigraph® film of DNA transferred from a 4% NuSieve® 3:1 Agarose gel (A) and a 4% MetaPhor® Agarose gel (B). Transfer time was 16 hours and was performed following a standard alkaline capillary technique. The five bands on the left of each blot are 150 bp PCR† fragments, and the 5 bands to the right are 300 bp PCR† fragments.

Southern blotting after alkaline gel electrophoresis

After Alkaline gel electrophoresis, the gel must be neutralized prior to transfer. Gels should be neutralized by soaking in a solution of 1.0 M Tris-HCl, pH 7.6, 1.0 M NaCl for 1 hour.

Southern blotting high concentration agarose gels

Southern transfer of DNA <1,000 bp is commonly done to detect DNA that is not visible by ethidium bromide staining, confirm the identity of PCR† products by hybridization or detect sequence tagged sites. Electrophoresis of these smaller DNA fragments requires the use of higher percentage agarose gels than those used when separating larger DNA fragments. As the agarose concentration increases, Southern transfer conditions become more critical.

For routine Southern transfers of small DNA, NuSieve® 3:1 Agarose is preferred over MetaPhor® Agarose. Downward blotting techniques (e.g., S&S® Turboblotter™ transfer system) can also be used to increase transfer efficiency.

MetaPhor Agarose is suitable for standard capillary transfer of DNA less than 300 bp in size, or when a high transfer efficiency is not required. For efficient transfer of the widest range of small-sized DNA fragments in this agarose, use semi-dry or tank electroblotting.

Alkaline Blotting onto a Nylon Membrane

Procedure for gel preparation

1. Cast a standard melting temperature agarose gel no thicker than 4 mm.
2. Electrophorese DNA following standard protocols.
3. Stain and photograph the gel.
4. Rinse the gel in distilled water.
5. Place the gel in a clean glass dish.
6. Optional step. If omitted go to step 7.
   - **6A.** Submerge the gel in 10 volumes of Depurination solution (0.25 N HCl).*
   - **6B.** Shake slowly on a shaker for 30 minutes at room temperature.
   - **6C.** Pour off the Depurination solution.
   - **6D.** Rinse the gel with distilled water.
7. Follow the guidelines below for further gel treatment:

IF the membrane is . . .	THEN soak with 10 volumes of Denaturing solution . . .
Charged nylon	0.4 M NaOH
Uncharged nylon	0.25 M NaOH

8. Shake slowly on a platform shaker for 20 minutes at room temperature.
9. Pour off the solution.
10. Proceed with transfer setup.

*This step results in partial depurination of the DNA fragments; it is not necessary for DNA fragments less than 15 kb.

Materials

- Positively charged or uncharged nylon membrane
- Glass dish larger than gel
- Oblong sponge (natural or synthetic) larger than the gel being blotted (wash with distilled water prior to use)
- Whatman® 3MM chromatography paper
- Plastic wrap
- UV transilluminator
- Paper towels
- Glass plate
- Orbital or rocking platform shaker
- <500.0 g weight
- Glass or plastic pipette

Reagents

- **Depurination solution**
  0.25 N HCl
- **Denaturing solution**
  0.4 M NaOH (for charged membrane)
  0.25 M NaOH (for uncharged membrane)
- **Transfer buffer**
  0.4 M NaOH (for charged membrane)
  0.25 M NaOH/1.5 M NaCl (for uncharged membrane)
- 2X SSC
- Distilled water

Caution

Wear gloves and protective equipment to avoid exposure to ethidium bromide.

Procedure for setting up the transfer

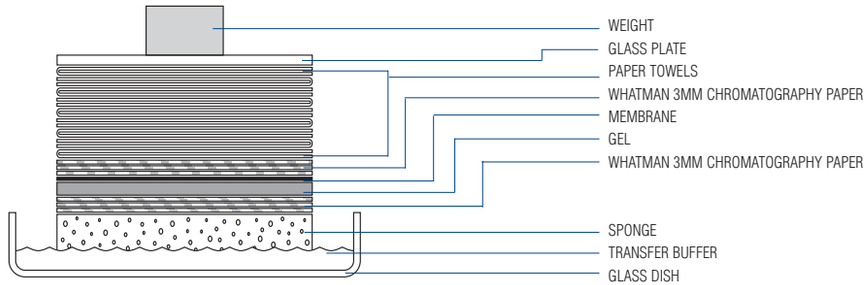
NOTE: See figure that follows for transfer setup using a sponge. See figure in High-Salt buffer section for transfer setup using Whatman® 3MM chromatography paper wick method.

1. Place the sponge in the glass dish.
2. Add Transfer buffer to the dish until the sponge is half submerged in buffer.
3. Cut three pieces of Whatman 3MM chromatography paper to the same size as the sponge.
4. Place the papers on top of the sponge.
5. Wet the papers with Transfer buffer.
6. Place the gel on the chromatography paper with the underside of the gel facing up.
7. Squeeze out any air bubbles between the Whatman 3MM chromatography paper and the gel by rolling a pipette over the surface of the gel.
8. Cut four pieces of plastic wrap. Each piece should be 2 cm wide and slightly longer than the four sides of the gel.
9. Place a piece of cut plastic wrap around each edge of the gel. This prevents the buffer from flowing around the gel rather than through the gel.
10. Cut the membrane large enough to cover the exposed surface of the gel and slightly overlap the plastic. Transfer membranes should be handled at the edges with gloves worn.
11. Treat the membrane following the guideline below:

IF the membrane is . . .	THEN . . .
Charged nylon	do not prewet
Uncharged nylon	wet in 0.25 M NaOH/1.5 M NaCl

12. Place the membrane on the surface of the gel.
13. Remove bubbles from between the membrane and the gel by gently rolling the pipette over the membrane.
14. Flood the surface of the membrane with Transfer buffer.
15. Cut 5 sheets of Whatman 3MM chromatography paper to the same size as the membrane.
16. Place the sheets on top of the membrane.
17. Cut paper towels to the same size as the membrane.
18. Stack the paper towels over the Whatman® 3MM chromatography paper to a height of 15 cm.
19. Lay a glass plate on top of the structure.
20. Place a weight on top to hold the assembly in place.
21. Leave to transfer for at least 2 hours. Alkaline transfer can also be performed following the procedure for downward capillary blotting (page 64).

Sponge method for transfer setup



Disassembly of the transfer setup

1. Remove the weight, glass plate, paper towels and filter papers.
2. Mark the wells of the gel on the membrane with a pencil for orientation.
3. Recover the membrane.
4. Rinse the membrane in 2X SSC.
5. Place the membrane on a sheet of Whatman 3MM chromatography paper.
6. Dry the membrane at room temperature.

Southern Blotting with High-Salt Buffer

Tips for setting up the blotting assembly

- Thoroughly wet the wicking paper, the membrane and the sheets of filter paper in distilled water, followed by equilibration in Transfer buffer.
- The blotting membrane should be in contact with the underside of the gel. Not only will contact with the gel be better, most of the DNA in the gel is concentrated toward the underside of the gel.
- Do not use more than a 500.0 g weight on the top of the stack. Excessive weight may increase gel compression and reduce capillary action. This will retard the blotting process and may result in incomplete transfer.
- Use the Transfer buffer recommended by the manufacturer of the selected membrane.

Blotting times

The time required for an efficient transfer depends on the gel thickness, the concentration of agarose, and the size of the fragments to be transferred. Although a few hours may be sufficient for a complete transfer, certain conditions require additional time.

Allow 15 - 20 hours for blotting if you are:

- Using gels thicker than 4 mm
- Using agarose concentrations >2%
- Detecting single-copy genes
- Transferring DNA fragments >15 kb

To improve the transfer efficiency during prolonged blotting, replace the wet toweling after it becomes saturated.

Blotting times can be shortened by replacing the stacking towels with commercially available, high-absorbent blotting pads.

Procedure for gel preparation

1. Cast a standard melting temperature agarose gel no thicker than 4 mm.

2. Electrophorese DNA following standard protocols.

3. Stain and photograph the gel.

4. Rinse the gel in distilled water.

5. Place the gel in a clean glass dish.

6. Optional step. If omitted go to Step 7.

6A. Submerge the gel in 10 volumes of Depurination solution.*

6B. Shake slowly on a shaker for 30 minutes at room temperature.

6C. Pour off the Depurination solution.

6D. Rinse the gel with distilled water.

7. Add 10 volumes of Denaturation solution.

8. Shake slowly on a platform shaker for 20 minutes at room temperature.

9. Remove solution and replace with a fresh 10 volumes of Denaturing solution.

10. Shake slowly on a platform shaker for 20 minutes at room temperature.

11. Pour off Denaturation solution.

12. Rinse the gel with distilled water.

13. Add 10 volumes of Neutralization solution.

14. Shake slowly on a platform shaker for 20 minutes at room temperature.

15. Remove solution and replace with a fresh 10 volumes of Neutralizing solution.

16. Shake slowly on a shaker for 20 minutes at room temperature.

*This step results in partial depurination of the DNA fragments; it is not necessary for DNA fragments less than 15 kb.

Materials

- **Nitrocellulose or charged nylon membrane**
- **Glass dish larger than gel**
- **Glass plate to serve as a platform for the gel**
- **Whatman® 3MM chromatography paper**
- **Plastic wrap**
- **UV transilluminator**
- **Paper towels**
- **Glass plate**
- **<500.0 g weight**
- **Orbital or rocking platform shaker**
- **Glass or plastic pipette**

Reagents

- **Depurination solution**
  0.25 N HCl
- **Denaturation solution**
  1.5 M NaCl
  0.5 M NaOH
  Store at room temperature
- **Neutralization solution**
  1.5 M NaCl
  0.5 M Tris-Cl, pH 7.5
  Store at room temperature
- **Transfer buffer**
  20X SSC; dilute as recommended by membrane manufacturer
- **Distilled water**

Caution

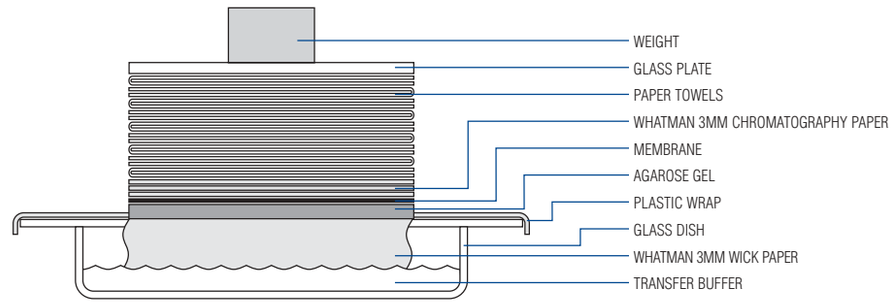
Wear gloves and protective equipment to avoid exposure to ethidium bromide.

Procedure for setting up the transfer

NOTE: See figure in Alkaline Blotting onto a Nylon Membrane section for transfer setup using a sponge or see figure on next page for using the Whatman® 3MM chromatography paper wick method.

1. Fill the dish with Transfer buffer until it is half full.
2. Cut a piece of Whatman 3MM chromatography paper so it will fit on the platform with both ends in the solution.
3. Place the glass plate across the dish to serve as a platform.
4. Wet the chromatography paper with Transfer buffer.
5. Lay the chromatography paper on the platform with both ends in the Transfer buffer.
6. Place the gel on the chromatography paper with the underside of the gel facing up.
7. Squeeze out any air bubbles between the Whatman 3MM chromatography paper and the gel by rolling a pipette over the surface of the gel.
8. Cut four pieces of plastic wrap. Each piece should be 2 cm wide and slightly longer than the four sides of the gel.
9. Place a piece of cut plastic wrap around each edge of the gel. This prevents the buffer from flowing around the gel.
10. Cut the membrane large enough to cover the exposed surface of the gel and slightly overlap the plastic. Transfer membranes should be handled at the edges with gloves worn.
11. Submerge the membrane in distilled water for 5 minutes. If using a nitrocellulose membrane, remove from distilled water and submerge for 5 minutes in Transfer solution.
12. Place the membrane on the surface of the gel.
13. Remove bubbles from between the membrane and the gel by gently rolling the pipette over the membrane.
14. Flood the surface of the membrane with Transfer buffer.
15. Cut 5 sheets of Whatman 3MM chromatography paper to the same size as the membrane.
16. Place the sheets on top of the membrane.
17. Cut paper towels to the same size as the membrane.
18. Stack the paper towels over the Whatman® 3MM chromatography paper to a height of 15 cm.
19. Lay a glass plate on top of the structure.
20. Place a weight on top to hold everything in place.
21. Leave in place overnight.

Whatman 3MM chromatography paper method for transfer setup



Disassembly of the transfer setup

1. Remove the weight, glass plate, paper towels and filter papers.
2. Invert the gel and membrane together.
3. Mark the positions of the wells on the membrane with a pencil for orientation then remove the gel from the transfer setup.
4. Rinse the membrane in the appropriate Transfer buffer dilution for your membrane.
5. Place the membrane on a sheet of Whatman 3MM chromatography paper.
6. Dry the membrane at room temperature.

Southern Blotting by Downward Capillary Transfer

Materials

- Nitrocellulose or charged nylon membrane
- Two glass dishes larger than gel
- Paper towels
- Whatman® 3MM chromatography paper
- UV transilluminator
- Glass or plastic pipette
- Support (see page 65)
- Orbital or rocking platform shaker
- Glass plate

Reagents

- Depurination solution
  0.25 N HCl
- Reagents for Alkaline blotting (page 57)
  OR
- Reagents for High-Salt buffer (page 61)
- 2X SSC
- Distilled water

Caution

Wear gloves and protective equipment to avoid exposure to ethidium bromide.

Introduction

Disadvantages of performing upward capillary transfer are that flow goes against gravity and the weight placed on top of the gel and filter papers can crush the gel. As transfer proceeds, compression of the gel increases, reducing capillary action. This will retard the blotting process and results in a transfer that must be carried out for 16 to 24 hours.

The downward capillary transfer system does not cause excessive pressure on the gel. Transfer can be complete in as little as 1 hour. This protocol can be used with all types of membranes and high-salt or alkaline buffer.

Procedure for gel preparation

1. Cast a standard melting temperature agarose gel no thicker than 4 mm.
2. Electrophorese DNA following standard protocols.
3. Stain and photograph the gel.
4. Rinse the gel in distilled water.
5. Place the gel in a clean glass dish.
6. Optional step. If omitted go to Step 7.
   - **6A.** Submerge the gel in 10 volumes of Depurination solution.*
   - **6B.** Shake slowly on a platform shaker for 30 minutes at room temperature.
   - **6C.** Pour off the Depurination solution.
   - **6D.** Rinse the gel with distilled water.
7. Follow the guidelines below for further gel treatment.

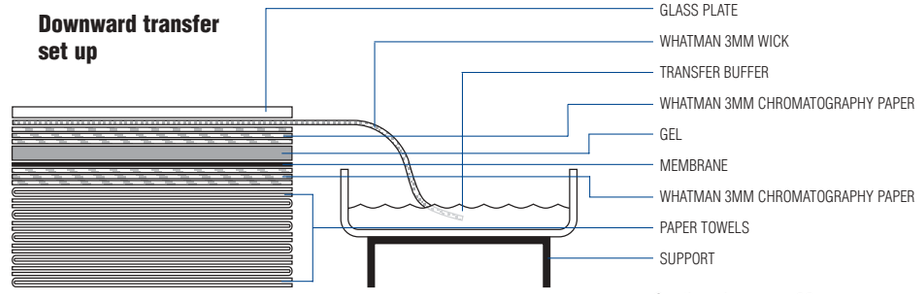
IF YOU ARE USING REAGENTS FOR . . .	THEN FOLLOW STEPS . . .
Alkaline blotting	7 - 10 on page 57
High-salt buffers	7 - 16 on page 61

*This step results in partial depurination of the DNA fragments; it is not necessary for DNA fragments less than 15 kb.

Procedure for setting up the transfer

1. Cut paper towels slightly larger than the agarose gel.
2. Stack the towels in a glass dish 2 cm - 3 cm high.
3. Cut 5 pieces of Whatman® 3MM chromatography paper to the same size as the towels.
4. Place 4 sheets of Whatman 3MM chromatography paper on top of the paper towels.
5. Wet 1 piece of chromatography paper in Transfer buffer.
6. Place the wetted paper on top of the other sheets.
7. Place the membrane on the top filter paper.
8. Remove bubbles between the filter paper and the membrane by rolling a pipette over the surface of the membrane.
9. Place the gel on the membrane so that the underside of the gel is in contact with the membrane.
10. Remove bubbles between the gel and the membrane.
11. Cut 3 pieces of Whatman 3MM chromatography paper to the same size as the gel.
12. Wet the chromatography paper with Transfer buffer.
13. Place the chromatography paper on top of the gel.
14. Place two larger pieces of Whatman 3MM chromatography paper together.
15. Soak the paper in Transfer buffer.
16. Fill a glass dish with Transfer buffer.
17. Place the glass dish containing the Transfer buffer on a support next to the stacked paper towels and gel.
18. Form a bridge with the two large pieces of Whatman 3MM chromatography paper between the glass dish containing the Transfer buffer and the Transfer pyramid.
19. Leave for 1 hour. Thick or high concentration gels may require longer transfer times.
20. Remove the paper towels and filter papers and recover membrane.

Downward transfer set up



Continued on page 66

Disassembly of Transfer setup

1. Remove the glass plate and chromatography paper.
2. Remove the gel and membrane together.
3. Mark the positions of the wells on the membrane with a pencil for orientation then remove the gel from the transfer setup.
4. Rinse the membrane in the appropriate Transfer buffer dilution for your membrane.
5. Place the membrane on a sheet of Whatman® 3MM chromatography paper.
6. Dry the membrane at room temperature.

Semi-Dry Blotting Agarose Gels

For more detailed information and protocols concerning semi-dry blotting, consult the blotting apparatus manufacturer's instructions or Ausubel, *et al.*

Materials and Reagents

- Positively charged nylon membrane
- Semi-dry blotting apparatus
- Whatman 3MM chromatography paper
- 1X TAE buffer

Procedure for semi-dry blotting

NOTE: The thinner the gel, the more efficient this process will be.

1. Run a 3 mm thick agarose gel in 1X TAE buffer following your standard procedure.
2. Soak 6 sheets of Whatman® 3MM chromatography paper in 1X TAE.
3. To make a semi-dry blotting sandwich, stack in the following order:
   - **3A.** Three sheets of soaked Whatman 3MM chromatography paper.
   - **3B.** A piece of positively charged nylon membrane.
   - **3C.** Agarose gel (underside of the gel in contact with the membrane).
   - **3D.** Three sheets of soaked Whatman 3MM chromatography paper.
4. Allow excess buffer to drain off the sandwich.
5. Load the sandwich into the semi-dry blotting apparatus.
6. Turn on the power and transfer by running at:
   - **6A.** 6 volts for 15 minutes
   - **6B.** 12 volts for 30 minutes
7. Remove the membrane from the blotting apparatus.
8. Treat the membrane as described in Immobilizing DNA on a Membrane (see page 69).

Electroblotting Agarose Gels

Materials and Reagents
- Transfer membrane
- Tank electroblot apparatus
- Whatman 3MM chromatography paper
- Glass dish
- Fiber pad
- 0.5X TBE Transfer buffer (chilled and room temperature)

For more detailed information and protocols concerning electroblotting, consult the blotting apparatus manufacturer's instructions or Ausubel, *et al*.

Procedure for electroblotting

1. Prepare 0.5X TBE Transfer buffer.
2. Chill the buffer to 4°C.
3. Run a 3 mm - 4 mm thick agarose gel following your standard procedure.
4. Remove enough cold Transfer buffer to cover the surface of the gel and place in the glass dish.
5. Equilibrate the gel in the transfer buffer for 1 hour in the cold room.
6. Soak the membrane for 20 minutes in the Transfer buffer.
7. Cut 2 pieces of Whatman® 3MM chromatography paper to the size of the gel.
8. Soak the chromatography paper in Transfer buffer.
9. Soak the fiber pads in Transfer buffer.
10. Make a sandwich of the filter paper, fiber pad and membrane following the equipment manufacturer's instructions.
11. Close the holder on the sandwich and latch.
12. Fill tank with Transfer buffer.
13. Attach to the power supply.
14. Transfer at 30 volts (constant) for 4 hours.
15. Remove the membrane from the apparatus.
16. Treat the membrane as described in Immobilizing DNA on Membrane (see page 69).

Immobilizing DNA on a Membrane

Immobilizing DNA by UV irradiation

This method is compatible with uncharged and charged nylon membranes.

Immobilizing DNA is not necessary if alkaline blotting was performed using a charged nylon membrane.

For best results with nylon membranes, we recommend baking at 80°C for 30 minutes, followed by exposure to a UV light source following the recommendations of the membrane manufacturer for exposure time and energy levels. Make sure that the side exposed to the UV light is the side containing the DNA. We also recommend that you titrate your UV source for optimal results.

Materials
- Membrane
- Plastic wrap
- UV transilluminator
- Whatman 3MM chromatography paper

Caution

Do not overexpose the membranes to UV light, as it will lower the ability of the DNA to hybridize.

1. Remove the membrane from the Whatman® 3MM chromatography paper.
2. Wrap the membrane in plastic wrap.
3. Place the wrapped membrane, DNA side down, on a UV transilluminator (254 nm wavelength).
4. Irradiate the DNA for 1 - 5 minutes.
5. Store membranes between dry sheets of Whatman 3MM chromatography paper for several months at room temperature.

Immobilizing DNA by baking

This method is compatible with nitrocellulose and nylon membranes.

Materials
- Membrane
- Vacuum gel dryer
- Whatman 3MM chromatography paper

1. Place membrane between two sheets of Whatman 3MM chromatography paper.
2. Bake under vacuum for 2 hours at 80°C.
3. Store membranes between dry sheets of Whatman 3MM chromatography paper for several months at room temperature.

References

Ausubel, F.M., *et al., Current Protocols in Molecular Biology,* Red Book, Wiley & Sons, 1995.

Chomczynski, P., *Anal. Biochem.* **201:** 134 - 139, 1991.

Sambrook. J., *et al., Molecular Cloning: A Laboratory Manual,* 2nd edition, Cold Spring Harbor Laboratory Press, 1989.

Notes

Chapter V

In-Gel Reactions

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Restriction Digestion in the Presence of Agarose 81
DNA Amplification in the Presence of Agarose 82
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Overview

Introduction

A variety of enzymatic reactions can be performed in the presence of agarose. In-gel reactions are an alternative approach to standard DNA recovery techniques and provide a multitude of benefits. The use of in-gel reactions will not only save time, but eliminate potential sample loss during DNA recovery from an agarose gel.

Advantages

- Saves time otherwise used for recovery
- Avoids recovery losses
- Avoids recovery damage, especially shearing of higher molecular weight DNA

Applications

Enzymes active in the presence of low melting temperature agaroses include:

- Alkaline phosphatase
- BAL 31 nuclease
- DNA polymerase I
- Klenow fragment
- Restriction endonucleases
- Reverse transcriptases
- T4 DNA ligase
- T4 DNA polymerase
- T4 Polynucleotide kinase
- T7 DNA ligase
- T7 DNA polymerase (Sequenase® polymerase)
- *Taq* DNA polymerase

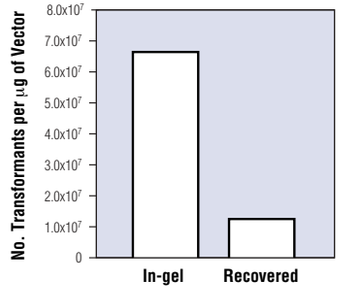
Compatible agaroses

In-gel reactions require the use of a low melting temperature (≤65°C) agarose. Cambrex has developed two high quality Genetic Technology Grade™ (GTG®) Agaroses for this application, specifically NuSieve® GTG and SeaPlaque® GTG Agarose. These agaroses are specifically designed and tested for compatibility with in-gel reactions.

- NuSieve GTG Agarose is the choice for separation of nucleic acids ≤1,000 bp with a resolving power of 4% - 6% difference in DNA size.
- SeaPlaque® GTG Agarose is the choice for separation of large nucleic acid fragments >1,000 bp.

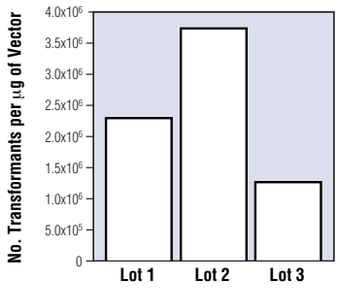
The figures below demonstrate the efficiency of in-gel cloning in our low melting temperature agaroses versus sample recovery and also the consistency between different agarose lots.

Efficiency of In-Gel Cloning from SeaPlaque GTG Agarose



Two lanes of *Bst* E II digest of lambda DNA were electrophoresed in a 1% SeaPlaque® GTG® Agarose gel prepared in 1X TAE. The 2.3 kb band was excised from each lane. One sample was ligated directly to pUC 19 in the presence of remelted agarose (In-gel) and the other sample was purified using a chaotrope-based recovery protocol (Recovered). Transformation into DH5α™ cells was done following standard protocols.

Efficiency of In-Gel Cloning from NuSieve GTG Agarose



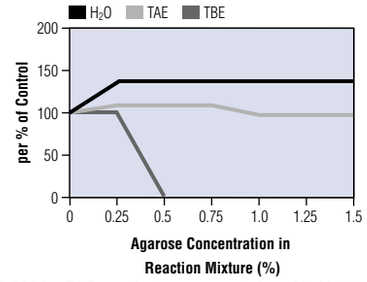
A 377 bp fragment was electrophoresed in three separate lots of a 3% NuSieve® GTG® Agarose gel in 1X TAE buffer then ligated to M13 in the presence of remelted agarose. Transformation into DH5αF′™ cells was done in remelted agarose following standard protocols. Results 1, 2 and 3 are from three separate lots of NuSieve GTG Agarose.

Tips for Increasing the Efficiency of In-Gel Reactions

Electrophoretic tips

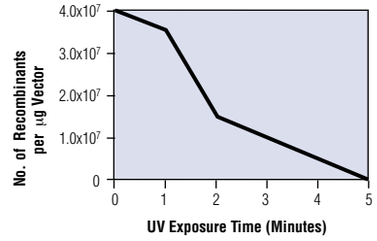
- Use a low EDTA (0.1 mM) 1X TAE buffer.
  Increases the availability of Mg^{+}, a necessary cofactor in many enzymatic reactions.
- Briefly stain the gel **after** electrophoresis with GelStar® or SYBR® Green I Nucleic Acid Gel Stain or ethidium bromide. The presence of ethidium bromide during electrophoresis can result in the degradation of DNA.
- Visualize your DNA with a UV light source that is ≥300 nm.
  Short UV wavelength light can damage DNA. Alternatively, DNA can be detected using Clare Chemical's Dark Reader™ transilluminator.
- Minimize exposure of DNA to UV light to less than 1 minute.
- The addition of 1 mM guanosine or cytidine to the gel and electrophoresis buffer is effective in protecting DNA against UV-induced damage.
  The nucleosides will not impede electrophoresis, detection or purification of DNA.
- Limit the amount of DNA in the band of interest to 100 ng if possible
  More DNA may cause smearing and a wide band will result in an unacceptably large piece of gel to be excised and melted.

Effect of Agarose Concentration and Buffer on In-Gel PCR



A 500 bp PCR† product was separated on 4% NuSieve® GTG® Agarose in either 1X TAE or 1X TBE buffer. Following electrophoresis, the PCR† product was reamplified with the same primers as in the original amplification. Relative efficiency was measured by comparing the products on an agarose gel.

Effect of UV Irradiation on In-Gel Cloning



A 2 kb fragment, separated in 1% SeaPlaque® GTG® Agarose and stained with ethidium bromide, was exposed to 300 nm UV irradiation for different lengths of time before excision from the gel, ligation into pBR322, and bacterial cell transformation. The mean of the number of white colonies per microgram vector on two plates is given.

Reaction tips

- Melt the gel slice at 65°C - 70°C. Do not exceed 70°C as this may cause melting of your DNA.
- Thoroughly mix the reaction components at 37°C - 40°C. This ensures proper mixing of DNA with reaction components.
- Do not exceed 0.5% SeaPlaque® GTG® or 1.5% NuSieve® GTG Agarose in the reaction mix.
- Increase the efficiency of blunt end ligations. The addition of larger amounts of vector, insert DNA and T4 DNA ligase or the addition of 5% (w/v) PEG-8000 to the ligation mixture and incubating the ligation reaction mixture for a longer time, can increase the efficiency of the reaction. An alternative strategy is to add linkers with sticky ends to the insert DNA.
- When transforming cells, dilute the melted ligation reaction mixture five-fold before adding to cells.
- When transforming bacterial cells, dilute the agarose containing the ligated DNA further with warmed (40°C) TAE or sterile distilled water before adding the ligation reaction to the competent cells. It is better to add 10 µl of a five-fold diluted mixture than 2 µl of a more concentrated reaction mixture. By doing this, it is less likely that your ligated DNA will be trapped in the agarose when it is added to the chilled cells. Also be sure that you do not add excessive DNA to the competent cells which can decrease the transformation efficiency.

Reference

Gründemann, D. and Schömig, E., *BioTechniques* **21:** 898 - 903, 1996.

Cloning in the Presence of Agarose

Materials

- Sterile microcentrifuge (≥1 ml) and polypropylene tubes (17 mm x 100 mm)
- Horizontal electrophoresis chamber
- Scalpel or razor blade
- Heating block or water bath
- Ice bucket and ice

Reagents

- SeaPlaque GTG or NuSieve GTG Agarose
- GelStar® or SYBR® Green I Nucleic Acid Gel Stain or ethidium bromide
- Distilled water
- T4 DNA ligase
- Competent cells
- Restriction enzymes
- Calf intestine alkaline phosphatase
- 1X TAE (0.1 mM EDTA) gel and running buffer
- Reagents for phenol extraction (See page 97)
- 10 mM Tris-HCl, pH 7.5
- Ethanol
- 10X T4 Ligation buffer

  500 mM Tris-HCl, pH 7.5
  100 mM $MgCl_2$
  50 mM Dithiothreitol
  10 mM ATP, pH 7.6
  200.0 µg/µl nuclease-free BSA (bovine serum albumin)

Timetable

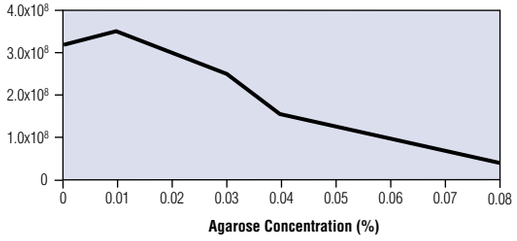
1. (Day 1) Preparation of vector. Preparation of insert. Preparation of competent cells.
2. (Day 2) Ligation reaction. Transformation reaction.
3. (Day 3) Assess the results obtained from the ligation-transformation reaction.

Tips

- Use 1X TAE buffer with 0.1 mM EDTA.
- Electrophorese the DNA without ethidium bromide in the gel.
- Minimize the exposure time of the DNA to less than 1 minute under UV light.
- Do not exceed a final concentration of 0.5% SeaPlaque® GTG® or 1.5% NuSieve® GTG Agarose in the reaction mixture.
- Dilute the agarose solution when running in-gel transformations. Do not exceed >0.02% agarose in the transformation reaction. See figure on the this page.

  NOTE: Electroporation can not be used with in-gel ligation/transformation procedures.

Effect of Agarose Concentration on Transformation



Aliquots of agarose solutions (0.25%, 0.5%, 1.0% and 2.0% in 1X TAE) were added to pUC19 monomer DNA which had been placed in labeled tubes. Aliquots (6 µl=0.01 ng DNA) of the samples containing agarose were added to 100 µl of GIBCO-BRL (LTI) frozen competent cells on ice. The agarose concentrations shown are those in the tubes of competent cells after samples were added. Transformations were carried out following standard protocol. Points shown on the graph represent the mean of three transformations.

Preparing the vector

1. Cut vector with appropriate restriction enzymes.
2. Dephosphorylate vector with calf intestine alkaline phosphatase.
3. Phenol extract vector.
4. Ethanol precipitate vector.

Preparing the insert

1. Cut DNA to be used as insert with appropriate restriction enzymes.
2. Electrophorese DNA (100 ng) in a low melting temperature agarose gel prepared in 1X TAE (0.1 mM EDTA).
3. Briefly stain DNA with GelStar® or SYBR® Green I Nucleic Acid Gel Stain or ethidium bromide.
4. Excise gel band containing insert and place in a sterile, preweighed tube.
5. Estimate the volume of the gel slice based upon the weight of the slice (e.g., 100 mg = 100 µl).
6. Determine the concentration of the DNA in the gel slice by assuming 100% recovery of DNA in the slice.
7. Store excised band at 4°C until ready for use.
8. Prepare agar plates with the appropriate selective media for the next day's use.

Preparing competent cells

If you prepare your own competent cells, set up a small overnight culture of cells. High transformation efficiencies can be obtained with frozen competent cells that are either prepared in the laboratory or purchased. Similar transformation efficiencies of 10^7 transformants/µg pUC18 DNA with insert have been obtained using the Hanahan procedure (see citation at end of section); transformation efficiencies are reduced when using the $CaCl_2$ method.

NOTE: Electroporation can NOT be used with in-gel ligation/transformation procedures.

Ligation reaction

1. Remelt the agarose slice by heating to 68°C for 10 minutes.
2. Place the remelted gel slice at 37°C until needed.
3. For a final reaction mixture of 50 µl, add the following components in the following order:

VOLUME	COMPONENT
18 µl	10 mM Tris-HCl, pH 7.5 or distilled H_20
5 µl	10X T4 DNA Ligation buffer
1 µl	Vector (amount sufficient to obtain a molar ratio of insert to vector of 3 - 4:1)
1 µl	T4 DNA ligase (between 1 - 2 units)

4. Mix the components gently with a pipette.
5. Add up to 25 µl of the remelted agarose gel slice [25 ng of insert DNA]. Do not exceed a final concentration of 0.5% SeaPlaque® GTG® or 1.5% NuSieve® GTG Agarose in the reaction mixture.
6. Mix components by resuspending with a pipette.
7. Ligate at room temperature for 2 - 3 hours.

Transformation reaction

The transformation procedure outlined below is a modification of the Life Technologies, Inc. procedure included with their DH5α™ competent cells. When using other frozen competent cells follow manufacturer's directions.

1. Prepare competent cells if working with fresh cells. If using frozen competent cells, remove cells from storage and thaw on ice.
2. Heat the ligation reaction at 68°C for 5 minutes to remelt the agarose.
3. Add competent cells into a prechilled polypropylene tube (17 mm x 100 mm). We use 100 µl of thawed DH5α cells.
4. Add 1 µl - 2 µl of diluted ligation reaction (70 pg - 140 pg vector) to competent cells.
5. Mix and incubate on ice for 30 minutes.

   NOTE: Exceeding 0.02% SeaPlaque® GTG® or NuSieve® GTG Agarose concentration in the final mixture may reduce transformation efficiencies.
6. Heat shock the cells by placing the tubes in a 42°C water bath for 45 seconds.
7. Place the cells back on ice for 2 minutes.
8. Add 0.9 ml of SOC medium to cells.
9. Incubate at 37°C with shaking for 30 - 60 minutes.
10. Spread 1 µl - 200 µl of the transformation mixture onto an agar plate with the appropriate selective medium.
11. Allow the liquid to soak in.
12. Invert the plates.
13. Incubate overnight at 37°C.

Reagents

- **SOC medium**

  2.0 g Bacto-tryptone
  0.5 g Yeast extract
  1.0 ml 1 M NaCl
  0.25 ml 1 M KCl

  Dissolve in a final volume of 100 ml distilled water; autoclave

  Add aseptically:

  1.0 ml 2 M Mg^{2+} (1 M $MgCl_2$, 1 M $MgSO_4$, filter sterilized)

  1.0 ml 2 M Glucose (filter sterilized)

(continued on page 80)

- **LB Agar Plates Supplemented with Ampicillin and X-gal (1 L)**
  - 10.0 g Tryptone
  - 5.0 g Yeast extract
  - 10.0 g NaCl
  - 15.0 g Agar
  - 800 ml distilled water
  - Stir to dissolve components
  - Adjust to pH 7.5 with NaOH
  - Adjust volume to 1 L with distilled water
  - Carefully heat while stirring until agar is dissolved
  - Remove stir bar and sterilize the medium by autoclaving 25 minutes
  - Place the flask of medium in a water bath set at 50°C and let the medium cool for at least 30 minutes
  - While medium is cooling, prepare ampicillin and X-gal stock solutions.
  - Add 1 ml of ampicillin stock and 2 ml of X-gal stock to the tempered medium and pour into plates
  - Allow plates to sit on bench overnight to dry; then package and store at 4°C
- **Ampicillin stock**
  - 100.0 mg of ampicillin to 1 ml of sterile distilled water
- **X-gal (5-bromo-4-chloro-3-indoyl-ß-D-galactoside) stock**
  - 40.0 mg of X-gal to 2 ml of dimethylformamide

Controls

We recommend the following controls be run in parallel with the test plates:

VECTOR	LIGASE	# OF BLUE COLONIES	# OF WHITE COLONIES	REASON FOR CONTROL
pUC 18-native	-	+++++	-	Cell competency test
pUC18-singly digested efficiency	-	+	-	Background for ligation
pUC18-singly digested	+	++++	-	Determines ligation efficiency
pUC18-doubly digested background	-	+	-	Unrestricted vector
pUC18-doubly digested	+	+	-	Determines the efficiency of double digestion
pUC18-native	+	+++++	-	Reagent check

Assessing the results

Count colonies and assess the results of ligation-transformation. Colonies containing recombinant plasmids should be white. Blue colonies result from nonrecombinant pUC18 plasmid.

References

Hanahan, D., *J. Mol. Biol.* **166:** 557 - 580, 1983.
Crouse, G.F., *et al., Meth. Enzymol.* **1:** 78 - 89, 1983.
Frischauf, A.M., *et al., Nucl. Acids Res.* **8:** 5541 - 5549, 1980.
Majumdar, D., *et al., BioTechniques* **7:** 188 - 191, 1989.
Murray, J.A.H., *Nucl. Acids Res.* **14:** 10118, 1986.
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Restriction Digestion in the Presence of Agarose

NOTE: The urea sample buffer prevents the samples from regelling after digestion. It will also result in good separation of the DNA during electrophoresis.

Procedure

1. Electrophorese DNA (several µg) in a low melting temperature agarose prepared in 1X TAE (0.1 mM EDTA).
2. Briefly stain the gel with GelStar® or SYBR® Green I Nucleic Acid Gel Stain or ethidium bromide.
3. Excise the gel slice containing the DNA of interest and place in a preweighed microcentrifuge tube.
4. Estimate the volume of the gel slice based upon the weight and determine the concentration of the DNA in the gel slice (e.g., 100 mg = 100 µl). Assume 100% recovery of the DNA.
5. Store excised band at 4°C until ready for use.
6. Add sterile distilled water to bring volume of the gel slice to 200 µl (200 mg).
7. Remelt the gel slice by heating to 68°C for 10 minutes.
8. Mix the melted gel slice by pipetting.
9. Remove a volume containing the quantity of DNA needed for restriction digestion.
10. Maintain the sample at 37°C to prevent gelling.
11. Dilute the restriction endonuclease with the appropriate reaction buffer.
12. Digest DNA at the appropriate temperature and time for your particular enzyme.
13. Stop the reaction by adding 30 µl of urea sample buffer to 50 µl of the digestion mixture and mixing.
14. Heat the sample to 65°C for 10 minutes if it has regelled.
15. Load the sample onto appropriate agarose gel for analysis.

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Hermann, R.G., *et al., Gene* **8:** 179 - 191, 1980.
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Peacock, A.C., *et al., Anal. Biochem.* **149:** 177 - 182, 1985.

Materials

- **Horizontal electrophoresis chamber**
- **Scalpel or razor blade**
- **Microcentrifuge tubes (≥1 ml)**
- **Water bath or heating block**

Reagents

- **SeaPlaque® GTG or NuSieve® GTG Agarose**
- **1X TAE (0.1 mM EDTA) gel and running buffer**
- **Sterile distilled water**
- **GelStar or SYBR Green I Nucleic Acid Gel Stain or ethidium bromide**
- **Restriction endonuclease(s)**
- **Urea sample buffer**
  - 8% Ficoll® (type 400) polymer
  - 27 mM EDTA
  - 0.27% Bromophenol blue
  - 5 M urea
  - Adjust to pH 8.0

DNA Amplification in the Presence of Agarose

Materials

- Horizontal electrophoresis chamber
- Scalpel or razor blade
- Vortex mixer
- Microcentrifuge
- Thin-walled microcentrifuge tube
- Microcentrifuge tubes (≥1 ml)
- Thermal cycler

Reagents

- SeaPlaque® GTG® or NuSieve® GTG Agarose
- 1X TAE (0.1 mM EDTA) gel and running buffer
- DNA amplification kit
- Forward and reverse primers
- Sterile distilled water
- GelStar or SYBR Green I Nucleic Acid Gel Stain or ethidium bromide

Procedure 1

1. Electrophorese PCR† products in low melting temperature agarose prepared in 1X TAE (0.1 mM EDTA) buffer.
2. Briefly stain the gel with GelStar® or SYBR® Green I Gel Stain or ethidium bromide.
3. Excise a gel slice containing the template DNA and place into a preweighed microcentrifuge tube.
4. Store the gel slice at 4°C protected from light until ready for use.
5. Melt the agarose gel slice containing template DNA at 65°C for 10 minutes.
6. Dilute with 65°C, sterile distilled water to a final DNA concentration of 0.1 ng/µl.
7. Add a portion of the DNA into the amplification reaction mixture (do not exceed 1 ng DNA/reaction).

Amplification Reaction Mixture

If the reaction buffer does not contain magnesium ion, add sufficient amount for your template/primer.

COMPONENT	VOLUME (µL)	FINAL CONCENTRATION
10X Reaction Buffer	5	1X
dNTP mix (1.25 mM each)	8	200 µM each
Primer #1, 10 µM	2.5	0.5 µM or 1.0 µM
Primer #2, 10 µM	2.5	0.5 µM or 1.0 µM
Template DNA + Agarose	5 - 10 (0.3 ng - 1.0 ng DNA)	
Polymerase	0.5 (2.5 units)	
Sterile distilled water	to 50 µl	

8. Combine the components of the reaction mixture.
9. Vortex the reaction mixture to mix the contents.
10. Spin briefly in a microcentrifuge.
11. Overlay the mixture with mineral oil if necessary.
12. Perform amplification reactions with conditions appropriate for the template DNA and primers.
13. Remove mineral oil.
14. Remelt the reaction mixture at 65°C prior to analyzing.

Procedure 2

1. Follow steps 1 - 2 in Procedure 1.
2. Turn the gel upside down while it is on the UV transilluminator.
3. Stab the band of interest with a glass Pasteur pipette. When the pipette is removed from the gel a small plug of agarose will be contained in the tip.
4. Remove the plug of agarose.
5. Add a portion of the plug of agarose to the amplification reaction. The plug does not require melting; it will melt during the first denaturing step.
6. Follow steps 8 - 14 in Procedure 1.

References

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Fors, L., *et al., Nucl. Acids Res.* **18:** 2793 - 2799, 1990.
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Kretz, K.A. and O'Brein, J.S., *Meth. Enzymol.* **218:** 72 - 79, 1993.

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Majumdar, D., *et al., BioTechniques* **7:** 188 - 191, 1989.
Murray, J.A.H., *Nucl. Acids Res.* **14:** 10118, 1986.

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Attaching Linkers

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Gap Filling

Majumdar, D., *et al., BioTechniques* **7:** 188 - 191, 1989.

Chapter VI

Recovery of DNA from Agarose Gels

IN THIS CHAPTER

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Tips for Increasing DNA Recovery Efficiency from Agarose Gels

Introduction

This section discusses various tips which will increase the efficiency of recovery of DNA from agarose gels. These tips and recommendations can be applied to all recovery techniques.

The section is divided into the following topics:

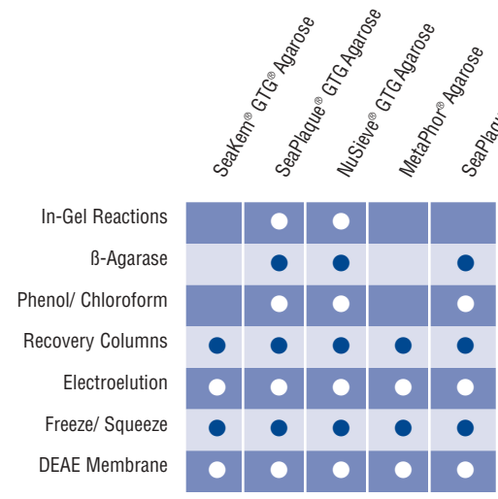
- Choosing the appropriate agarose
- Choosing the appropriate electrophoresis buffer
- Gel casting and DNA loading tips
- Staining and recovery tips

Choosing the appropriate agarose for recovery

When recovering DNA, the choice of agarose is one of the most important factors. To avoid recovery altogether one can choose to perform in-gel reactions.

Cambrex offers Genetic Technology Grade™ (GTG®) products that are specially prepared for demanding molecular biology applications. Cambrex's GTG quality tests go beyond standard nuclease assays to include enzymatic performance measurements. Our additional testing provides a more realistic index of overall product quality and reliability. You no longer need to screen agarose lots to find those which yield biologically active DNA.

Cambrex Agaroses and compatible recovery techniques



Buffer types

When recovering DNA from agarose gels, 1X Tris-acetate (TAE) buffer is recommended for electrophoresis.

Casting and DNA loading tips

- Prepare the gel in 1X TAE buffer.
- Do not cast the gel with ethidium bromide.
- Cast a gel 3 mm - 4 mm thick.
- Use a comb ≤1 mm thick.
- Load no more than 100 ng of DNA per band.

Continued on page 88

Staining and recovery tips

When recovering DNA from agarose gels, we recommend the following:

- Stain the gel for 15 - 20 minutes.
- Destain the gel in distilled water for two, 20-minute washes.
- Do not expose the DNA to UV light for any longer than 1 minute. Long exposure of DNA to UV light can nick the DNA.
- The addition of 1 mM guanosine or cytidine to the gel and electrophoresis buffer is effective in protecting DNA against UV-induced damage.
- Cut the smallest gel slice possible.

It is possible to avoid staining samples which will be used for recovery by running an additional lane containing a small amount of your sample immediately next to the molecular weight marker. However, DNA is damaged by UV light in the absence of ethidium bromide so keep exposure to UV light as brief as possible. Cut the lanes containing the marker and the small amount of the sample from the rest of the gel and stain. To recover the preparative loading, line up the stained portion of the gel with the unstained portion. Check by placing on UV transilluminator and cut out the area that lines up with your sample on the unstained portion of the gel.

Reference

Grundemann, D. and Schomig, E., *BioTechniques* **21(5):** 898 - 903, 1996.

ß-Agarase Recovery of DNA from Agarose Gels

Introduction

ß-Agarase is an enzyme that digests the polysaccharide backbone of agarose to alcohol-soluble oligosaccharides. DNA in a low melting temperature agarose gel can be recovered after the gel is melted and digested with this enzyme. The remaining oligosaccharides will not gel or interfere with subsequent DNA manipulations such as cloning, labeling, restriction digestion or sequencing. Cambrex's ß-agarase is free of any detectable DNase, RNase and phosphatase activities.

ß-Agarase recovery is particularly useful for recovering large DNA (>10 kb) which could be sheared by other methods of recovery.

Compatible agaroses

- SeaPlaque® GTG® Agarose (certified and tested for the recovery of DNA)
- NuSieve® GTG Agarose (certified and tested for the recovery of DNA)
- SeaPlaque Agarose

Tips

- Transfer no more than 200 mg of the agarose gel to a microcentrifuge tube for equilibration with digestion buffer.
- Completely melt the gel slice prior to the addition of enzyme.
- Ethanol precipitations should be incubated at room temperature with ammonium acetate rather than sodium acetate to decrease the likelihood of co-precipitation of agarose-oligosaccharides with the DNA.
- Polynucleotide kinase is inhibited by ≥7 mM ammonium ion. Use sodium acetate for your precipitation if you will be phosphorylating your DNA after recovery.
- The enzyme preparation retains full activity for several hours at 45°C. However, it will gradually lose activity upon longer incubations. For this reason, incubate at 40°C for overnight digestions.
- For efficient recovery of small nucleic acids (<500 bp) and/or very dilute samples (<0.05 µg/ml), we recommend either carrier tRNA or nuclease-free glycogen be added. In addition, overnight precipitation at room temperature can be helpful.

Continued on page 90

- The enzyme preparation has maximum activity between pH 6 - 7 and is relatively unaffected by salt concentrations between 0.1 M and 0.25 M. The equilibration of the gel slice with ß-agarase buffer is necessary to provide the enzyme with optimal buffer conditions. This equilibration is more important for gels prepared in TBE than in TAE buffer due to the greater buffering capacity of TBE buffer.
- Ethanol precipitate the DNA at room temperature or on ice. The most effective DNA precipitation can be achieved at 0°C to 22°C, rather than at -20°C to -70°C. At higher temperatures, yields are more consistent and precipitation of oligosaccharides will be avoided.

ß-Agarase unit definition

One unit of Cambrex ß-Agarase will completely digest 200 mg of a molten 1% SeaPlaque® GTG® Agarose gel which has been dialyzed in 1X ß-Agarase buffer in 1 hour at 40°C. Similar activities are obtained for other low melting temperature agaroses such as NuSieve® GTG Agarose.

Concentration: 1 unit per µl

Procedure for ß-Agarase digestion

BEFORE YOU BEGIN: Set one water bath or heating block for 70°C. Set another water bath or heating block for 45°C.

1. Electrophorese DNA in a low melting temperature agarose gel (such as SeaPlaque®, SeaPlaque GTG®, MetaPhor® or NuSieve® GTG Agarose) which has been prepared in TAE or TBE buffer.
2. Briefly stain the gel with GelStar® or SYBR® Green I Nucleic Acid Stain or ethidium bromide.
3. Photograph the gel.
4. Excise a gel slice containing the DNA of interest.
5. Place the gel slice containing the DNA sample into a microcentrifuge tube.
6. Equilibrate the gel slice with 10 volumes of 1X ß-Agarase buffer for 60 minutes at room temperature OR follow steps 6A - 6D.
   - **6A**. Melt the gel slice at 70°C for 15 minutes. Make sure that the gel slice is completely melted as the enzyme preparation will not digest agarose in the gelled state.
   - **6B.** Add 2 µl of the 50X ß-Agarase buffer to approximately 100 µl of melted gel solution.
   - **6C.** Mix the solution.
   - **6D.** Proceed to steps 9, 10 and 11.
7. Discard the buffer.
8. Melt the gel slice at 70°C (approximately 15 minutes). Make sure the gel slice is completely melted as the enzyme preparation will not digest agarose in the gelled state.
9. Cool the melted agarose solution to 45°C.
10. Add 1 unit of ß-Agarase for 200 mg (approximately 200 µl) of 1% agarose gel (add proportionally more or less of the enzyme preparation for larger or smaller gel slices, or with higher or lower agarose concentrations, respectively). Skip to step 12, page 92.

Continued on page 92

Materials

- Microcentrifuge tubes (≥1 ml)
- Two water baths or heating blocks
- Scalpel or razor blade
- Microcentrifuge

Reagents

- SeaPlaque, SeaPlaque GTG, NuSieve GTG or MetaPhor Agarose
- GelStar or SYBR Green I Nucleic Acid Gel Stain or ethidium bromide
- ß-Agarase buffer:
  Supplied as a 50X concentrate. 1X concentration is:
  40 mM Bis Tris/HCl
  40 mM NaCl
  1 mM EDTA (pH 6.0)
- ß-Agarase enzyme
- 10 M Ammonium acetate
- 100% Ethanol

11. If you have followed the protocol in steps 6A - 6D, the amount of enzyme will need to be adjusted.

IF THE GEL WAS PREPARED WITH . . .	THEN YOU WILL NEED TO ADD . . .
TAE buffer	Twice the amount of enzyme
TBE buffer	Seven times the amount of enzyme

12. Mix.

13. Incubate at 45°C for 60 minutes.

14. If the DNA is ≤30 kb...
Place the solution at -20°C for 15 minutes. Centrifuge the solution for 15 minutes at 4°C.
Continue with Step 15
OR
The sample can be concentrated without ethanol precipitation by centrifugation in a molecular weight-cutoff-spin column (e.g., Amicon®'s Microcon™).
If the DNA is >30 kb...
The DNA/ß-Agarase solution may be used directly without precipitation for subsequent enzymatic manipulations.
OR
The sample can be concentrated without ethanol precipitation by centrifugation in a molecular weight-cutoff-spin column (e.g., Amicon's Microcon or Centricon™ microconcentrators).

15. Transfer the supernatant to a new microcentrifuge tube leaving behind any undigested agarose in the pellet.

16. Add ammonium acetate to the supernatant to a final concentration of 2.5 M.

17. Add 2 to 3 volumes of 100% ethanol.

18. If the DNA is ≤0.05 µg/ml. . .
Precipitate at room temperature for 24 hours
OR
Add 10 µg of RNA.
If the DNA is >0.05 µg/ml. . .
Precipitate at room temperature for 30 minutes.

19. Collect the precipitate by centrifugation.

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Electroelution of DNA from Agarose Gels

Introduction

Electroelution is a reliable and consistent recovery method. The recovered DNA is suitable for a wide range of applications. For small fragments, a typical yield would be 50% - 85%, but as fragment length increases, yield can drop as low as 20%. The procedure below describes the electroelution of DNA into dialysis bags and has been adapted from Sambrook, *et al*. Alternatively, if you have a commercially available apparatus, follow the manufacturer's instructions.

Compatible agaroses

- SeaKem® GTG® Agarose (certified and tested for the recovery of DNA)
- SeaPlaque® GTG Agarose (certified and tested for the recovery of DNA)
- NuSieve® GTG Agarose (certified and tested for the recovery of DNA)
- SeaPlaque Agarose
- NuSieve 3:1 Agarose
- MetaPhor® Agarose

Tips

- Electrophorese DNA in 1X TAE buffer.
- Have 1 µg of DNA in your band of interest.
- Minimize exposure of DNA to UV light for no more than 1 minute.
- Cut the smallest gel slice possible.

Materials

- Scalpel or razor blade
- Spatula
- Dialysis tubing
- Dialysis tubing clips
- Electrophoresis chamber
- Hand-held, long-wavelength ultraviolet lamp
- Disposable plastic tube
- Pasteur pipette

Reagents

- 1X TAE buffer
- 2% w/v Sodium bicarbonate, 1 mM EDTA pH 8.0, prepare in distilled water
- 1mM EDTA, pH 8.0
- Distilled water
- GelStar or SYBR Green I Nucleic Acid Gel Stain or ethidium bromide

Caution

Wear gloves and protective equipment to avoid exposure to ethidium bromide.

Procedure for preparing dialysis tubing

1. Cut the tubing into 10 cm to 20 cm long pieces.
2. Boil for 10 minutes in a large volume of 2% sodium bicarbonate/1 mM EDTA, pH 8.0.
3. Rinse the tubing with distilled water.
4. Boil for 10 minutes in 1 mM EDTA, pH 8.0.
5. Cool the tubing.
6. Store at 4°C submerged in 1 mM EDTA, pH 8.0.

Procedure for electroeluting DNA from agarose gels

1. Electrophorese DNA in an agarose gel.
2. Stain the gel with GelStar® or SYBR® Green I Nucleic Acid Stain or ethidium bromide.
3. Locate the band of interest using a UV light source. Minimize exposure of DNA to UV light to less than 1 minute.
4. Excise the band of interest using a scalpel or razor blade.
5. Wet a spatula with 1X TAE buffer.
6. Place the agarose slice containing the DNA on a wetted spatula or a scalpel.
7. Photograph the gel for a record of which band was eluted.
8. Seal one end of treated dialysis tubing with a dialysis clip.
9. Fill the bag to the top with 1X TAE buffer.
10. Transfer the slice of agarose into the bag with the spatula.
11. Allow the slice of agarose to sink to the bottom of the bag.
12. Remove the buffer from the dialysis bag, leaving just enough to keep the gel slice in constant contact with the buffer.
13. Clip the dialysis bag above the gel slice, avoiding air bubbles.
14. Place a shallow layer of 1X TAE buffer in an electrophoresis chamber.
15. Immerse the bag in the electrophoresis chamber.
16. Pass electric current through the bag (4 - 5 V/cm for 2 - 3 hours). During this time the DNA is eluted out of the gel and onto the inner wall of the dialysis tubing. This process can be monitored with a hand-held, long-wavelength UV lamp. Expose to UV light briefly.
17. Reverse the polarity of the current for 1 minute. This will remove the DNA from the wall of the bag.
18. Recover the bag from the electrophoresis chamber.
19. Gently massage the side of the bag with gloved fingers where the DNA has accumulated. This will remove the DNA from the wall of the bag. This process can be monitored with a hand-held UV lamp. Expose to UV light briefly.
20. Open the dialysis bag.
21. Transfer all of the buffer surrounding the gel slice to a plastic tube.
22. Wash out the bag with a small amount of 1X TAE buffer.
23. Transfer the solution to the plastic tube.
24. Remove the gel slice from the bag.
25. **OPTIONAL:** Stain the slice with GelStar® or SYBR® Green I Nucleic Acid Stain or ethidium bromide to ensure all the DNA has been eluted from the slice.
26. Purify the DNA from 1X TAE solution using phenol/chloroform extractions.*

*After electroelution, it is recommended that the DNA is further purified with a phenol/chloroform extraction followed by ethanol precipitation. Oligosaccharides and other contaminants (found in low-grade agarose) can copurify with the DNA. Phenol extractions will remove any oligosaccharides, avoiding their coprecipitation during ethanol precipitations.

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Phenol/Chloroform Extraction of DNA from Agarose Gels

Compatible agaroses

- SeaPlaque® GTG® Agarose (certified and tested for the recovery of DNA)
- NuSieve® GTG Agarose (certified and tested for the recovery of DNA)
- SeaPlaque Agarose

Tips

Recovery failures when extracting DNA from agarose using phenol/chloroform most often result from either extracting too large a piece of agarose, or precipitating agarose along with the DNA at the ethanol precipitation step. To address these difficulties, we recommend the following:

- No more than 200 mg (200 µl) of agarose should be extracted in a single tube. If your gel slice containing the DNA is larger than this, separate it into smaller pieces, then combine the extracted solutions prior to ethanol precipitation.
- Ethanol precipitation of agarose can be avoided by chilling the extracted solution on ice for 15 minutes, then centrifuging the sample(s) in a cold room for 15 minutes at maximum speed in a microcentrifuge prior to adding salts and ethanol. The supernatant is then carefully decanted, and the DNA in the supernatant is precipitated following standard protocols.
- Not useful for large DNA (>10 kb). Vortexing will shear the DNA.

Procedure

This protocol has been adapted from Sambrook, *et al*.

1. Electrophorese DNA in a low melting temperature agarose gel prepared in 1X TAE.
2. Set a water bath for 67°C.
3. Prewarm the TE at 67°C.
4. Excise the gel fragment containing the DNA.
5. Place the DNA slice in a microcentrifuge tube.
6. Estimate the volume of the slice. If the slice is significantly greater than 200 mg, break the agarose slice into smaller pieces and place each agarose piece in a separate microcentrifuge tube.
7. Melt the gel slice at 67°C for 10 minutes.
8. Add the appropriate volume of TE buffer (prewarmed to 67°C) so that the final concentration of agarose is ≤0.5%.
9. Maintain the samples at 67°C until you are ready to phenol extract.
10. Add an equal volume of buffer-equilibrated phenol.
11. All subsequent steps can be done at room temperature.
12. Vortex for 15 seconds.
13. Centrifuge at maximum speed in a microcentrifuge for 3 minutes.
14. Carefully remove the top aqueous phase. The interface of white debris is the agarose, which can contain some trapped DNA. This can be back-extracted with TE to maximize yield.
15. Place aqueous phase in a clean microcentrifuge tube.
16. Repeat steps 10 - 15.
17. Add an equal volume of phenol/chloroform to the aqueous phase.
18. Vortex for 15 seconds.
19. Centrifuge at maximum speed in a microcentrifuge for 3 minutes.
20. Remove the aqueous phase and place in a clean microcentrifuge tube.
21. Repeat the extraction with an equal volume of chloroform.
22. Remove the aqueous phase and place in a clean microcentrifuge tube.
23. Chill the aqueous phase for 15 minutes on ice.
24. Centrifuge in a microcentrifuge at high speed for 15 minutes at 4°C.
25. Carefully decant the supernatant into a clean microcentrifuge tube.
26. Ethanol precipitate the DNA in the supernatant following standard protocols.

Materials

- Water bath set to 67°C
- Microcentrifuge tubes (≥1 ml)
- Ice bucket and ice
- Vortex mixer
- Microcentrifuge
- Scalpel or razor blade

Reagents

- SeaPlaque® GTG®, NuSieve® GTG or SeaPlaque Agarose
- TE buffer warmed to 67°C
- Buffer-equilibrated penol (See p. 166)
- Chloroform
- Phenol/ chloroform (1:1)

Caution

Phenol causes severe burns. Gloves, safety glasses and a lab coat should be worn when working with phenol solutions.

References

Benson, S.A., *BioTechniques* **2:** 66 - 67, 1984.

Sambrook, J., *et al., Molecular Cloning: A Laboratory Manual,* 2nd Edition, Cold Spring Harbor Laboratory Press, 1989.

"Modified Freeze/Squeeze" Extraction of DNA from Agarose Gels

Materials

- Microcentrifuge tubes (≥1 ml)
- Ice bucket and ice
- Vortex mixer
- Microcentrifuge
- Scalpel or razor blade
- Glass or plastic stir rod

Reagents

- TE buffer warmed to 67°C
- Buffer equilibrated phenol (See p. 166)
- Chloroform
- Phenol/ chloroform (1:1)

Introduction

This protocol is relatively fast and will work with low and standard melting temperature agaroses. However, like many recovery techniques, the DNA yield decreases with increasing DNA size, but should be approximately 50% for DNA ≤5 kb.

Compatible agaroses

- SeaKem® GTG® Agarose (certified and tested for the recovery of DNA)
- SeaPlaque® GTG Agarose (certified and tested for the recovery of DNA)
- NuSieve® GTG Agarose (certified and tested for the recovery of DNA)
- SeaPlaque Agarose
- MetaPhor® Agarose
- NuSieve 3:1 Agarose
- SeaKem Gold Agarose

Procedure

This protocol has been adapted from Benson.

For more detailed protocols on phenol/chloroform extractions refer to the previous section.

1. Electrophorese DNA in an agarose gel prepared in 1X TAE.
2. Excise gel slice containing DNA.
3. Place in a microcentrifuge tube.
4. Estimate volume based on weight.
5. Mash the gel slice with the stir rod.
6. Add an equal volume of buffer-equilibrated phenol.
7. Vortex for 10 seconds.
8. Freeze at -70°C for 5 to 15 minutes.
9. Centrifuge for 15 minutes at room temperature.
10. Remove the supernatant which contains the DNA.
11. Place the supernatant, in a clean microcentrifuge tube.
12. Phenol/chloroform extract the supernatant.
13. Follow with a chloroform extraction.
14. Ethanol precipitate following standard procedures.

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Ethanol Precipitation of DNA Recovered from Agarose Gels

Materials

- Ice bucket and ice
- Vortex mixer
- Microcentrifuge
- Vacuum gel dryer
- Microcentrifuge tubes (≥1 ml)

Reagents

- 100% ethanol (ice cold)
- 70% ethanol (room temperature)
- TE buffer, pH 8.0
- 10 M Ammonium acetate

Introduction

This method is compatible with all the recovery techniques listed in this chapter.

Tips

- Prior to adding salts and ethanol, precipitation of agarose can be avoided by chilling the supernatant on ice for 15 minutes, then centrifuging the sample(s) in a cold room for 15 minutes at maximum speed in a microcentrifuge. The supernatant is then carefully decanted, and the DNA in the supernatant is ethanol precipitated following standard protocols.
- Ethanol precipitations should be incubated at room temperature with ammonium acetate rather than sodium acetate in order to decrease the likelihood of coprecipitation of agarose-oligosaccharides with the DNA or RNA.

Procedure

This protocol has been adapted from Sambrook, *et al*.

1. Chill the supernatant on ice for 15 minutes.
2. Centrifuge the sample(s) in a cold room for 15 minutes at maximum speed in a microcentrifuge.
3. Carefully decant the supernatant.
4. Place in a clean microcentrifuge tube.
5. Measure the volume of the sample.
6. Add 0.2 volumes of 10 M ammonium acetate to the sample.
7. Add 2 volumes of 100% ice-cold ethanol.
8. Briefly vortex.
9. Store the mixture for 30 minutes to overnight at room temperature.
10. Centrifuge for 30 minutes at 12,000 rpm.
11. Decant the supernatant.
12. Wash the pellet three times with 70% ethanol.
13. Allow to air-dry at room temperature on the bench top.
14. Dry under vacuum for 5 - 10 minutes.
15. Dissolve the DNA in TE buffer.

References

Life Technologies, Inc., *GIBCO-BRL Focus* **7(4):** 1 - 2, 1985.

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References for Recovery using glass beads

Ausubel, F.M., *et al., Current Protocols in Molecular Biology,* Red Book, Wiley & Sons, 1995.
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Vogelstein, B. and Gillespie, D., *Proc. Natl. Acad. Sci.* **76:** 616 - 619, 1979.

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Chapter VII

Separation of DNA in Polyacrylamide Gels

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Separation of DNA in Polyacrylamide Gels

Overview

Polyacrylamide gels can separate DNA that differs by 0.2% in length, well beyond the resolving capabilities of agarose (2% difference in DNA length). Another advantage to using polyacrylamide gels is that they can accommodate large amounts of DNA (up to 10 µg) without any loss in resolution.

Depending upon the application, polyacrylamide gels can be prepared as denaturing or nondenaturing gels.

Applications

Denaturing gels: concentrations range from 8% - 20%

- Oligonucleotide purification
- Separation of single-stranded DNA
- Isolate radiolabeled DNA probes
- S1 nuclease assay
- DNA footprinting
- RNase protection assays

Nondenaturing gels: concentrations range from 3% - 20%

- Separation of di-nucleotide repeats
- Separation of DNA ranging from 20 bp - 2000 bp in length
- Study DNA-Protein interactions (Gel Shift Assays)

Buffers for Electrophoresis

To ensure adequate buffering power during vertical electrophoresis, TBE buffer is used for polyacrylamide gel electrophoresis at a working strength of 1X. Lower dilutions of the buffer or the use of TAE buffer may cause gels to overheat and result in band smiling throughout the gel.

TBE is commercially available as 5X or 10X solutions (Cambrex AccuGENE® TBE Buffer). Alternatively, it can be prepared as follows:

10X TBE Stock Solution (890 mM Tris base, 890 mM Boric acid, 20 mM EDTA)
1X = 89 mM Tris base, 89 mM Boric acid, 2 mM EDTA)

108.0 g Tris base
55.0 g Boric acid
7.44 g $Na_2EDTA \bullet 2H_20$

Adjust volume to 1 liter with distilled water
Filter through a 0.45 µm filter
pH adjustment is not necessary

Suggested polyacrylamide concentrations

NONDENATURING POLYACRYLAMIDE GELS

GEL PERCENTAGE	FRAGMENT SIZES
3.5%	1,000 bp - 2,000 bp
5.0%	80 bp - 500 bp
6.0%	75 bp - 2,000 bp
8.0%	60 bp - 400 bp
10.0%	30 bp - 1,000 bp
12.0%	40 bp - 200 bp
15.0%	25 bp - 150 bp
20.0%	5 bp - 100 bp
4 - 20%	10 bp - 2,000 bp

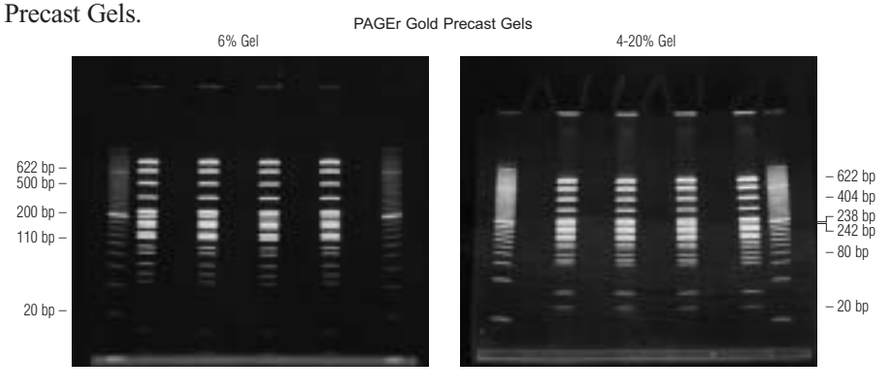
Preparation and Casting of Polyacrylamide Gels

Overview

Acrylamide is a potent neurotoxin and is absorbed through the skin. The effects of acrylamide are cumulative. Gloves, safety glasses, labcoat and a mask should be worn when weighing powdered acrylamide and bis acrylamide. Although polyacrylamide is considered to be nontoxic, it should be handled with care because of the possibility that it might contain small quantities of unpolymerized acrylamide.

Precast gels have become widely used as they improve gel to gel consistency and save the time required for gel preparation. Cambrex offers PAGEr® Gold Precast Gels in TBE buffer. These nondenaturing gels are available in 6%, 10% and 4 - 20% gradient gel concentrations.

The figure below demonstrates the performance of Cambrex's PAGEr Gold Precast Gels.



Separation of DNA Markers in PAGEr® Gold Precast Gels. Outside lanes: Cambrex 20 bp DNA Ladder; 6-10 ng/band. Interior lanes: Msp I digested pBR322 (New England BioLabs); 0.5 µg/lane. 6% Gel: 10 x 10 cm gel run at 200 volts in 1X TBE buffer (Prepared from AccuGENE® 10X TBE Buffer). 4-20% Gel: 9 x 10 cm gel run at 180 volts in 1X TBE buffer (Prepared from AccuGENE 10X TBE Buffer). Gels were stained in a 1 µg/ml solution of ethidium bromide for 10 minutes, rinsed briefly in distilled water and photographed.

Advantages of using PAGEr Gold Precast Gels

- Save gel preparation time
- Improved gel to gel consistency
- Compatible with most vertical minigel boxes
- High resolution
- No toxic chemicals to work with
- Multi-channel pipette compatible comb formats
- Easy-to-load and open

Tips for casting and preparing polyacrylamide gels

- Ensure plates are clean and free of debris prior to use.
- Spacers and combs range in thickness from 0.5 mm to 2.0 mm. Thinner gels are preferable because they produce the sharpest bands. Thicker gels may overheat and result in band smiling throughout the gel.
- Prepare solutions of 10% ammonium persulfate (APS) just prior to use.

Preparing the casting assembly

There are several different electrophoresis chambers commercially available, and the arrangement of the glass plates and spacers varies slightly depending on the manufacturer. It is always advisable to consult with the manufacturer for the appropriate cassette assembly specific to your system. A procedure for Cassette Assembly can be found on page 24.

Preparing the Gel Solution

1. Ensure the glass plates are clean and free of dried gel and soap residues. To remove any residues, apply ethanol to both plates and wipe dry.
2. Assemble glass plates according to manufacturer's instructions or follow the procedure outlined on page 24.
3. Prepare a 10% solution (w/v) of APS by adding 0.10 g of ammonium persulfate to 1 ml of distilled water. Gently mix.
4. Place the specified quantity of the first three or four components from the table below into a clean flask. If including Urea: facilitate dissolving by warming solution. Cool to room temperature prior to the addition of TEMED and APS.

Preparation of Polyacrylamide Gel (100 ml Total Volume)**

Components	3.5%	5.0%	8.0%	12.0%	20.0%
Urea*	42.0 g	42.0 g	42.0 g	42.0 g	42.0 g
10X TBE	10 ml	10 ml	10 ml	10 ml	10 ml
30% Acrylamide Solution	11.6 ml	16.6 ml	26.6 ml	40.0 ml	66.6 ml
Distilled-deionized Water	Fill to 100 ml				
TEMED	35 µl	35 µl	35 µl	35 µl	35 µl
10% APS	700 µl	700 µl	700 µl	700 µl	700 µl

*Include urea if preparing a denaturing gel. **Adjust volumes proportionately.*

Continued on page 108

Materials

- Flask with side arm

Reagents

- 30% acrylamide stock solution (Commercially available or prepare as follows:
  - 29.0 g Acrylamide
  - 1.0 g N,N´-methylenebis-acrylamide,
  - Adjust volume to 100 ml with distilled water,
  - Heat the solution to 37°C to dissolve the chemicals
- Distilled water
- TEMED
- Ammonium persulfate (APS)
- Urea if preparing a denaturing gel
- AccuGENE® 10X TBE Buffer

5. Mix gently by swirling.
6. If desired, degas the solution for 5 minutes.
7. Add the specified amount of TEMED and 10% APS to the solution.
8. Mix gently by swirling.

Casting the gel

1. Immediately after solution preparation, draw the solution into a 50-cc syringe.
2. Invert the syringe to remove any air bubbles.
3. Insert the needle between the two glass plates.
4. Gently expel the solution between the glass plates until it reaches the top of the smaller plate.

   NOTE: Avoid creating air bubbles by tilting the cassette to one side and slowly and consistently expel the solution into the glass plates.

5. Place the remainder of the acrylamide solution at 4°C.
6. Immediately insert the comb, being careful not to allow air bubbles to become trapped under the teeth.
7. Clamp the comb in place.
8. If necessary, use the remainder of the acrylamide solution to fill any gaps.
9. Ensure that no acrylamide solution is leaking from the cassette assembly.

   NOTE: Leaks may be stopped by using tape to seal.

10. Allow the gel to completely polymerize for 60 minutes at room temperature.

    NOTE: Gels may be stored for 1 - 2 days prior to use once polymerized. After polymerization is complete, surround the comb and the top of the gel with paper towels that have been soaked in 1X TBE buffer. Seal the gel in a plastic bag and store at 4°C.

Materials
- 50-cc syringe
- 18-gauge needle
- Clamps

Loading and Running DNA in Polyacrylamide Gels

DNA loading

The amount of DNA to load on a polyacrylamide gel is variable. Most important are the quantities of DNA in the bands of interest. The highest amount of DNA that yields a clean sharp band is approximately 100 ng. The lowest amount of DNA that can be reliably detected with ethidium bromide is 10 ng, and 60 pg if using GelStar® or SYBR® Green Nucleic Acid Gel Stains.

Loading buffers

The loading buffers used for DNA electrophoresis in polyacrylamide gels are the same as those used for electrophoresis in agarose gels. Refer to Loading Buffers on page 30 for a brief description of each.

Dye mobility tables

The following table is a migration table of double-stranded DNA in relation to bromophenol blue (BPB) and xylene cyanol (XC) in 29:1 nondenaturing polyacrylamide gels. Dye migrations may vary slightly if using precast polyacrylamide gels and may vary from manufacturer to manufacturer.

Gel Percentage	Xylene Cyanol	Bromophenol Blue
3.5%	460 bp	100 bp
8.0%	160 bp	45 bp
12.0%	70 bp	20 bp
15.0%	60 bp	15 bp
20.0%	45 bp	12 bp

The following table is a migration table of single-stranded DNA in relation to bromophenol blue (BPB) and xylene cyanol (XC) in denaturing polyacrylamide gels (7 M Urea). Dye migrations may vary slightly depending on denaturant concentration and if using precast polyacrylamide gels they may vary from manufacturer to manufacturer.

Gel Percentage	Xylene Cyanol	Bromophenol blue
5.0%	35	140
6.0%	26	106
8.0%	19	75
10.0%	12	55
20.0%	8	28

Materials
- Pasteur pipette
- Power supply
- Electrophoresis chamber

Reagents
- Distilled water
- AccuGENE® 10X TBE Buffer

Follow the steps below to run polyacrylamide gels

1. Slowly remove the comb from the gel.
2. Rinse the wells with distilled water.
3. If tape was used to prepare the cassette assembly, remove the tape from the bottom of the gel.
4. Use 1X TBE running buffer in the electrophoresis chamber.
5. Attach the gel to the electrophoresis tank following the manufacturer's instructions.
6. Fill the reservoirs of the electrophoresis tank with 1X TBE.
7. Flush out the wells with 1X TBE electrophoresis buffer.
8. Ensure buffer is not leaking from the upper buffer chamber.
9. Mix the sample with the appropriate amount of 6X gel loading buffer.
10. Load DNA.
11. Connect the electrodes to the power supply.
12. Run the gel between 15 - 20 V/cm (interelectrode distance).

    NOTE: If higher voltages are used, band smiling may occur.
13. Run the gel until the marker dyes have migrated the desired distance through the gel.

Detecting DNA in Polyacrylamide Gels with GelStar® or SYBR® Green Nucleic Acid Gel Stains

GelStar and SYBR Green Nucleic Acid Gel Stains are highly sensitive fluorescent stains for detecting nucleic acids. These stains provide high sensitivity detection of double-stranded or single-stranded DNA. See table below for a comparison of staining sensitivities and limits of detection.

Stain	ssDNA	dsDNA	Native RNA
GelStar® Stain	25 pg	20 pg	10 ng
SYBR® Green I Stain	–	20-30 pg	–
SYBR® Green II Stain	60 pg	–	15 ng
Ethidium Bromide	350 pg	100 pg	30 ng

Tips for staining gels with GelStar and SYBR Green Nucleic Acid Gel Stains

- The powder used on some laboratory gloves may contribute to background fluorescence. We recommend using powder-free gloves and rinsing gloves prior to handling gels.
- Fibers shed from clothing or lab coats may be fluorescent; be cautious when handling gels.
- Staining of nucleic acids with these dyes has minimal impact on blotting efficiency. To ensure efficient hybridization, use of prehybridization and hybridization solutions containing 0.1% - 0.3% SDS is important to remove stain retained during transfer.
- These stains can be removed from nucleic acids by ethanol precipitation. Isopropyl alcohol precipitation is less effective at removing the dye; butyl alcohol extraction, chloroform extraction and phenol do not remove the dye efficiently.
- Allow time for the stock solution to thaw completely. Removal of stain from partially thawed solutions will result in depletion of stain over time.
- These stains may be diluted in most common electrophoresis buffers with a pH range from 7.0 - 8.5 or in TE buffer. Staining solutions prepared in water or in buffer with a pH below 7.0 or above 8.5 are less stable and show reduced staining efficiency.

Continued on page 112

Materials

- Clear polypropylene container (e.g., Rubbermaid® recycling #5 plastics)
- Microcentrifuge
- UV transilluminator
- Photographic Filter for GelStar® Stain or SYBR® Green Stain (Wratten® #15 or Wratten® #9 respectively)

Reagents

- GelStar Nucleic Acid Gel Stain or SYBR Green I or II Nucleic Acid Gel Stain
- Buffer between pH 7.5-8.5 (TBE or TE)

Caution

These stains should be handled with care and disposed of properly. Gloves should be worn when handling solutions of these dyes and stained gels. Avoid skin and eye exposure to UV light.

- Prepare and store the stain in polypropylene containers such as Rubbermaid containers or pipette-tip box lids. The stain may adsorb to glass surfaces and some plastic surfaces, particularly if the surfaces carry residues of anionic detergents or reagents.
- As an alternative to the protocol presented for staining gels on the cassette plate, smaller gels such as mini gels may be removed from both plates then stained using the protocol for post-staining agarose gels found on page 45.
- Treatment of one plate with a "release" agent, such as Gel Slick® Solution, increases the ease of separation the glass plates while keeping the gel in place on the other plate for staining.
- Handling or compression of gels (particularly polyacrylamide-type gels) can lead to regions of high background after staining. If possible gels should not be handled directly; use a spatula (or other tool) and a squirt bottle to slide the gel off the plates and into the stain or onto the light box.

Procedure for staining

Incorporating these dyes into the gel or prestaining the nucleic acid in a vertical format is not recommended. The dye binds to glass or plastic plates and DNA may show little to no signal. Gels should be post-stained as described below.

1. Remove the concentrated stock solution from the freezer and allow the solution to thaw at room temperature.
2. Spin the solution in a microcentrifuge to collect the dye at the bottom of the tube.
3. Dilute the 10,000X concentrate to a 1X working solution for DNA and a 2X working solution for RNA, in a pH 7.5 - 8.5 buffer, in a clear plastic polypropylene container.
4. Open the cassette, and leave the gel in place on one plate.
5. Place the plate, gel side up, in a staining container.
6. Gently pour the stain over the surface of the gel; a disposable pipette may be used to help distribute the stain evenly over the gel surface.
7. Stain the gel for 5 - 15 minutes. No destaining is required.
8. Remove the gel from the staining solution and view with a 300 nm UV transilluminator, Dark Reader™ transilluminator or CCD imaging system.

For highest sensitivity the gel should be carefully removed from the plate and placed directly on the transilluminator or scanning stage. Alternatively, if a relatively low fluorescence plate is used, the results may be visualized by placing the gel and plate gel side down on the transilluminator and photographing or by scanning the gel directly on the plate.

NOTE: More detailed information on photographing gels and decontaminating staining solutions is described in Detecting DNA with GelStar® or SYBR® Green I Stains (see page 47).

Detecting DNA in Polyacrylamide Gels with Ethidium Bromide

The procedure for post-staining DNA in polyacrylamide gels with ethidium bromide is identical to the procedures used for post-staining agarose gels. Follow the procedures described in Detecting DNA with Ethidium bromide (see page 48).

Electroblotting Polyacrylamide Gels

Materials

- Uncharged nylon membrane
- Tank electroblot apparatus
- Whatman 3MM Chromatography paper
- Glass dish
- Fiber pad

Reagents

- 0.5X TBE transfer buffer (chilled and room temperature)

Because the pore sizes of polyacrylamide gels are small, capillary transfer is ineffective and gels must be electrophoretically transferred. For more detailed information and protocols concerning electroblotting, consult the blotting apparatus manufacturer's instructions or Ausubel, *et al.*

1. Prepare 0.5X TBE transfer buffer.
2. Chill the buffer to 4°C.
3. Run a polyacrylamide gel following your standard procedure.
4. Place gel in the glass dish.
5. Equilibrate the gel in cold transfer buffer for 1 hour in the cold room.
6. Soak the membrane for 20 minutes in the Transfer buffer.
7. Cut 2 pieces of Whatman® 3MM chromatography paper to the size of the gel.
8. Soak the chromatography paper in Transfer buffer.
9. Soak the fiber pads in Transfer buffer.
10. Make a sandwich of the filter paper, fiber pad and membrane following the equipment manufacturer's instructions.
11. Close the holder on the sandwich and latch.
12. Fill tank with Transfer buffer.
13. Attach to the power supply.
14. Transfer at 30 volts (constant) for 4 hours.
15. Remove the membrane from the apparatus.
16. Treat the membrane as described in Immobilizing DNA on Membrane (see page 69).

Recovery of DNA in Polyacrylamide Gels

The two primary methods for recovering DNA from polyacrylamide gels are the "Crush and Soak" method or electroelution. Described below is the "Crush and Soak" procedure. The procedure for electroeluting DNA from polyacrylamide gels is similar to the procedures used for agarose gels with one exception; 0.5X TBE buffer should be used rather than TAE buffer. Follow the procedures described in Electroelution of DNA from Agarose Gels (see page 93).

Tips for increasing DNA recovery from polyacrylamide gels

When recovering DNA from polyacrylamide gels we recommend the following:

- Stain the gel for no more than 15 minutes.
- If ethidium bromide staining, destain the gel in distilled water for two, 20-minute washes.
- Do not expose the DNA to UV light for any longer than 1 minute. Longer exposures may result in DNA nicking.
- Cut the smallest gel slice possible.
- If recovering small amounts of DNA, the addition of 10 µg of carrier RNA prior to ethanol precipitation may improve recovery yields.

Materials

- Scalpel or razor blade
- Microfuge tube
- Disposable pipette tip
- Rotary wheel or platform
- 37°C oven
- Microcentrifuge at 4°C
- Vortex
- Disposable plastic column or a syringe barrel containing a Whatman GF/C filter or packed siliconized glass wool
- Ice

Reagents

- Elution Buffer
  (3.85 g Ammonium acetate, 0.215 g Magnesium acetate, 0.2 ml AccuGENE® 0.5 M EDTA Solution, 1.0 ml AccuGENE 10% SDS Solution, Fill to 100 ml with distilled water)
- 100% and 70% Ethanol
- AccuGENE® 1X TE, pH 7.6
- AccuGENE 3 M Sodium acetate, pH 5.2

Crush and soak procedure

1. While the gel is on the transilluminator cut out the band of interest using a razor blade or scalpel. Cut the smallest size gel slice possible.

   NOTE: If the gel has been covered with plastic wrap, do not remove the plastic wrap before cutting.

2. Peel the small piece of gel containing the DNA from the plastic wrap.
3. Transfer the gel slice to a microfuge tube.
4. Crush the gel slice against the wall of the microfuge tube with the disposable pipette tip.
5. Add 1 - 2 volumes of elution buffer to the gel slice (e.g., if the estimated gel slice volume is 200 µl add 200 µl - 400 µl of elution buffer).
6. Incubate the tube at 37°C on a rotating wheel or rotary platform. For fragments less than 500 bp incubate for 3 - 4 hours. For fragments greater than 500 bp incubate for 12 - 16 hours.
7. Centrifuge the samples at 12,000 rpm for 1 minute at 4°C.
8. Transfer the supernatant to a fresh microfuge tube, being careful to avoid transferring fragments of polyacrylamide.
9. Add 0.5 volumes of elution buffer to the pellet of polyacrylamide.
10. Vortex briefly.
11. Centrifuge at 12,000 rpm for 1 minute at 4°C.
12. Combine the two supernatants.
13. Remove any remaining polyacrylamide by passing the supernatant through a disposable plastic column or a syringe barrel containing a Whatman® GF/C filter or packed siliconized glass wool.
14. Add 2 volumes of cold ethanol.
15. Store the solution on ice for 30 minutes.
16. Recover the DNA by centrifugation at 12,000 rpm for 10 minutes at 4°C.
17. Decant the supernatant.
18. Redissolve the DNA in 200 µl of AccuGENE® 1X TE, pH 7.6.
19. Add 25 µl of AccuGENE 3 M Sodium acetate, pH 5.2.
20. Repeat steps 14 - 17.
21. Rinse pellet once with 70% Ethanol.
22. Add 10 µl of AccuGENE 1X TE, pH 7.6 to pellet and dissolve.

References

Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory Press, 1989.
Rickwood, D. and Hames, B.D., *Gel Electrophoresis of Nucleic Acids: A Practical Approach*, 2nd edition, IRL Press, 1990.
Ausubel, F.M., *et al.*, *Short Protocols in Molecular Biology*, 4th edition, John Wiley & Sons, Inc., 1999.
Brown, T.A., *Essential Molecular Biology: A Practical Approach*, volume 1, 2nd edition, Oxford University Press, 2000.

Notes

Chapter VIII

Separation of RNA in Agarose Gels

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Separation of RNA in Agarose Gels

Introduction

Separation of RNA in agarose gels is used for a number of different purposes, including Northern blots to monitor RNA expression levels, checking RNA integrity and size-selection of RNA for cloning experiments. Separation of RNA based on fragment length requires conditions that are different from DNA analysis. These include sample preparation, the use of sample and gel denaturants, electrophoresis buffers, and visualization.

Denaturing systems

The purpose of the experiment and the size of the RNA being separated are the primary drivers in determining which denaturing system to use. The most frequently used denaturants for RNA agarose gel electrophoresis are formaldehyde, formaldehyde/formamide, and glyoxal plus DMSO. In each system, the denatured RNA migrates through the agarose gel in a linear relation to the log of its molecular weight (similar to DNA). The most efficient RNA denaturant is methylmercury hydroxide. Because of the hazards associated with this denaturant, it is the least used system for RNA analysis.

The resolving powers of the glyoxal/DMSO and the formaldehyde buffer systems are nearly identical. For detection by Northern analysis, glyoxal/DMSO denaturant is preferable because these gels tend to produce sharper bands than the formaldehyde system. Glyoxal gels require more care to run than formaldehyde gels and because of the lower buffering capacity of glyoxal, these gels must be run at lower voltages than gels containing formaldehyde. Glyoxal gels require a phosphate electrophoresis buffer and the buffer must be recirculated during electrophoresis. If the pH of the buffer rises above 8.0, glyoxal dissociates from RNA, causing the RNA to renature and migrate in an unpredictable manner.

For staining purposes, either denaturant can be used. Ethidium bromide, GelStar® Nucleic Acid Gel Stain and SYBR® Green II Gel Stain bind formaldehyde-denatured RNA more efficiently than glyoxal-denatured RNA. Glyoxal denaturant can interfere with binding of the stain, but gel backgrounds are often lower in these gels than with formaldehyde-denatured gels.

It is important to minimize RNase activity when running agarose gels by following certain precautions. There are several agents on the market that effectively remove RNase's or consult Sambrook, *et al.*

Preparation of RNA Samples

Which sample denaturation method to choose depends on the final goal of the experiment and the secondary structure of the RNA. There are several procedures to choose from, the most useful of which are described here. Any sample denaturation method can be used with any of the gel buffering systems. If simply checking the integrity of cellular RNA, no sample denaturation is necessary and TAE or TBE buffer can be used.

Common denaturants	Application
Formamide	Retain biological activity
Formaldehyde	Sample recovery
Glyoxal	Northern blotting; significant secondary structure

Formamide-only denaturation

Formamide denaturation is suitable for almost all RNA samples and is recommended if you need to retain biological activity. Gels can be cast and run in standard TAE or TBE buffer systems or MOPS buffer. If there is a significant amount of secondary structure, another sample denaturation method should be chosen.

Formamide denaturation of RNA samples

1. Bring the RNA volume up to 8 µl with RNase-free water.
2. Add 2 µl of 10X MOPS buffer.
3. Add 9 µl of deionized formamide.
4. Mix thoroughly.
5. Heat at 70°C for 10 minutes.
6. Chill on ice for at least 1 minute before loading.

Materials

- **Water bath set to 70°C**
- **Ice**

Reagents

- **AccuGENE® Molecular Biology Water (RNase-free)**
- **Deionized formamide**
- **AccuGENE 10X MOPS Buffer** (200 mM MOPS, pH 7.0, 50 mM sodium acetate, 10 mM EDTA, 10 mM EGTA) pH 7.0

Continued on page 122

Materials

- **Water bath set to 70°C**
- **Ice**

Reagents

- **AccuGENE® Molecular Biology Water (RNase-free)**
- **AccuGENE 10X MOPS Buffer**
  (200 mM MOPS, pH 7.0, 50 mM sodium acetate, 10 mM EDTA, 10 mM EGTA) pH 7.0
- **37% (v/v) formaldehyde**
- **Deionized formamide**

Formaldehyde denaturation

Formaldehyde denaturation is suitable when samples are to be recovered. It is necessary to ensure that the formaldehyde is fully removed from the recovered RNA prior to subsequent studies. Some enzymatic reactions, such as *in vitro* transcription, may be problematic even after complete removal of the formaldehyde.

Formaldehyde denaturation of RNA samples

1. Bring the RNA volume up to 6 µl with RNase-free water.
2. Add 2 µl of 10X MOPS buffer.
3. Add 2 µl of 37% formaldehyde.
4. Add 9 µl deionized formamide.
5. Mix thoroughly.
6. Heat at 70°C for 10 minutes. Chill on ice for at least 1 minute before loading.

Materials

- **Water bath set to 50°C**
- **Ice**

Reagents

- **AccuGENE Molecular Biology Water (RNase-free)**
- **DMSO**
- **100 mM Sodium phosphate, pH 7.0**
  Mix 5.77 ml of 1 M Na_2HPO_4 with 4.23 ml of 1 M NaH_2PO_4
  Adjust volume to 100 ml with RNase-free water.
- **6 M Glyoxal, 40% (v/v) solution, deionized immediately before use.**
  Pass solution through a small column of mixed-bed ion exchange resin until the pH is >5.0. Large volumes can be deionized then stored frozen in aliquots at –20°C.

Glyoxal denaturation

Glyoxal is a very efficient denaturant, but should not be used if samples are to be recovered. Glyoxal denatures RNA by introducing an additional ring into the guanosine residues, thus interfering with G-C base pairing. Glyoxal denaturation would be the recommend procedure for Northern blotting. Typically a phosphate electrophoresis buffer is recommended with recirculation to prevent formation of a pH gradient. Alternatively, a 10 mM PIPES, 30 mM bis-Tris buffer, or a 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 1 mM EGTA buffer systems can also be used without recirculation.

Glyoxal denaturation of RNA samples

1. Bring the RNA volume up to 11 µl with RNase-free water.
2. Add 4.5 µl of 100 mM Sodium phosphate.
3. Add 22.5 µl of DMSO.
4. Add 6.6 µl of deionized glyoxal.
5. Mix thoroughly.
6. Heat at 50°C for 1 hour. Chill on ice for at least 1 minute before loading.

Buffers for Electrophoresis

The two commonly used buffer systems for RNA electrophoresis are a phosphate buffer for glyoxal/DMSO denatured RNA and a MOPS buffer for formaldehyde or formamide denatured RNA. These buffers are very low in ionic strength. During electrophoresis, a pH gradient may be generated along the length of the gel, resulting in the hydrolysis (melting) of the agarose gel. This problem can be avoided by recirculating the buffer. For glyoxal-denatured gels, if the pH of the buffer rises above pH 8.0, the glyoxal will dissociate from the RNA, causing the RNA to renature and migrate in an unpredictable manner.

Buffer preparation

NOTE: Use RNase-free chemicals, water and containers

100 mM Sodium Phosphate Buffer, pH 7.0

(GLYOXAL/DMSO DENATURED RNA)	g/l
1 M Na_2HPO_4	57.7 ml
1 M NaH_2PO_4	42.3 ml

Adjust volume to 1 liter with RNase-free water.

Adjust volumes accordingly to prepare more buffer.

10X MOPS Buffer

(FORMALDEHYDE OR FORMAMIDE DENATURED RNA)	g/l
200 mM MOPS (free acid)	41.86 g
50 mM Sodium acetate	6.80 g
10 mM EDTA•$2H_2O$	3.72 g
10 mM EGTA (free acid)	3.80 g

Mix with 850 ml of distilled water.

Adjust pH to 7.0 with 10 M NaOH.

Adjust volume to 1 liter with RNase-free water.

Filter through a 0.2 µm nitrocellulose filter and store in the dark.

Electrophoresis of RNA

The choice of an agarose free of RNase contamination is of major importance. Cambrex offers a variety of agarose products for RNA electrophoresis including Reliant® and Latitude® Precast RNA Gels, Cambrex Agarose, AccuGENE® 10X MOPS Buffer, RNA Marker, GelStar® and SYBR® Green Nucleic Acid Gel Stains.

General guidelines

- Northern blotting requires a standard melting temperature agarose such as SeaKem® LE or NuSieve® 3:1 Agarose or Reliant or Latitude Precast RNA Gels.
- If samples are to be recovered, a low melting temperature agarose can be used such as NuSieve GTG® or SeaPlaque® GTG Agarose.
- A 1.5% - 2.0% gel made with SeaKem GTG or SeaKem Gold Agarose or Reliant or Latitude Precast RNA Gels will work for RNA molecules of 500 - 10,000 nucleotides.
- For RNA smaller than 500 nucleotides, use a 3% or 4% NuSieve 3:1 or MetaPhor® Agarose gel.
- For RNA larger than 10,000 nucleotides, SeaKem Gold Agarose and Reliant or Latitude Precast RNA Gels will be a better choice for tighter bands and better resolution.
- If a low melting temperature agarose is required, a 1.5% or 2.0% SeaPlaque GTG gel can be used for separation of RNAs from 500 - 10,000 nucleotides, while a 3.0% or 4.0% NuSieve GTG gel should be used for fine resolution of RNAs smaller than 500 nucleotides. NuSieve GTG Agarose is not recommended for formaldehyde/MOPS gels.

Agarose selection guide for RNA electrophoresis

	Separation	Northern Blotting	Preparative
Fragments <500 bases	NuSieve® 3:1 Agarose MetaPhor® Agarose Reliant® RNA Gel System Latitude® RNA Midigels	NuSieve 3:1 Agarose Reliant RNA Gel System Latitude® RNA Midigels	NuSieve GTG Agarose
Fragments between 500-10,000 bases	SeaKem® LE Agarose SeaKem GTG Agarose Reliant RNA Gel System Latitude RNA Midigels	SeaKem LE Agarose SeaKem GTG Agarose Reliant RNA Gel System Latitude RNA Midigels	SeaPlaque GTG Agarose
Fragments >10,000 bases	SeaKem Gold Agarose Reliant RNA Gel System Latitude RNA Midigels	SeaKem Gold Agarose Reliant RNA Gel System Latitude RNA Midigels	SeaPlaque GTG Agarose

Dye mobilities for RNA gels

The following table is a migration table of single-stranded RNA in relation to bromophenol blue (BPB) and xylene cyanol (XC) in formaldehyde or glyoxal agarose gels.

	Formaldehyde gels		Glyoxal gels	
Agarose (% w/v)	**XC**	**BPB**	**XC**	**BPB**
SeaKem® Gold Agarose				
1.0	6,300	660	9,500	940
1.5	2,700	310	4,300	520
2.0	1,500	200	2,300	300
SeaKem GTG® and SeaKem LE Agarose				
1.0	4,200	320	7,200	740
1.5	1,700	140	2,800	370
2.0	820	60	1,600	220
SeaPlaque® and SeaPlaque GTG Agarose				
1.0	2,400	240	4,400	400
1.5	800	80	1,900	180
2.0	490	30	1,050	120
NuSieve® 3:1 Agarose				
2.0	950	70	1,600	155
3.0	370	20	740	75
4.0	190	5	370	40
NuSieve GTG and MetaPhor® Agarose				
2.0	Not applicable		1,300	150
3.0	Not applicable		480	60
4.0	Not applicable		260	40

Materials
- Water bath set to 60°C
- Fume hood
- Accessories to cast an agarose gel
- Electrophoresis chamber and power supply
- Flask or beaker

Reagents
- AccuGENE® 10X MOPS Buffer prewarmed to 60°C
- 1X MOPS Buffer 37% (v/v), Formaldehyde prewarmed to 60°C
- Formaldehyde loading buffer (1 mM EDTA, pH 8.0, 0.4% bromophenol blue and xylene cyanol, 50% glycerol)
- Distilled water

Caution
Formaldehyde is a confirmed carcinogen. Solutions of formaldehyde should be handled in the fume hood. Gloves, labcoat, and safety glasses should be worn when handling solutions containing formaldehyde. Electrophoresis tanks should be kept covered during electrophoresis.

Electrophoresis of gels containing formaldehyde

A formaldehyde denaturant with a MOPS buffer is the most commonly used system for RNA electrophoresis. Care should be taken when handling gels containing formaldehyde. These gels are less rigid than other agarose gels. NuSieve® GTG® Agarose is not compatible with the MOPS buffering system.

Formaldehyde electrophoresis of RNA

NOTE: Do not exceed 20 µg of RNA per lane as larger amounts can result in loss of resolution.

1. For a 1% gel, dissolve 1.0 g of agarose in 72 ml of water. Adjust the amounts for different percent gels.
2. Cool agarose to 60°C in hot water bath.
3. Place in fume hood.
4. Immediately add 10 ml of prewarmed 10X MOPS buffer.
5. Add 5.5 ml of prewarmed 37% formaldehyde.
6. Cast gel in a fume hood.
7. Denature the RNA sample following one of the methods previously described.
8. Add 2 µl of formaldehyde loading buffer per 20 µl of sample.
9. Mix thoroughly.
10. Remove the gel comb.
11. Place the gel in the electrophoresis chamber.
12. Cover surface of the gel to a depth of 1 mm with 1X MOPS buffer.
13. Load the samples.
14. Electrophorese at a maximum of 5 V/cm (interelectrode distance) until the bromophenol blue has traveled at least 80% of the way through the gel (see mobility table on page 125).

Electrophoresis of gels containing glyoxal/DMSO

These gels should be run slower than formaldehyde gels with buffer recirculation to avoid the formation of a pH gradient. Glyoxylated RNA will give sharper bands than formaldehyde-treated RNA.

Glyoxal/DMSO electrophoresis of RNA

1. For a 1% gel, dissolve 1.0 g of agarose in 100 ml of 10 mM sodium phosphate, pH 7.0. Adjust amounts for different percent gels.
2. Cool agarose to 60°C in hot water bath.
3. Cast the gels to a thickness that will accommodate a loading volume of 60 µl.
4. Remove the comb.
5. Place the gel in the electrophoresis chamber.
6. Cover gel with 10 mM sodium phosphate buffer to a depth of 1 mm.
7. Add 12 µl of glyoxal loading buffer per 45 µl of sample.
8. Mix thoroughly.
9. Load 0.5 µg - 1.0 µg of RNA per lane.
10. Electrophorese at 4 V/cm (interelectrode distance) while the buffer is recirculated. If no recirculation apparatus is available, pause electrophoresis every 30 minutes and remix the buffer.
11. Electrophorese until the bromophenol blue has traveled at least 80% of the way through the gel (see mobility table on page 125).

Materials
- Water bath set to 60°C
- Fume hood
- Accessories to cast an agarose gel
- Electrophoresis chamber and power supply
- Flask or beaker
- Recirculating unit

Reagents
- 100 mM Sodium phosphate pH 7.0
- Glyoxal loading buffer
  (10 mM Sodium phosphate, pH 7.0, 0.25% bromophenol blue and xylene cyanol, 50% glycerol)

Detection of RNA in Agarose Gels

Detecting RNA with GelStar® or SYBR® Green II Gel Stains

Introduction

Detection of RNA in agarose gels varies depending on the denaturant, stain and photographic conditions used. Cambrex offers two highly sensitive stains, GelStar and SYBR Green II Gel Stains for the detection of RNA in agarose gels. These stains exhibit higher RNA detection sensitivity than ethidium bromide, allowing you to load less RNA sample on your gel. Unlike ethidium bromide, GelStar and SYBR Green II Stain only fluoresce upon binding to the nucleic acid resulting in lower background fluorescence which is particularly useful when including the stain in the gel for glyoxal denatured samples or when concentrations of formaldehyde in the gel exceed 2 M. The chart below shows the detection sensitivity of various dyes for RNA using in-gel or post staining techniques.

Stain	Method	Native RNA	Glyoxal RNA
GelStar® Stain	in-gel	10 ng	10 ng
Ethidium bromide, no destain	in-gel	30 ng	150 ng
Ethidium bromide, with destain	in-gel	30 ng	50 ng
SYBR® Green II Stain	post stain	15 ng	50 ng

Limits are based on optimal detection methods for each stain. Samples detected with SYBR Green Stain were post stained, samples detected with ethidium bromide or GelStar Stain were detected by in-gel staining (post-stained gels showed similar results).

Tips for staining gels with GelStar® or SYBR® Green II Gel Stains

Follow the guidelines below to increase the detection sensitivity of GelStar or SYBR Green II Gel Stains.

- New clear polypropylene containers (e.g., Rubbermaid® recycling #5 plastics) should be obtained for use with GelStar and SYBR Green Stains. When stored in the dark in polypropylene containers, the diluted stain can be used for up to 24 hours and will stain 2 to 3 gels with little decrease in sensitivity. The containers should be rinsed with distilled water (do not use detergents) after each use and dedicated to GelStar or SYBR Green Stain use only.
- These stains bind to glass and some non-polypropylene (polystyrene) plastics, resulting in reduced or no signal from the nucleic acid.
- A 2X working solution of GelStar or SYBR Green Stain should be prepared just prior to use from the 10,000X stock solution by diluting in 1X MOPS buffer or a pH 7.5 to 8.5 buffer (e.g., TAE, TBE or TE).
- For nondenaturing or denaturing polyacrylamide gels dilute GelStar or SYBR Gel Stain 1:10,000X in 1X electrophoresis buffer.
- Agarose gels should be cast no thicker than 4 mm. As gel thickness increases, diffusion of the stain into the gel is decreased, lowering the efficiency of RNA detection.
- Optimal sensitivity for GelStar and SYBR Green Stains is obtained by using the appropriate photographic filters for each stain.

  GelStar Stain: Wratten® or Tiffen® #9 filter

  SYBR Green Stains: Wratten or Tiffen #15 filter
- We do not recommend photographing gels with a 254 nm transilluminator. An outline of the UV light source may appear in photographs. A filter that will allow a 525 nm transmission and exclude infrared light is required.

Materials

- Clear polypropylene container (e.g., Rubbermaid® recycling #5 plastics)
- GelStar Photographic Filter (Wratten® #9 equivalent) or SYBR Green Photographic Filter (Wratten® #15 equivalent)
- Microcentrifuge
- UV transilluminator Dark Reader transilluminator or CCD imaging system.

Reagents

- 1X MOPS buffer
- GelStar or SYBR Green II Gel Stain stock solution

Cautions

GelStar and SYBR Green II Gel Stains should be handled with care and disposed of properly. Gloves, labcoat, and safety glasses should be worn when handling solutions of this dye and stained gels. Avoid skin and eye exposure to UV light.

Procedure for staining RNA with GelStar® or SYBR® Green II Gel Stains

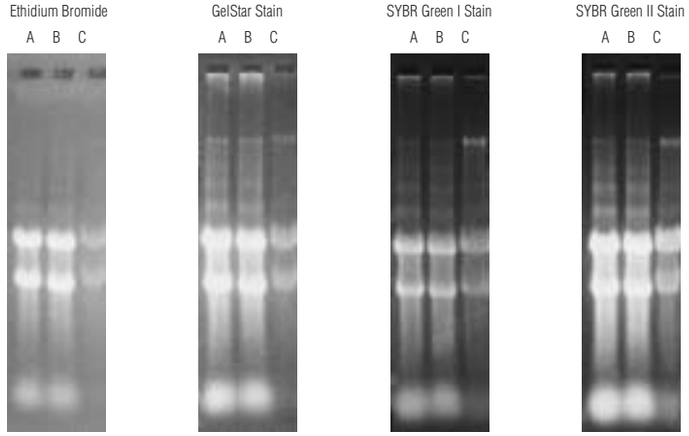
For optimal resolution, sharpest bands and lowest back ground, stain the gel with GelStar or SYBR Green II Gel Stain following electrophoresis.

The photographs below demonstrate the detection sensitivity of various stains with different sample denaturants.

Follow the steps below to stain RNA after electrophoresis

1. Remove the concentrated stock solution of the stain from the freezer and allow the solution to thaw at room temperature.
2. Spin the solution in a microcentrifuge to collect the dye at the bottom of the tube.
3. Dilute the 10,000X concentrate to a 2X working solution (2 µl/10 ml) in 1X MOPS buffer, in a clear plastic polypropylene container. Prepare enough staining solution to just cover the top of the gel.
4. Remove the gel from the electrophoresis chamber.
5. Place the gel in staining solution.
6. Gently agitate the gel at room temperature.
7. Stain the gel for 60 minutes.
8. Remove the gel from the staining solution and view with a transilluminator, CCD camera or Dark Reader™ transilluminator.

NOTE: Gels stained with GelStar or SYBR Green II Gel Stains do not require destaining. The dyes' fluorescence yield is much greater when bound to RNA than when in solution.

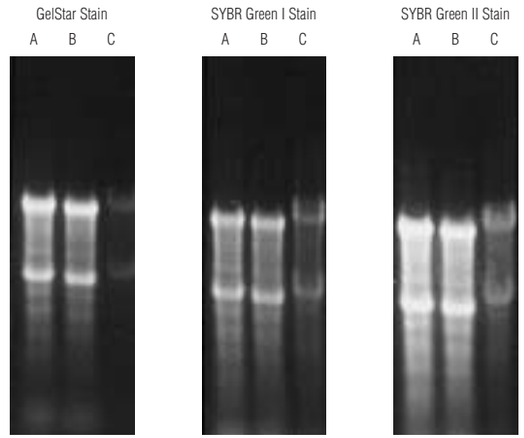


Follow this procedure when including GelStar® Nucleic Acid Gel Stain in the agarose gel

NOTE: Unlike ethidium bromide, GelStar Nucleic Acid Gel Stain does not interact with glyoxal.

1. Remove the concentrated stock solution of GelStar Stain from the freezer and allow the solution to thaw at room temperature.
2. Spin the solution in a microcentrifuge tube.
3. Prepare the agarose solution.
4. Once the agarose solution has cooled to 70°C, add the stain by diluting the stock 1:5,000 into the gel solution prior to pouring the gel (2 µl per 10 ml).
5. Slowly swirl the solution.
6. Pour the gel into the casting tray.
7. Load samples onto the gel.
8. Run the gel.
9. Remove the gel from the electrophoresis chamber.
10. View with a 300 nm UV transilluminator, CCD camera or Clare Chemical's Dark Reader™ transilluminator.

NOTE: GelStar stained gels do not require destaining. The dye's fluorescence yield is much greater when bound to RNA than when in solution.



Samples of *E. coli* total RNA were denatured using the following denaturants: Lane A: Formaldehyde/Formamide; Lane B: Formamide; Lane C: Glyoxal. Samples were loaded at 2 mg/lane for the formaldehyde/formamide and formamide only denatured samples, and 4 mg/lane for the glyoxal denatured samples. Reliant® RNA Precast Agarose Gels were run at 7 V/cm for 40 minutes in 1X MOPS buffer (prepared from AccuGENE® 10X MOPS Buffer) and post stained using GelStar® or SYBR® Green II Stain or ethidium bromide. The left series of photographs were taken using a Polaroid® camera, SYBR® Green Photographic Filter on a UV light box. The right series of photographs were taken using the SYBR Green Photographic Filter on the Clare Chemical's Dark Reader™ transilluminator.

Materials

- Staining vessel larger than the gel
- UV transilluminator, Dark Reader™ transilluminator or CCD imaging system
- Magnetic stir plate
- Magnetic stir bar

Reagents

- Ethidium bromide stock solution (10 mg/ml)
  (1.0 g Ethidium bromide,100 ml distilled water, stir on magnetic stirrer for several hours, transfer the solution to a dark bottle, store at room temperature)
- Distilled water
- 0.1 M ammonium acetate

Caution

Ethidium bromide is a powerful mutagen. Gloves should be worn when handling solutions of this dye and stained gels. Also, avoid skin and eye exposure to UV light; See Sambrook, *et al.*, p. 6.19 (1989).

Visualization by photography

Gels stained with GelStar® and SYBR® Green II Gel Stains exhibit negligible background fluorescence, allowing long film exposures when detecting small amounts of RNA. Use the appropriate photographic filter for the stain you are using (see page 129).

The table below provides suggested film types and photographic conditions

Polaroid® Film	f-stop	Exposure time
Type 57 or 667	4.5	0.5-2 seconds
Type 55	4.5	15-45 seconds

Visualization by image capture system

GelStar and SYBR Green II Nucleic Acid Gel Stains are compatible with most CCD and video imaging systems. Due to variations in the filters for these systems you may need to purchase a new filter. Cambrex does not sell filters for this type of camera. Contact your systems manufacturer and using the excitation and emission information listed they can guide you to an appropriate filter.

Stain	Emission (nm)	Excitation (nm)
GelStar Stain	527	493
SYBR Green I Stain	521	494
SYBR Green II Stain	513	497

Application notes

- The fluorescent characteristics of GelStar and SYBR Green II Gel Stains make them compatible with argon ion lasers.
- Gels previously stained with ethidium bromide can subsequently be stained with GelStar or SYBR Green II Gel Stain following the standard protocol for post-staining. There will be some decrease in sensitivity when compared to a gel stained with only GelStar or SYBR Green II Gel Stain.
- We recommend the addition of 0.1% to 0.3% SDS in the prehybridization and hybridization solutions when performing Northern blots on gels stained with these dyes.

Detecting RNA with Ethidium Bromide

Introduction

Ethidium bromide does not stain RNA as efficiently as it does DNA, so be certain that sufficient RNA is loaded to see the band of interest. Although as little as 10 ng - 20 ng of DNA can usually be visualized by ethidium bromide fluorescence, as much as 10-fold more RNA may be needed for good visualization. Visualization of poorly staining RNA is made even more difficult by the higher background fluorescence of RNA gels. In formaldehyde gels, background fluorescence can be minimized by dropping the formaldehyde concentration in the gel from 2.2 M to 0.66 M. Staining with ethidium bromide is not recommended when performing Northern blot analysis onto nylon membranes.

Follow the steps below to stain RNA after electrophoresis

1. Prepare enough working solution of ethidium bromide (0.5 mg/ml of ethidium bromide in 0.1 M ammonium acetate) to cover the surface of the agarose gel.
2. Remove the gel from the electrophoresis chamber.
3. Submerge the gel for 30 - 45 minutes in the ethidium bromide solution.
4. Remove the gel from the solution.
5. Submerge the gel for 60 minutes in a new container filled with distilled water.

   NOTE: For gel concentrations of 4% or greater, these times may need to be doubled. If after destaining the background is still too high, continue to destain.
6. Repeat in fresh distilled water.
7. Gels can be viewed with a UV light transilluminator, Dark Reader™ transilluminator or CCD imaging system.

Continued on page 134

Follow the steps below when including ethidium bromide in the agarose gel

1. Prepare agarose solution.
2. While the agarose solution is cooling, add ethidium bromide to a final concentration of 0.5 µg/ml to the solution.
3. Slowly swirl the solution.
4. Pour the gel into the casting tray.
5. Add ethidium bromide to the running buffer to a final concentration of 0.5 µg/ml.
6. Load and run the gel.
7. Destain the gel by submerging the gel in distilled water for 60 minutes.
8. Repeat in fresh distilled water.
9. Gels can be viewed with a UV light transilluminator, Dark Reader™ transilluminator or CCD imaging system.

Decontamination of GelStar® and SYBR® Green Gel Stains and ethidium bromide solutions

Staining solutions should be disposed of by passing through activated charcoal followed by incineration of the charcoal. For absorption on activated charcoal, consult Sambrook, *et al.*, pp. 6.16 - 6.19, (1989). Solutions can also be passed through Schleicher & Schuell®'s S&S® Extractor™ Ethidium Bromide Waste Reduction System, followed by the incineration of the filter.

Northern Blotting

Recommended agaroses for Northern blotting

When transferring RNA from an agarose gel to a membrane, a standard melting temperature agarose should be used. Standard melting temperature agaroses have higher gel strength than low melting temperature agaroses and do not fracture during transfer. The table below is a list of Cambrex Agaroses that are recommended for blotting.

Agarose	Size Range (bases)
NuSieve® 3:1 Agarose	<500
SeaKem® LE Agarose	500 - 10,000
SeaKem GTG® Agarose	500 - 10,000
SeaKem Gold Agarose	500 - 10,000
Reliant® & Latitude® RNA Gel Systems	25 - 10,000

Tips for agarose gel preparation

- Use the lowest agarose concentration required to resolve your fragments.
- If staining with ethidium bromide prior to transfer, thoroughly destain the gel with distilled water.
- Avoid casting thick gels (>4 mm) unless absolutely necessary. Thick gels not only require longer electrophoretic times but may interfere with the free transfer of nucleic acids to the hybridization membrane.
- Do not use more than a 500.0 gm weight on top of the stack. Excess weight can compress the gel. This will increase the agarose concentration and decrease the pore size, which can inhibit movement of the buffer and RNA.

Continued on page 136

Choosing the appropriate membrane

When transferring RNA, nylon membranes have several advantages over nitrocellulose:

Nylon	Nitrocellulose
Uncharged/charged	Supported/unsupported
High strength	Good strength*
Good for reprobing	Poor for reprobing
UV crosslinking	Baking in vacuum oven at 80ºC
50 nucleotides**	500 nucleotides**

**Good for supported nitrocellulose, poor for unsupported nitrocellulose*
***Lower size limit for efficient nucleic acid retention*

Choosing a blotting method

Consider the following when choosing a blotting method:

- Gel concentration
- Fragment size
- Speed and transfer efficiency

Capillary transfer

Traditional passive capillary transfer uses paper towels to draw the transfer buffer from a reservoir through the gel. Passive capillary transfer takes 16 - 24 hours to complete. The most common complaints are poor transfer efficiency for larger RNA molecules and long transfer time.

Vacuum transfer

Vacuum transfer systems use negative pressure to pull the transfer buffer through the gel along with the nucleic acid. These systems take approximately 30 - 60 minutes to complete. Vacuum blotting can be useful for high concentration gels where compression of the gel is a concern.

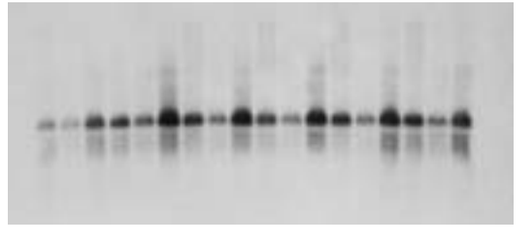
Electroblotting

Electroblotting is most often used for polyacrylamide gels. A voltage gradient pulls the sample out of the gel and on to a membrane. Semi-dry electroblotting requires minimal buffer with a low voltage and current. Care should be taken so smaller fragments are not pulled completely through the membrane.

The Schleicher & Schuell® TurboBlotter™ system

The Schleicher & Schuell TurboBlotter system is based on a traditional downward capillary transfer that takes advantage of gravity and does not cause excessive pressure on the gel resulting in high transfer efficiencies. With this system, precut blotting paper and a reusable tray are provided and transfer can be complete in 3 hours.

The photograph below demonstrates a Northern blot from Cambrex's Latitude® RNA Midigels using the Schleicher & Schuell TurboBlotter system.



Samples of mouse total RNA were denatured using a formaldehyde/formamide sample buffer system and then separated by electrophoresis at 5 V/cm for 1 hour in a 20 well precast Latitude® RNA Midigel. Sample loads consisted of 625 ng of total RNA per lane loaded in a repeating pattern of samples from brain, spleen, and liver (from left to right across gel). Samples were transferred to a nylon membrane using a 3 hour downward transfer protocol. The membrane was hybridized overnight using a biotin labeled probe for b-actin. Final detection was carried out using a chemiluminescent substrate and exposure to x-ray film (2 minute exposure).

Materials

- Nitrocellulose or nylon membrane
- 2-3 glass dishes larger than gel
- Paper towels
- Whatman® 3MM chromatography paper
- Glass or plastic pipette
- Flat ended forceps
- Glass plate to serve as a platform for the gel
- Plastic wrap
- <500.0 g weight
- Orbital or rocking platform shaker

Reagents

- AccuGENE® Molecular Biology Water
- AccuGENE 20X SSPE or AccuGENE MOPS Buffer

General guidelines for Northern blotting

- It is important to minimize RNase activity by following certain precautions. There are several agents on the market that effectively remove RNase's or consult Sambrook, *et al.*
- **Wear gloves** throughout the procedure. RNAs are not safe from nuclease degradation until they have been immobilized on the membrane.
- Cut or mark membrane for orientation. Do this before wetting the membrane.
- The blotting membrane should be in contact with the underside of the gel. Since nucleic acids will concentrate near the bottom of the gel there is less distance for them to travel during the transfer.
- Cut all papers and membranes to the correct size, such that, the only thing pulling the transfer buffer is the buffer solution.
- Avoid bubbles, ensure that there is even contact between all the layers of the blotting system.
- Incorrect denaturation of probe can cause poor transfer results.
- Glyoxal gels can be transferred immediately after electrophoresis. The glyoxal will be removed in the post transfer, prehybridization wash.
- Destain gels that contain formaldehyde or ethidium bromide to avoid sample loss and inefficient transfer efficiency.

  NOTE: Soak gel 3 times for 5 - 10 minutes each in either 1X MOPS, transfer buffer, or sterile water. This will remove most of the formaldehyde and excess background fluorescence seen with ethidium bromide. Complete destaining usually takes 2 hours or longer.

- Cambrex's Reliant® and Latitude® Precast RNA Gels do not contain any denaturants or stains and do not require destain prior to transfer.

Procedure for RNA transfer by passive capillary electrophoresis

Transfer buffer

The concentration of the transfer buffer will vary depending on the method and type of membrane used. The capillary method outlined here uses a nylon membrane and 5X SSPE buffer.

20X SSPE
(3 M NaCl, 0.2 M $NaH_2PO_4 \bullet H_2O$, 0.2 M EDTA • $2H_2O$)

175.3 g	NaCl
27.6 g	$NaH_2PO_4 \bullet H_2O$
7.4 g	EDTA • $2H_2O$

Adjust to pH 7.4 with 10 N NaOH
To 1 liter with distilled water

Continued on page 140

Materials

- Membrane
- Plastic wrap
- UV light source
- Vacuum dryer set to 80°C or hot oven at 65°C.

Reagents

- AccuGENE® 1 M Tris pH 8.0
- RNase free water
- AccuGENE Molecular Biology Water or AccuGENE 10X Mops Buffer
- AccuGENE 20X SSPE

Follow the steps below for gel preparation and setting up the transfer

1. Cast a standard melting temperature agarose gel no thicker than 4 mm.
2. Electrophorese RNA following standard protocols.
3. For formaldehyde gels follow the steps below prior to transfer. For glyoxal gels, proceed to step 4.

   3A. Soak the gel in an excess of 1X MOPS buffer, distilled water or 5X SSPE for 10 minutes.

   3B. Repeat three times with new wash solution each time.
4. Float membrane in RNase-free water for 5 minutes.
5. Equilibrate membrane in 5X SSPE for 5 minutes. The membrane may remain in the transfer buffer until it is used.
6. Set up transfer using either an upward capillary transfer set up or downward capillary transfer set up (see chapter IV).
7. Allow the transfer to proceed as follows:

Transfer set-up	Time
Upward	16 - 24 hours
Downward	1 - 4 hours depending on gel thickness and concentration
Turboblotter™	1 - 3 hours

8. Remove the paper toweling or chromatography paper.
9. Remove the gel and membrane together.
10. Mark the positions of the wells on the membrane with a pencil for orientation then remove the gel from the transfer setup.
11. Rinse the membrane for 30 - 60 seconds in transfer buffer.
12. Place the membrane on a sheet of Whatman® 3MM chromatography paper.
13. Treat membrane as described in Immobilizing RNA on a Membrane, next section.

Immobilizing RNA on a membrane

The methods and procedures for immobilizing RNA on the membrane are essentially the same as they are for DNA. Refer to Immobilizing DNA on a Membrane (see page 69).

If samples have been denatured under the formaldehyde system, immobilization can take place immediately after transfer without any pretreatment steps. The formaldehyde has been removed from the system prior to transfer during the gel destaining steps. If the glyoxal denaturing system was used, the glyoxal must now be removed from the filter. Follow one of the procedures below to remove glyoxal from the membrane.

Option 1

1. Air dry the membrane.
2. Bake the membrane as follows:

Membrane Type	Temperature	Time
Nitrocellulose	80°C	2 hours under vacuum
Nylon	65°C	1 - 1.5 hours

3. Immerse the filter in 200 ml of preheated 20 mM Tris, pH 8.0.
4. Cool to room temperature.

Option 2

1. Immobilize RNA on membrane either by UV irradiation or baking.
2. Wash membrane 65°C for 15 minutes in 20 mM Tris, pH 8.0.

References

Ausubel, F.E., *et al.*, *Current Protocols in Molecular Biology*, J. Wiley and Sons, Inc.,1998.
Farrell, R.E. Jr., *RNA Methodologies: A Laboratory Guide for Isolation and Characterization*, 2nd Edition, Academic Press, 1998.
Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory Press, 1989.
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Notes

Chapter IX

Special Applications in Agarose Gels

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Alkaline Gel Electrophoresis

Introduction

Sodium hydroxide is added to agarose gels to make them denaturing for the following reasons:

- To analyze single-stranded DNA
- To size cDNA strands in DNA/RNA hybrids
- To size first and second strands of cDNA
- To check for nicking activity in enzyme preparations used for molecular cloning

Procedure for preparing and running alkaline agarose gels

1. Prepare agarose solution in distilled water. Do not add NaOH to a hot agarose solution, as it causes hydrolysis of the agarose polysaccharide.
2. Cool the solution to 50°C - 60°C.
3. Add NaOH to the agarose solution to a final concentration of 50 mM.
4. Add EDTA (pH 8.0) to the agarose solution to a final concentration of 1 mM.
5. Cast the gel as described in Chapter I.
6. Allow the gel to set at room temperature.
7. Place the gel in the electrophoresis chamber.
8. Add 1X Alkaline electrophoresis buffer until it is 3 mm - 5 mm over the surface of the gel.
9. Add 0.2 volumes of 6X Alkaline loading buffer to DNA sample.
10. Load the samples onto the gel.
11. Electrophoresis at 0.25 V/cm until the bromocresol green has migrated out of the well and into the gel. Alkaline gels draw more current than neutral gels at comparable voltages and will heat up during electrophoresis.
12. Place a glass plate on top of the gel. This will keep the bromocresol green from diffusing into the buffer.
13. Electrophoresis at 0.25 - 1.8 V/cm until the dye has migrated approximately two thirds of the length of the gel.

Materials

- **Horizontal electrophoresis chamber**
- **Power supply**
- **Glass plate**

Reagents

- **Standard melting temperature agarose**
- **Distilled water**
- **0.5 M EDTA (pH 8.0)**
- **10 N NaOH**
- **6X Alkaline loading buffer**
  - 300 mN NaOH
  - 6 mM EDTA
  - 18% Ficoll® (type 400) polymer in distilled water
  - 0.15% Bromocresol green*
  - 0.25% Xylene cyanol FF
- **1X Alkaline electrophoresis buffer**
  - 50 mN NaOH
  - 1mM EDTA
  - Prepared fresh.

* Bromocresol green is used in place of bromophenol blue because it displays a more vivid color than bromophenol blue under alkaline pH.

Preparing alkaline gels for autoradiography

1. Remove the gel from the electrophoresis chamber.
2. Soak in 7% trichloroacetic acid (TCA) for 30 minutes at room temperature.
3. Mount the gel on a glass plate.*
4. Place several layers of paper towels on top of the gel.
5. Weigh down the paper towels with the second glass plate.
6. Allow the gel to dry for several hours.
7. Remove top glass and paper towels
8. Cover the gel with plastic wrap.
9. Expose to X-ray film.

*Alternatively gels can be dried under vacuum. Do not apply heat as this could melt the gel.

Materials and Reagents

- **Two glass plates larger than gel**
- **Paper towels**
- **OR a vacuum gel dryer**
- **Plastic wrap**
- **X-ray film**
- **7% Trichloroacetic acid (TCA)**

Continued on page 146

Staining and recovering fragments from alkaline gels

1. Remove gel from chamber and cover with plastic wrap.
2. Expose wet gel to X-ray film.
3. Excise band of interest.

OR

1. Remove gel from chamber.
2. Soak in 1 M Tris-HCl (pH 7.6), 1.5 M NaCl for 45 minutes.
3. Stain gel in GelStar® or SYBR® Green I stain or 0.5 µg/ml ethidium bromide in 1X TAE.
4. Excise band of interest.

Materials and Reagents

- Plastic wrap
- X-ray film
- Scalpel or razor blade
- 1 M Tris-HCl (pH 7.6), 1.5 M NaCl
- GelStar or SYBR Green I Nucleic Acid Gel Stain or 0.5 µg/ml of ethidium bromide in 1X TAE solution

Preparing gels for alkaline blotting

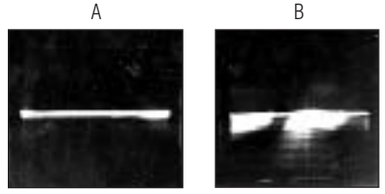
If the DNA will be detected by hybridization, gels should be neutralized in 1 M Tris-HCl (pH 7.6), 1.5 M NaCl for 45 minutes. The DNA can then be transferred to a nitrocellulose or nylon membrane.

Oligonucleotide Purification from MetaPhor® Agarose Gels

Introduction

Synthetic oligonucleotides often consist of the product of the desired length, and smaller failure sequences. Some protocols, for example gel shift analysis, require that the full length oligonucleotide is purified from failure sequences. Denaturing polyacrylamide gel electrophoresis is commonly used for fractionating oligonucleotides because of its resolving properties. However, acrylamide is toxic and preparation of denaturing polyacrylamide gels is laborious. Cambrex's fine-sieving MetaPhor Agarose can be used to purify synthetic oligonucleotides with an efficiency equal to that of denaturing polyacrylamide gel electrophoresis. MetaPhor Agarose has the advantage over polyacrylamide gel purification in that horizontal MetaPhor Agarose gels are convenient to cast, faster to run, and denaturant systems are not required. In addition, the recovery of oligonucleotides from MetaPhor Agarose gels is easier than from polyacrylamide gels.

The photographs below show that oligonucleotides can be purified by electrophoresis in MetaPhor Agarose gels as well as those fractionated in denaturing polyacrylamide.



Fractionation of oligonucleotides by agarose gel electrophoresis. A 60 base oligonucleotide was separated on a MetaPhor® Agarose gel (A) and on a denaturing polyacrylamide gel (B). Gels were stained with ethidium bromide and photographed on a UV transilluminator. *Courtesy of Shu Huang at Lifecodes Corporation.*

Isolation of oligonucleotides by gel electrophoresis. The 60 base crude sample (lane 1), DNA recovered from polyacrylamide (lane 2) and MetaPhor® Agarose (lane 3) were fractionated by gel electrophoresis in 10% denaturing polyacrylamide and visualized by ethidium bromide staining. *Courtesy of Shu Huang at Lifecodes Corporation.*

Materials

- Horizontal electrophoresis chamber to accommodate at least a 20 cm long gel
- Power supply
- Preparative comb
- Magnetic stir plate
- Magnetic stir bar
- Microwave

Reagents

- MetaPhor Agarose
- 1X TBE buffer (chilled)
- Distilled water
- GelStar® or SYBR® Green I Nucleic Acid Gel Stain or ethidium bromide

Applications

Oligonucleotides purified by electrophoresis in MetaPhor® Agarose gels perform as well as those fractionated in denaturing polyacrylamide in several commonly used techniques, such as:

- Radiolabeling
- Nonisotopic labeling for chemiluminescent detection
- Sequencing
- PCR† primers
- Hybridization experiments

Agarose concentration

IF THE OLIGONUCLEOTIDE IS . . .	PREPARE A . . .
≤50 bases	6% gel
>50 bases	5% gel

Procedure for gel preparation and casting

Stage 1 To disperse MetaPhor Agarose in 1X TBE buffer.

1. Chill buffer to 4°C prior to addition of agarose.
2. Slowly sprinkle the desired amount of agarose into the proper volume of cold TBE buffer. Prevent clumping by continually stirring the buffer during the addition of the agarose powder.
3. Allow the agarose/buffer solution to sit on the bench top at room temperature for 15 - 30 minutes. (This helps prevent agarose from boiling over when dissolving).

Stage 2 To dissolve MetaPhor Agarose.

1. Microwave on high for 1 minute.
2. Remove solution from microwave and allow to sit on bench top for 15 minutes. (This helps prevent agarose from foaming over).
3. Microwave the agarose on high in 1 minute pulses (swirl during pauses) until the agarose is completely dissolved.
4. Reconstitute to the desired volume with hot distilled water.

Stage 3 To cast MetaPhor® Agarose gel.

1. Allow the agarose solution to cool to 70°C - 75°C.
2. Cast solution in horizontal chamber.
3. Allow gel to set at room temperature.
4. Chill gel at 4°C for 30 minutes prior to use.

Procedure for gel loading and electrophoresis

1. While the comb is in position, flood the surface of the gel with 3 mm of 1X TBE buffer.
2. Place the gel in the electrophoresis chamber.
3. Add electrophoresis buffer to the chamber.
4. Slowly remove the comb.
5. Load the oligonucleotide onto the gel.
6. Electrophorese the oligonucleotide at 5 V/cm of interelectrode distance.
7. Stop electrophoresis when the bromophenol blue tracking dye is approximately 1 cm from the bottom of the gel.

Detection

Oligonucleotides can be detected by the following methods:

- GelStar® Nucleic Acid Gel Stain — see page 42
- SYBR® Green I Nucleic Acid Gel Stain — see page 42
- Ethidium bromide detection — see page 48

Purification from MetaPhor® Agarose

Oligonucleotides can be purified from MetaPhor Agarose by the following methods:

- Electroelution — see page 93
- Used “In-Gel” — see page 72

Amplification of Plasmid cDNA Libraries with SeaPrep® Agarose

Materials

- Autoclave
- 2 L Erlenmeyer Flasks
- Water bath set to 37°C
- pH meter
- Sterile 50 ml polypropylene centrifuge tubes
- Ice water bath
- Sterile 500 ml centrifuge bottle
- Centrifuge
- Sterile Nunc® tubes
- 0.2 µm filter

Reagents

- SeaPrep® Agarose
- 2X L Broth (LB)
- 50 mg/ml ampicillin
- Double distilled water
- Bacto-tryptone
- Bacto-yeast extract
- NaCl
- 1 N NaOH or 1 N HCl
- pH standards
- LB-amp plates
- Filter sterilized glycerol

Introduction

This section describes an amplification technique developed to address the common problem of disproportionate amplification of plasmids seen when expression cloning mRNAs of very low abundance encoding cytokines, receptors, and cell surface molecules from plasmid cDNA libraries derived from highly complex tissue sources. This amplification technique allows amplification of a plasmid cDNA library in a representative fashion, decreasing the possibility that less abundant clones would vanish during the amplification due to differential rates of replication.

The technique below describes a method where bacterial transformants are suspended, not plated, in low gelling temperature agarose. With this method, it has been found that, from generation to generation of amplification, the relative abundance of bacterial cells containing plasmids encoding selected hematopoietic factors varies less than two fold.

Preparing the bacterial growth media plus agarose

NOTE: Alternatively, prepared solutions of L Broth, LB Broth (Cambrex) or Super Broth (Cambrex) can also be used.

1. In a 2 liter Erlenmeyer flask, add the following reagents to 1 liter of double distilled water:

1A. 20.0 g bacto-tryptone

1B. 10.0 g bacto-yeast extract

1C. 10.0 g NaCl

2. Measure pH of solution.

3. If necessary, adjust to pH 7.0 using 1 N NaOH or 1 N HCl.

4. Add 3.0 g of SeaPrep Agarose to the solution.

5. Autoclave the solution on a liquid cycle for 15 - 20 minutes.

6. Cool the solution to 40°C.

7. Store in a 37°C water bath until ready-for-use.

8. Prior to use add 2 ml of 50 mg/ml ampicillin.

cDNA/vector annealing reactions

1. Prepare vector using a tailing method.

2. Prepare and purify cDNA following standard procedures.

3. Optimize the tailing of cDNA inserts following standard procedures.

4. Scale up the appropriate cDNA/vector-annealing reaction to generate up to 2.5 x 10^6 cfu/2000 ml.

5. Perform the appropriate number of standard bacterial transformations following standard procedures.

6. Pool all the transformations after the 37°C incubation.

NOTE: General procedures for vector and cDNA preparation, ligation and transformation can be found in Sambrook, J., *et al.* and Kriegler, M.

Plasmid cDNA library amplification

1. Add ampicillin to a final concentration of 100 µg/ml (2.0 ml of 50 mg/ml stock solution) to the 2X LB-agarose solution.

2. Add transformants (≤1.25 x 10^6 cfu) to the LB-agarose solution.

3. Gently swirl to avoid foaming.

4. Aliquot the transformation mix into 25 ml aliquots in 50 ml polypropylene centrifuge tubes.

5. Place the tubes in an ice-water bath for 20 - 60 minutes to allow the agarose gel to set.

6. Incubate overnight at 37°C undisturbed.

7. Plate 100 µl of the cell-agarose suspension directly on LB-amp plates for titer determination or prepare the suspension for library storage.

Continued on page 152

Library Storage

1. Prepare 12.5% glycerol by diluting in double distilled water.
2. Filter solution through a 0.2 µm filter into a sterile container.
3. Pour the colony-containing gel into a sterile 500 ml centrifuge bottle.
4. Pellet the cells at 8,000 rpm for 20 minutes at room temperature. It is not necessary to melt the gel, the cells will pellet through the soft agarose.
5. Decant the media from the cell pellets.
6. Resuspend the cell pellets in 100 ml of 12.5% glycerol in 2X L broth.
7. Aliquot into sterile Nunc® tubes.
8. Store library at –70°C.

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Perez, C. and Kriegler, M., *Resolutions*, **6(4)**: 1 - 2, 1990.

Ausubel, F.M., *et al.*, *Current Protocols in Molecular Biology*, Wiley & Sons, 1995.

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Drying Agarose Gels without a Vacuum Gel Dryer

Introduction

Occasionally, agarose gels are dried for the purpose of autoradiography, in-gel hybridization or as a preservation measure. In this section, we discuss techniques for drying gels without using a vacuum gel dryer.

Materials

- Scalpel or razor blade
- GelBond Film
- Glass plate or press board
- Clamps or elastic bands
- A forced hot-air oven

Reagents

- SeaKem® LE or GTG® Agarose
- SeaKem Gold Agarose
- NuSieve® 3:1 or NuSieve GTG Agarose
- MetaPhor® Agarose

Drying at room temperature

To dry an agarose gel at room temperature, the gel must have first been cast onto GelBond® Film. This will prevent the gel from shrinking during the drying process.

1. Remove the gel from the electrophoresis chamber.
2. Remove 0.5 cm to 1 cm of agarose from each side of the gel, leaving the GelBond Film exposed.
3. Clamp the exposed GelBond Film, gel side up, to a glass plate or particle board using clamps or elastic bands. This will prevent the gel from curling during drying.
4. Allow to dry on the bench top at least 8 hours.

Drying in a forced hot-air oven

To dry an agarose gel in a forced hot-air oven, the gel must have first been cast onto GelBond Film. This will prevent the gel from shrinking during the drying process.

1. Set the temperature of the forced hot-air oven to 55°C.
2. Remove the gel from the electrophoresis chamber.
3. Remove 0.5 to 1 cm of agarose from each side of the gel, leaving the GelBond Film exposed.
4. Clamp the exposed GelBond Film, gel side up, to a glass plate or particle board using clamps or elastic bands. This will prevent the gel from curling.
5. Place in the oven and allow to dry for 1 to 4 hours, depending on the thickness of the gel.

Drying Agarose Gels with a Vacuum Gel Dryer

Materials
- Vacuum gel dryer with variable temperature control
- Whatman 3MM chromatography paper
- Plastic wrap
- GelBond Film

Reagents
- SeaKem® LE or GTG® Agarose
- SeaKem Gold Agarose
- NuSieve® 3:1 Agarose
- Distilled water

Introduction

Agarose gels can be dried in a standard vacuum gel dryer. This method has the advantage that the gels do not necessarily need to be cast on GelBond® Film. However, adhering the gel to GelBond Film will provide a stable support for the brittle, dried gel.

Drying gels cast on GelBond Film on a vacuum gel dryer

1. Set the temperature on the vacuum gel dryer to 60°C if you are not adhering GelBond Film to the gel during drying.

   WARNING: Use of a temperature of greater than 60°C may cause the gel to melt.

2. Place three sheets of Whatman® 3MM chromatography paper onto the vacuum gel dryer.
3. Remove the gel from the electrophoresis chamber.
4. Place the gel onto the Whatman 3MM chromatography paper.

   NOTE: If the gel is adhered to GelBond Film, the gel must be between the GelBond Film and the vacuum source.

5. Cover the gel with plastic wrap.
6. Turn on the vacuum and dry for 1 to 2 hours.

   WARNING: Radioactive DNA may leach out of agarose gels more easily than from polyacrylamide gels during vacuum drying. Test the vacuum drying equipment for radioactivity following drying.

7. After drying, remove the chromatography paper from the agarose gel by moistening the paper with distilled water, and carefully peeling it off the gel.

   NOTE: Agarose gels do not adhere to chromatography paper as well as polyacrylamide gels. The gel may spontaneously disadhere.

Adhering agarose gels to GelBond® Film while vacuum drying

Agarose gels not cast on GelBond Film prior to electrophoresis can be permanently adhered to GelBond Film during the drying process.

Materials and Reagents
- Whatman 3MM chromatography paper
- GelBond Film
- Plastic wrap
- Vacuum gel dryer with variable temperature control
- Distilled water

1. Place three sheets of Whatman® 3MM chromatography paper onto the vacuum gel dryer.
2. Remove the gel from the electrophoresis chamber.
3. Place the gel, bottom side facing up, onto the Whatman 3MM chromatography paper.
4. Place a sheet of GelBond Film onto the gel, hydrophilic side in contact with the gel.
5. Cover gel and GelBond Film with plastic wrap.
6. Turn on vacuum without heat for 30 minutes.
7. Set dryer temperature to 60°C and dry for 40 minutes.
8. After drying, remove the Whatman 3MM chromatography paper from the agarose gel by moistening the paper with distilled water, and carefully peeling it off the gel.

Preparing Agarose for use in Cell Culture Applications

Introduction

Agarose has unique properties that make it especially useful in cell culture media applications. Agarose can form stable gels at concentrations as low as 0.3% (w/v), has very low ionic concentrations and is free of contaminating impurities that may affect the growth characteristics of cells in culture. Agarose could be used directly without supplementation for plaque assays ensuring high cell variability. The use of agarose is suggested for media where the absence of a nutrient is mandatory.

Advantages

- Highly purified, eliminating contaminants
- Excellent optical transparency which enhances colony observation
- Gelling temperatures below 28°C allowing manipulation of cells in solution
- Can be used to produce a 'defined' media system

Applications

- Cell culture media
  - Mammalian
  - Plant
  - Bacterial
  - Viral
- Overlay assays
- Plaque hybridization assays
- Soft agarose hybridoma cloning
- Attachment dependent cell culture
- Colony lift assays
- Analysis of matrix and cell matrix interactions

Preparing the Agarose

Introduction

The concentration and type of agarose to use largely depends on your cell system, the application, and the lot specific gel strength of the agarose. There are three types of agarose which can be used for cell culture media, each having unique properties making one more suitable for a given application than another.

- **SeaKem® LE Agarose –** A standard melting and gelling temperature agarose which can be used as a solid medium to support cell growth by supplementation with growth factors and nutrients, top agar enriched with magnesium for baculovirus screening, substrate for bacterial growth and for colony lifts.
- **SeaPlaque® Agarose –** A low gelling temperature agarose (28°C) that remains a liquid at 37°C allowing the manipulation of cells within the solution. SeaPlaque Agarose can be used as a semi-solid media for anchorage independent assays, plaque assays or overlays. SeaPlaque Agarose has also been found to be very effective as a medium for protoplast culture.
- **SeaPrep® Agarose –** Unique ultra low gelling temperature (15°C) and gel strength agarose (>75 g/cm^2). SeaPrep Agarose is ideal for hybridoma cloning. Cells can be recovered from the gel by increasing the temperature slightly allowing transfer to a viable cell suspension for subsequent growth in liquid medium.

Suggested agarose products and concentration guidelines

The table below provides general guidelines on agaroses and gel concentrations for given applications. General Guidelines and specific information pertaining to a given cell type and application can be obtained from the current literature and *Cell Biology: A Laboratory Manual.*

Application	SeaKem® LE Agarose	SeaPlaque® Agarose	SeaPrep® Agarose
Mammalian cell culture	0.3% - 1%	0.25% - 1.5%	
Baculovirus screening		0.3% - 0.6%	
Bacterial culture	0.7% - 1.0%	1.0%	
Colony Lifts	0.7% - 1.0%	1.0%	
Anchorage independent assays	0.25% - 0.5%		
Plaque assays	0.3% - 1%	0.50% - 1.5%	
Overlays	0.3% - 0.6%	0.3% - 1%	
Protoplast culture		0.35% - 0.7%	
Hybridoma cloning			0.8% - 1.5%
Virus Enumeration			1%
Cell matrix interactions		1.0% - 3.0%	
Attachment Dependent Cell culture		0.6%	
In vitro cloning – mammalian	0.5% - 2%		1% - 4%

NOTES: Higher agarose concentration gels can affect and possibly restrict cell proliferation. Optimal gel concentrations should be determined for each culture system on a case-by-case basis.
In some cases, such as hybridoma cloning using SeaPrep Agarose it is advisable to sample different lots of agarose for the desirable gel strength qualities.

Agarose preparation

When preparing agarose for cell culture work, it is always best to prepare the agarose in water suitable for cell culture and separate from any growth media or nutrients. Agarose solutions and media solutions should be prepared at 2X concentrations (i.e., if desired final agarose concentration is 0.6%, prepare a 1.2% agarose solution), autoclaved separately, and aliquoted into useable aliquots.

Procedure for Autoclaving Agarose

Materials
- Flask that is 2-4 times the volume of the solution
- Autoclave
- Aluminum foil
- Sterile flasks or culture tubes

Reagents
- Agarose powder
- Water suitable for cell culture

1. Choose a flask that is 2 - 4 times the volume of the solution.
2. Add water to the flask.
3. Sprinkle in the pre-measured agarose powder at a 2X final agarose concentration.
4. Cover the flask with aluminum foil.
5. Place the flask in the autoclave.
6. Sterilize the agarose by autoclaving for 10 minutes at 15 lb/in^2. If using SeaPrep® Agarose, autoclave for no longer than 5 minutes.

   NOTE: Agarose may lose gel strength when exposed to longer periods of time in the autoclave.
7. Once the agarose solution has cooled, aliquot into useable aliquots and store at 4°C prior to use.

General Procedure for Using Agarose in Culture Medium

Materials
- Microwave or hot water bath

Reagents
- Gelled agarose solution
- 2X media solution

1. Remelt the agarose by placing in a hot water bath or microwave.
2. Allow the agarose solution to cool to 37°C.
3. Prewarm the 2X media solution to 37°C.
4. Mix equal volumes of the sterile 2X agarose solution with sterile 2X media containing growth factors and nutrients.
5. Cast the agarose/media solution into plates or sterile culture tubes.
6. Allow the agarose solution to gel for 20 minutes if using as a feeder or overlay or maintain the solution at 37°C if using as a liquid culture.

NOTE: A solution containing 1% SeaPlaque® Agarose will stay liquid for approximately 18 hours. The amount of time an agarose solution will stay in a liquid state at 37°C, largely depends on the agarose concentration (increased agarose concentrations will decrease the time the solution stays in a liquid state), the age of the agarose and the particular lot of agarose you are using. When purchasing a new lot of agarose, we recommend you test this prior to culturing cells.

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Chapter X

Reagents and Solutions for Nucleic Acid Separation

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Common Electrophoresis Buffers

TBE-Tris-borate, EDTA buffer*

5X STOCK SOLUTION	g/l FOR 5X STOCK SOLUTION
445 mM Tris base	54.0 g Tris base
445 mM Boric acid	27.5 g Boric acid
10 mM EDTA	3.72 g $Na_2EDTA \bullet 2H_2O$ (MW=372.24)
	Adjust to 1 liter with distilled water

1X WORKING SOLUTION	0.5X WORKING SOLUTION
89 mM Tris-borate	45 mM Tris-borate
2 mM EDTA	1 mM EDTA

TAE-Tris-acetate, EDTA buffer*

50X STOCK SOLUTION	g/l FOR 50X STOCK SOLUTION
2 M Tris base	242.2 g Tris base
2 M Glacial acetic acid	57.1 ml Glacial acetic acid
50 mM EDTA	18.61 g $Na_2EDTA \bullet 2H_2O$ (MW=372.24)
	Adjust to 1 liter with distilled water

1X WORKING SOLUTION
40 mM Tris-acetate
1 mM EDTA

TPE-Tris-phosphate, EDTA buffer

10X STOCK SOLUTION	g/l FOR 10X STOCK SOLUTION
900 mM Tris base	109.0 g Tris base
900 mM Phosphoric acid	15.5 ml 85% Phosphoric acid
20 mM EDTA	7.44 g $Na_2EDTA \bullet 2H_2O$ (MW=372.24)
	Adjust to 1 liter with distilled water

1X WORKING SOLUTION
90 mM Tris-phosphate
2 mM EDTA

MOPS buffer*

10X STOCK SOLUTION	g/l FOR 10X STOCK SOLUTION
200 mM MOPS (free acid)	41.86 g
50 mM Sodium acetate	6.80 g
10 mM EDTA $\bullet 2H_2O$	3.72 g
10 mM EGTA (free acid)	3.80 g
Bring volume to 850 ml with distilled water	
Adjust to pH 7.0 with 10 M NaOH	
Adjust volume to 1 liter with distilled water	
Filter through a 0.2 µm nitrocellulose filter	
Store solution protected from light	

1X WORKING SOLUTION
20 mM MOPS (free acid)
5 mM Sodium acetate
1 mM EDTA $\bullet 2H_2O$
1 mM EGTA (free acid)

* Alternatively Cambrex offers ready-to-use electrophoresis buffers; AccuGENE® TBE Buffer, AccuGENE TAE Buffer, and AccuGENE MOPS Buffer.

Stock Buffer Solutions

0.5 M EDTA, pH 8.0*
186.1 g $Na_2EDTA \bullet 2H_2O$ (MW=372.24)
Bring volume to 800 ml with distilled water
Mix
Adjust to pH 8.0 with NaOH pellets (approximately 20.0 g)
Adjust volume to 1 liter with distilled water

1 M Tris-HCl*
121.1 g Tris to 800 ml of distilled water
Adjust to desired pH with concentrated HCl
Adjust volume to 1 liter with distilled water

TE, pH 7.4*
10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0
10 ml 1 M Tris-HCl
2 ml 0.5 M EDTA, pH 8.0
Adjust volume to 1 liter with distilled water

1 N HCl
Mix in the following order:
913.8 ml distilled water
86.2 ml concentrated HCl

CAUTION: Always add the acid to the water. This reaction is exothermic and the solution should be prepared in the fume hood.

5 M NaCl*
292.0 g NaCl in 800 ml distilled water
Adjust volume to 1 liter with distilled water

1 M NaCl
58.4 g NaCl in 800 ml of distilled water
Adjust volume to 1 liter with distilled water

10 M NaOH
400.0 g NaOH in 800 ml distilled water
Adjust volume to 1 liter with distilled water

10 M Ammonium acetate
385.4 g Ammonium acetate in 150 ml distilled water
Adjust volume to 500 ml with distilled water

3 M Sodium acetate*
408.3 g Sodium acetate ($3H_2O$) in 800 ml distilled water
Adjust to pH 4.8 or 5.2 with 3 M acetic acid
Adjust volume to 1 liter with distilled water

0.1 M Sodium phosphate buffer, pH 7.0
57.7 ml of 1 M Na_2HPO_4
42.3 ml of 1 M NaH_2PO_4
Adjust volume to 1 liter with distilled water

1 M Na_2HPO_4 (Sodium phosphate-dibasic)
142.0 g Na_2HPO_4 in 800 ml distilled water
Adjust volume to 1 liter with distilled water

1 M NaH_2PO_4 (Sodium phosphate-monobasic)
120.0 g NaH_2PO_4 in 800 ml distilled water
Adjust volume to 1 liter with distilled water

* Alternatively Cambrex offers ready-to-use molecular biology solutions; AccuGENE® 0.5 M EDTA, AccuGENE Tris-HCl, AccuGENE TE, AccuGENE 5 M NaCl, and AccuGENE 3 M Sodium Acetate.

How to Prepare Buffer Equilibrated Phenol

Materials
- Magnetic stir plate
- Magnetic stir bar
- 2 L glass beaker
- Glass container
- Aluminum foil
- 25 ml glass pipette
- pH indicator paper

Reagents
- 8-hydroxyquinoline
- Molecular biology-grade phenol
- 50 mM Tris base (unadjusted pH)
- 50 mM Tris adjusted to pH 8.0 with HCl

Caution
Phenol causes severe burns. Gloves, safety glasses and a lab coat should be worn when working with phenol solutions. All manipulations should be carried out in a fume hood.

Procedure
1. Melt phenol crystals in a 65ºC water bath.
2. Place a stir bar in a 2 liter beaker.
3. Add 0.5 g of 8-hydroxyquinoline to the glass beaker.
4. Gently pour in 500 ml of melted phenol. The phenol will turn yellow.
5. Add 500 ml of 50 mM Tris base.
6. Cover the beaker with aluminum foil.
7. Stir 15 minutes at low speed on magnetic stir plate at room temperature.
8. Remove from the stir plate.
9. Let the phases separate.
10. Aspirate the top phase with the glass pipette into a suitable waste receptacle.
11. Add 500 ml of 50 mM Tris, pH 8.0.
12. Repeat steps 5 through 9.
13. Check the pH of the phenol phase with indicator paper. If it is not pH 7.8, steps 4 through 12 should be repeated.
14. Add 250 ml of 50 mM Tris-HCl, pH 8.0 or TE buffer, pH 8.0.
15. Place in glass container.
16. Store at 4°C (stable for 2 months) or -20°C.

Staining Solutions

GelStar® and SYBR® Green I & II Nucleic Acid Gel Stains
1:10,000 or 1:5,000 dilution of the dye in TAE, TBE or TE
Store in the dark in a polypropylene staining container

Ethidium bromide stock solution
10 mg/ml prepared in distilled water
1.0 g in 100 ml of distilled water
Stir for several hours to ensure dye is dissolved
Store protected from light at room temperature

Ethidium bromide working solution
0.5 µg/ml in electrophoresis buffer or distilled water
Store protected from light at room temperature

CAUTION: Ethidium bromide is a mutagen. Wear proper personal protective equipment and handle with care.

Loading Buffers

6X Ficoll® buffer
0.25% w/v Bromophenol blue
0.25% w/v Xylene cyanol
15% Ficoll (type 400) polymer in distilled water
Store at room temperature

6X Glycerol buffer
0.25% w/v Bromophenol blue
0.25% w/v Xylene cyanol
30% Glycerol in distilled water
Store at 4°C

6X Sucrose buffer
0.25% w/v Bromophenol blue
0.25% w/v Xylene cyanol
40% (w/v) Sucrose in distilled water
Store at 4°C

6X Alkaline buffer
300 mM NaOH
6 mM EDTA
18% Ficoll (type 400) polymer in distilled water
0.15% w/v Bromocresol green
0.25% w/v Xylene cyanol
Store at 4°C

Urea buffer
8% Ficoll (type 400) polymer in distilled water
5 M Urea
0.27 M EDTA
0.027% w/v
Adjust to pH 8.0
Store at room temperature

Formaldehyde loading buffer
1 mM EDTA • $2H_2O$
0.4% Bromophenol Blue
0.4% Xylene Cyanol
50% (v/v) Glycerol

Glyoxal loading buffer
10 mM Sodium phosphate
0.25% Bromophenol Blue
0.25% Xylene Cyanol
50% (v/v) Glycerol

Blotting and Hybridization Solutions

SSPE*

20X STOCK SOLUTION	g/l FOR 20X STOCK SOLUTION
3 M NaCl	175.3 g NaCl
0.2 M NaH_2PO_4 • H_2O	27.6 g NaH_2PO_4 • H_2O
0.02 M EDTA	07.4 g EDTA
Adjust to pH 7.4 with 10 N NaOH.	
Adjust volume to 1 liter with distilled water.	

SSC*

20X STOCK SOLUTION	g/l FOR 20X STOCK SOLUTION
3 M NaCl	175.3 g NaCl
0.3 M sodium citrate	88.2 g Na_3citrate • $2H_2O$
Adjust to pH 7.0 with 1 M HCl	
Adjust volume to 1 liter with distilled water	

High salt reagents – Nylon or nitrocellulose transfer membranes

DENATURATION SOLUTION	NEUTRALIZATION SOLUTION
1.5 M NaCl, 0.5 M NaOH	1.5 M NaCl, 0.5 M Tris-HCl, pH 7.0
87.7 g NaCl	87.7 g NaCl
20.0 g NaOH	500 ml of 1 M Tris-HCl, pH 7.0
Adjust volume to 1 liter with distilled water	Adjust volume to 1 liter with distilled water
Store at room temperature	Store at room temperature

Alkaline reagents – Charged nylon transfer membranes

DENATURATION SOLUTION	TRANSFER SOLUTION
0.4 M NaOH	0.4 M NaOH
16.0 g NaOH	16.0 g NaOH
Adjust volume to 1 liter with distilled water	Adjust volume to 1 liter with distilled water

Alkaline reagents – Uncharged nylon transfer membranes

DENATURING SOLUTION	TRANSFER SOLUTION
0.25 M NaOH	0.25 M NaOH,1.5 M NaCl
10.0 g NaOH	10.0 g NaOH
Adjust volume to	87.7 g NaCl
1 liter with distilled water	Adjust volume to 1 liter with distilled water

* Alternatively Cambrex offers ready-to-use AccuGENE® 20X SSPE, and AccuGENE 20X SSC,

Miscellaneous Stock Solutions

2X L broth (LB)

20.0 g	Bacto-tryptone
10.0 g	Bacto Yeast extract
10.0 g	NaCl
1,000 ml	Double distilled water

Adjust pH to 7.0 using 1 N NaOH or 1 N HCl
Autoclave on a liquid cycle for 15 - 20 minutes

50 mg/ml Ampicillin
50.0 mg ampicillin in 1 ml of double distilled water

Elution buffer

3.85 g	Ammonium acetate
0.215 g	Magnesium acetate
0.2 ml	AccuGENE® 0.5 M EDTA
1.0 ml	AccuGENE 10% SDS solution

Adjust volume to 100 ml with distilled water

30% 29:1 Acrylamide Solution

29.0 g	Acrylamide
1.0 g	N,N´-methylenebisacrylamide

Bring volume to 100 ml with distilled water
Mix thoroughly
Heat solution to 37°C until chemicals are completely dissolved

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Chapter XI

Protein Separation in Polyacrylamide Gels

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Protein Separation in Polyacrylamide Gels

Overview

Introduction

Polyacrylamide gel electrophoresis (PAGE) is a powerful tool for separating and identifying mixtures of proteins and peptides. Several systems exist for performing PAGE and consideration should be given to which system best suits a given sample prior to running the samples.

Gradient vs. homogeneous (straight percentage) gels

Gradient gels are suitable for a wide range of size resolutions, and will result in tighter band separation than single concentration gels. A homogeneous, or single concentration gel is appropriate where the proteins of interest are known to be within a narrow size range.

Discontinuous and continuous buffer systems

A discontinuous buffer system utilizes a large-pore gel (the stacking gel) layered on top of a small pore gel (the resolving gel). In this system, the buffer used to prepare the gels is different from the buffer used in the tank. The different buffers create two ion fronts concentrating the proteins into a very tight zone.

A continuous buffer system uses the same buffer in the gel and the tank. This system uses a single separating gel (no stacking gel) and has a single ion front. This system is less used than discontinuous systems, however, the separation of specific proteins and protein complexes that precipitate or aggregate may require the use of a continuous system.

Buffers for Electrophoresis

The Laemmli Buffer System (Tris-Glycine) is a discontinuous buffer system, widely used for fine resolution of a broad molecular weight range of proteins. In this system, the gel is prepared with Tris-HCl buffer and the Tris-glycine is used as the running buffer.

In the Tris-Tricine buffer system, tricine replaces glycine in the running buffer. The result is more efficient stacking and destacking, and higher resolution of proteins and peptides with lower molecular weights (under 10 kDa - 15 kDa).

Buffer Preparation

Tris-Glycine SDS Buffer, pH 8.3

10X STOCK SOLUTION	g/l FOR 10X STOCK SOLUTION
.25 M Tris base	30.3 g Tris Base
1.92 M Glycine	144.0 g Glycine
1.0% SDS*	10.0 g SDS
	Adjust volume to 1 liter with distilled water

(1X = 25 mM Tris base, 192 mM Glycine, 0.1% SDS)*
**Omit SDS if running native proteins.*

Tris-Tricine SDS Buffer, pH 8.3

10X STOCK SOLUTION	g/l FOR 10X STOCK SOLUTION
1 M Tris base	121.1 g Tris base
1 M Tricine	179.0 g Tricine
1.0% SDS*	10.0 g SDS
	Adjust volume to 1 liter with distilled water

(1X = 100 mM Tris base, 100 mM Tricine, 0.1% SDS)*
**Omit SDS if running native proteins.*

Casting Polyacrylamide Gels

Materials

- Vertical electrophoresis plate assembly with comb
- Sidearm flask assembly or filter unit for degassing solutions
- Paper towels

Reagents

- 30% Acrylamide mix, 29% (w/v) Acrylamide, 1% (w/v) bisacrylamide, prepared in distilled water
- 1.5 M Tris pH 8.8
- 10% SDS
- 10% Ammonium Persulfate (APS)
- TEMED
- Distilled water
- Water saturated isobutanol

Follow the steps below to cast a straight percentage polyacrylamide gel with a stacking gel. Additional information for polyacrylamide gel casting can be found in Appendix C. Alternatively, Cambrex offers PAGEr® Gold Precast Gels in a wide variety of concentrations and well formats.

Casting the resolving gel

1. Assemble plates according to the manufacturer's instructions.
2. Place the specified quantity of the first four components from the table on page 175 into a side arm flask.
3. Mix gently by swirling.

   NOTE: Omit SDS if running a native gel.
4. Degas the solution for 15 minutes.
   A high concentration of APS (0.3%) can be used to speed polymerization and skip the degassing step.
5. Add the specified amounts of TEMED and 10% APS.
6. Mix gently by swirling.
7. Pour the resolving gel, leaving space for the stacking gel.
8. Gently overlay the acrylamide with water-saturated isobutanol.
   The overlay blocks oxygen from inhibiting polymerization of the resolving gel.
9. Allow the gel to polymerize for 30 - 60 minutes.
   A very sharp liquid-gel interface will be visible when the gel has polymerized.
10. Pour off the overlay.
11. Rinse the top of the gel several times with water.
12. Blot any remaining water with a paper towel.

Preparation of Tris-Glycine Resolving Gel

	COMPONENT VOLUMES (ML)		
SOLUTION COMPONENTS	10 ML	20 ML	50 ML
6%			
Distilled water	5.3	10.6	26.5
30% acrylamide mix	2.0	4.0	10.0
1.5 M Tris (pH 8.8)	2.5	5.0	12.5
10% SDS	0.1	0.2	0.5
10% ammonium persulfate	0.1	0.2	0.5
TEMED	0.008	0.016	0.04
8%			
Distilled water	4.6	9.3	23.2
30% acrylamide mix	2.7	5.3	13.3
1.5 M Tris (pH 8.8)	2.5	5.0	12.5
10% SDS	0.1	0.2	0.5
10% ammonium persulfate	0.1	0.2	0.5
TEMED	0.006	0.012	0.03
10%			
Distilled water	4.0	7.9	19.8
30% acrylamide mix	3.3	6.7	16.7
1.5 M Tris (pH 8.8)	2.5	5.0	12.5
10% SDS	0.1	0.2	0.5
10% ammonium persulfate	0.1	0.2	0.5
TEMED	0.004	0.008	0.02
12%			
Distilled water	3.3	6.6	16.5
30% acrylamide mix	4.0	8.0	20.0
1.5 M Tris (pH 8.8)	2.5	5.0	12.5
10% SDS	0.1	0.2	0.5
10% ammonium persulfate	0.1	0.2	0.5
TEMED	0.004	0.008	0.02
15%			
Distilled water	2.3	4.6	11.5
30% acrylamide mix	5.0	10.0	25.0
1.5 M Tris (pH 6.8)	2.5	5.0	12.5
10% SDS	0.1	0.2	0.5
10% ammonium persulfate	0.1	0.2	0.5
TEMED	0.004	0.008	0.02

NOTE: Adjust proportionally based on the amount of gel needed.

Preparation of a 5% stacking gel

1. Place the specified quantity of the first four components from the table below into a side arm flask.

	Component volumes (ml)		
SOLUTION COMPONENTS	1 ML	5 ML	10 ML
Distilled water	0.68	3.4	6.8
30% acrylamide mix	0.17	0.83	1.7
1.5 M Tris (pH 8.8)	0.13	0.63	1.25
10% SDS	0.01	0.05	0.1
10% ammonium persulfate	0.01	0.05	0.1
TEMED	0.001	0.005	0.01

2. Mix gently by swirling.

NOTE: Omit SDS if running a native gel.

3. Degas the solution for 15 minutes.
4. Add the specified amounts of TEMED and 10% APS.
5. Mix gently by swirling
6. Pour the stacking gel directly onto the resolving gel.
7. Insert the comb immediately.
8. Allow the gel to polymerize for at least one hour.
9. Prepare samples while the gel is polymerizing.
10. Carefully remove the comb.

NOTE: Gels may be stored overnight at 4°C with the comb in place and wrapped in plastic wrap.

Casting gradient polyacrylamide gels

Hand casting gradient gels is not covered in this resource. Due to the complexity involved in hand casting gradient gels, precast gels have become a popular alternative. Detailed discussions and protocols for preparing gradient gels can be found in *Electrophoresis in Practice*, 2nd edition.

Loading and Running Proteins on Polyacrylamide Gels

Introduction

Protein load levels will vary depending upon sample purity and staining method used. For highly purified proteins, 0.5 µg to 5 µg protein per lane on a minigel is generally sufficient. Complete mixtures such as cell lysates may require as much as 50 µg protein per lane. The table below provides lower detection limits for protein detection.

Protein stain detection limits

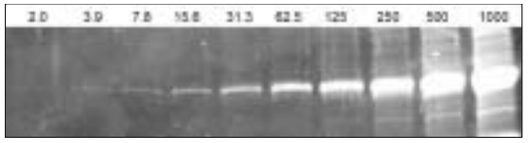
PROTEIN STAIN	LOWER DETECTION LIMIT (PROTEIN/BAND)
Coomassie® blue stain	100 ng
Silver stain	1 ng
SYPRO® Orange Protein Gel Stain	1 ng–2 ng
SYPRO Red Protein Gel Stain	1 ng–2 ng
SYPRO Tangerine Protein Gel Stain	4 ng–8 ng

NOTE: Limits are based on optimal detection methods for each stain.

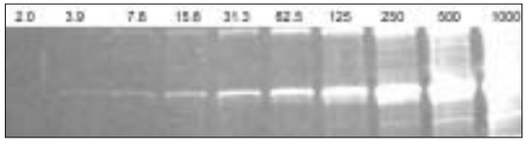
The photographs below demonstrate the detection sensitivities of commonly used stains

Coomassie® blue stain (CCD camera)

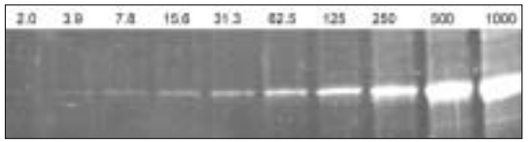
SYPRO® Orange Protein Gel Stain diluted in 7.5% acetic acid (Dark Reader™/CCD Camera)



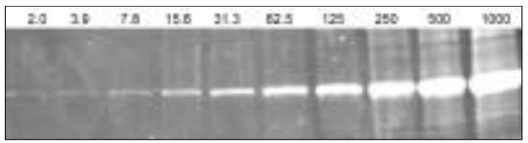
SYPRO® Orange Protein Gel Stain diluted in 7.5% acetic acid (Polaroid® photo UV light)



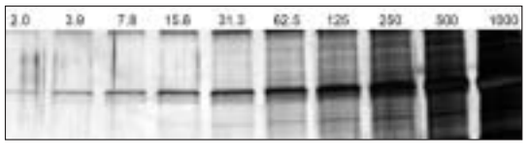
SYPRO Red Protein Gel Stain diluted in 7.5% acetic acid (Polaroid photo UV light)



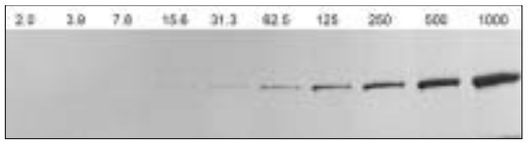
SYPRO Tangerine Protein Gel Stain diluted in PBS (Polaroid photo UV light)



Silver Stain (Amersham Plus™ One kit)



Silver Stain (Pierce GelCode® Silver Snap™ kit)



Serial dilutions of ProSieve® Protein Markers were separated on 12% PAGEr® Gold Precast Gels, stained and photographed as noted. The figure shows the staining sensitivity seen for the 50 kDa band of the marker. The level of protein present is indicated in nanograms. Staining was performed following the manufacturer's instructions provided with the stains. Exposure times for photography were adjusted to obtain highest possible detection levels. Note that in both silver stain examples shown, development was allowed to proceed for an extended time period relative to the range given in the instructions to maximize detection. In the case of the Amersham kit this results in overexposure of higher protein loading levels. Images of the silver stained gels were captured using a CCD camera system.

Loading Buffers

Introduction

In general loading buffers for protein electrophoresis contain Tris-HCl, pH 6.8; 2% SDS; a reducing agent such as dithiothreitol (DTT), β-mercaptoethanol (βME), or Tris[2-Carboxyethylphosphine]hydrochloride (TCEP); glycerol (a sinking agent) and a marker dye. An alternative reducing agent to βME, Bond-Breaker™ TCEP Solution from Pierce Chemical is an odor-free, ready-to-use solution added to the sample buffer prior to denaturation.

Gel loading buffers serve four purposes in protein electrophoresis:

- Reduction of protein complexes if performing denaturing PAGE
- Dissociation of proteins to allow them to run through the gel
- Increase the density of the sample ensuring samples drop evenly into the well
- Addition of a dye to the sample to simplify loading and monitor the electrophoretic process

2X Tris-Glycine SDS sample buffer

2X CONCENTRATE	AMOUNT TO ADD FOR 2X CONCENTRATE
126 mM Tris-HCl, pH 6.8	2.5 ml of 0.5 M Tris-HCl, pH 6.8
20%Glycerol	2 ml Glycerol
4% SDS	4 ml of 10% SDS
0.005% Bromophenol blue	0.5 ml of 0.1% Bromophenol blue
	Adjust volume to 10 ml with distilled water

(1X = 63 mM Tris-HCl, 10% glycerol, 2% SDS, 0.0025% Bromophenol blue, 2.5% βME)

Sample preparation

Tips for sample preparation

- Keep samples on ice prior to adding the sample buffer
- Add room temperate sample buffer to the cold samples
- If preparing samples for future use, aliquot treated samples into usable aliquots to avoid freeze thawing
- Do not leave the samples in SDS sample buffer at room temperature without first heating to 95°C to inactivate proteases. A loss of high-molecular weight bands and general smearing of the bands are indications of protease activity.

Continued on page 180

Materials
- Boiling water bath
- Ice

Reagents
- βME or TCEP
- 10 ml 2X Tris-Glycine SDS sample buffer

Procedure for sample preparation

Follow the guidelines below for preparing protein samples for electrophoresis.

1. Add 0.5 ml βME or 1 ml TCEP to 10 ml of 2X Tris-Glycine SDS sample buffer.
2. Add 1 part 2X sample buffer to 1 part sample on ice.
3. Mix well.
4. Heat sample at 95°C - 100°C in a boiling water bath for 4 minutes.
5. Place on ice until ready-to-use or store at –20°C for up to 6 months.

Loading the samples

Tips for loading samples

- Load the same sample volume in each well.
- If the well is not needed for a sample, load with 1X sample buffer.

NOTE: If a well is left empty, adjacent samples may spread.

Materials
- Electrophoresis chamber
- Gel

Reagents
- Samples
- 1X Electrophoresis buffer

Procedure for sample loading

1. Slowly and gently lift the comb straight up from the gel. Allow air to enter the well area to release the vacuum which forms between the gel and the comb.
2. Rinse each well with 1X electrophoresis buffer.
3. Place the gel into the electrophoresis chamber.
4. Add 1X electrophoresis buffer to cover the wells.
5. Gently load the desired volume of sample beneath the buffer in each well.

NOTE: Loading the sample too fast will lead to diffusion of the sample in the well.

Optimal Voltage, Running Times and Power Settings

Optimal voltage

Tris-Glycine polyacrylamide minigels are typically run at constant voltage between 125 - 200 volts. During electrophoresis, the current drops and heat decreases. Voltage set too high, or not limited causes excessive heating, resulting in band distortion and potential damage to the gel and apparatus. Constant voltage allows the same voltage to be used with multiple gels in an apparatus. Gel thickness is not a factor when using constant voltage. For large format gels, a constant current setting with a voltage limit set slightly higher (5 volts) than the expected voltage for the run may also be used to maintain sample velocity.

Optimal electrophoretic time

The gel should be run until the bromophenol blue dye has migrated to the bottom of the gel. Gel running times are dependent upon the buffer system used, the length of the gel and the polyacrylamide concentration. Typically minigels will take approximately 30 - 90 minutes to run. Whereas large format gels may take as long as 5 hours to run.

PAGEr® Gold Precast Polyacrylamide Minigels

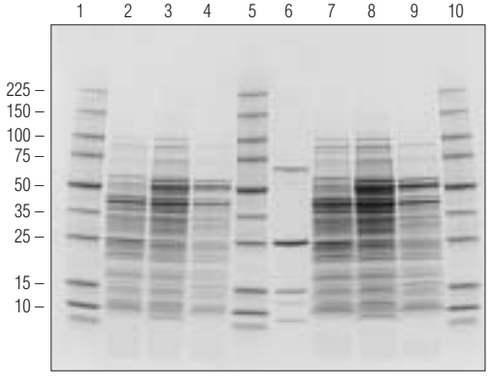
Introduction

PAGEr® Gold Precast Gels are Tris-Glycine (Tris-HCl) gels with a 4% stacking gel. The gels contain no SDS, so can be used for native gel electrophoresis or SDS may be added to the sample and running buffer for denaturing conditions.

Advantages

- Eliminates gel preparation time
- Printed well markings for ease of sample loading
- Easy open cassette
- Compatible with most commonly used vertical mini-gel apparatuses

The photograph below demonstrates the resolution performance of PAGEr® Gold Precast Gels.

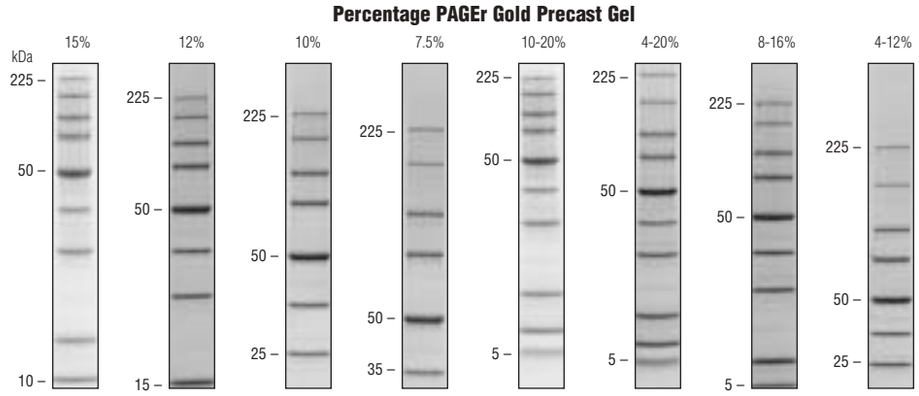


Separations were run using a 10 well 4-20% Tris-Glycine PAGEr® Gold Precast Gel. The gel was run at 120 volts until the tracking dye reached the base of the gel (approximately 90 minutes). ProSieve® Protein Markers were loaded in lanes 1, 5 and 10; aqueous extracts of three different strains of *E. coli* cells were run in lanes 2 - 4 and 7 - 9. Lanes 2 - 4 were loaded at one half the load level of lanes 7 - 9. Lane 6 contains a mixture of purified proteins. Proteins were detected by Coomassie® blue stain.

Separation ranges for proteins in PAGEr Gold Tris-Glycine Gels

Polyacrylamide Concentration	Size Separation Range
7.5%	50 kDa - 200 kDa
10%	25 kDa - 200 kDa
12%	20 kDa - 100 kDa
15%	10 kDa - 50 kDa
4 - 20%	5 kDa - 200 kDa
10 - 20%	5 kDa - 150 kDa
4 - 12%	25 kDa - 250 kDa
8 - 16%	15 kDa - 200 kDa

The figure below demonstrates the separation patterns of ProSieve® Protein Markers on PAGEr® Gold Precast Gels at various concentrations. Smaller molecular weight bands do not separate on some lower concentration gels.



ProSieve® Protein Markers contain 10 proteins with exact masses of 225, 150, 100, 75, 50, 35, 25, 15, 10 and 5 kDa. Gels were run at 175 volts until the dye front reached the bottom of the gel (approximately 60 minutes). 8 ml - 10 ml of ProSieve Protein Marker was loaded per lane (0.8 µg - 1 µg per band). Gels were stained with Coomassie® blue stain.

Chamber Compatibility

Manufacturer and Model	Gel Size 9 x 10 cm	Gel Size 10 x 10 cm
FISHERBIOTECH™		
Fisher FB-VE 10-1		x
Fisher FB-VRE 12-1		x
Fisher EC 120-2	x	x
INVITROGEN/NOVEX®		
XCell SureLock™ MiniCell		x
XCell I & II		x
BIO-RAD®		
Mini-PROTEAN® I & II + 3	x	
Ready Gel™ Cell	x	
HOEFER® MIGHTY SMALL		
SE 260	x	x
SE 250	x	x
SE 280	x	x
LIFE TECHNOLOGIES		
Mini V	x	
SIGMA ALDRICH		
Mini Techware	x*	x**
CBS SCIENTIFIC		
MGV System	x*	x**
OWL SEPARATIONS SYSTEMS		
Penguin™		x
DAIICHI/ISS		
2 &6 Systems (1 gel/run)		x
ZAXIS		
System 200		x

** 11.3 x 10 cm unit* *** 10 x 8 cm unit*

Some chambers may require adjustment for optimal fit, see page 184.

Chamber Modification Instructions

The following guidelines will ensure optimal performance of PAGEr® Gold Precast Gels in these systems.

Bio-Rad® Mini-PROTEAN® II, Mini-PROTEAN III or Ready Gel™ Cell Systems

Remove the rubber gasket from the inner core. Replace the gasket in the reverse orientation into the unit so the flat side faces outward.

Daiichi 2

To run one gel: Place one cassette on wedge side of chamber. Use the taller half of an Owl glass cassette or an equivalent as a buffer dam on the other side. Use Daiichi wedges. The PAGEr Gold cassettes cannot be used as the dam in this system.

To run two gels: Widen the hole on the yellow port of the inner core. Replace the long arm wedges with modified wedges, which are thicker and shorter. This chamber modification and the new wedges are available from Cambrex free of charge. Contact Cambrex Technical Service for details.

FisherBioTech™ Vertical Minigel Protein System: FB-VE10-1 mini chamber

Cambrex offers an adapator for this chamber, contact Cambrex Technical Service for details. The adapter only works if the inner gasket is white.

Replace black-plastic side spacer with Cambrex adapator. Use one on each side of the inner core.

FisherBioTech Vertical Minigel Protein System: FB-VE12-1

Chamber comes with 2 sets of wedges. Use the thinner wedges with PAGEr Gold Precast Gels.

Hoefer Mighty Small™ (SE250)

Replace the lower buffer chamber with a Deep Lower Buffer Chamber for the SE260. Available from Amersham Pharmacia (Part No. 80-6148-78). The extra depth of the SE260 buffer chamber allows the lid to lock into place.

Novex XCell SureLock™ Mini-Cell

Cambrex offers a spacer for this chamber, contact Cambrex Technical Service for details.

To run one gel: Put the gel in the front of the chamber. Put the buffer dam on the back. Place the Cambrex spacer between the buffer dam and the buffer core, on the side of the chamber with the gel tension wedge. Lock the gel tension wedge.

To run two gels: Put a gel on each side of the buffer core. Place the Cambrex spacer between the gel and the buffer core, on the side of the chamber with the gel tension wedge. Lock the gel tension wedge.

Owl Separation Systems Penguin® Model P8DS-1

Cambrex offers an adaptor for this chamber, contact Cambrex Technical Service for details. The Cambrex adaptor for the Penguin only works if the inner gasket is white. Replace black-plastic side spacer with Cambrex adaptor. Use one on each side of the inner core.

Materials
- Scissors
- Pipette
- Electrophoresis apparatus
- Power Supply
- Container
- Spatula

Reagents
- Distilled water
- Running Buffer AccuGENE® 10X Tris-Glycine or Tris-Glycine SDS Buffer

Procedure for electrophoresis using PAGEr® Gold Precast Gels

1. Cut open pouch and remove gel.

2. Rinse the gel with distilled or deionized water.

3. Slowly and gently lift the comb straight up.

NOTE: Put the comb aside so it can be used to separate the cassette plates at the end of the run.

4. Remove white tape from bottom of the cassette.

NOTE: The sharp end of the comb can be used to peel off the tape.

5. Mount the cassette(s) into the electrophoresis chamber so the printed side faces the outer (anode) buffer chamber. If running only one gel, mount the appropriate buffer dam.

6. Fill the buffer chambers with 1X running buffer.

7. Wash the wells with 1X running buffer, displacing any air bubbles in the wells.

8. Load samples.

NOTE: For best results, load 1X sample buffer in the wells without samples.

9. Run the gels at a constant voltage of 125 - 200 volts until the dye front is near the bottom of the gel (approximately 30 - 90 minutes).

10. Remove the gel(s) from electrophoresis chamber.

11. Place the cassette on a flat surface with the short side of the cassette facing up.

12. Using the end of the comb and starting at the top of the cassette separate the two plates by using a twisting motion to crack the cassette.

13. Carefully remove the short plate.

14. Hold the plate with the gel over an open container.

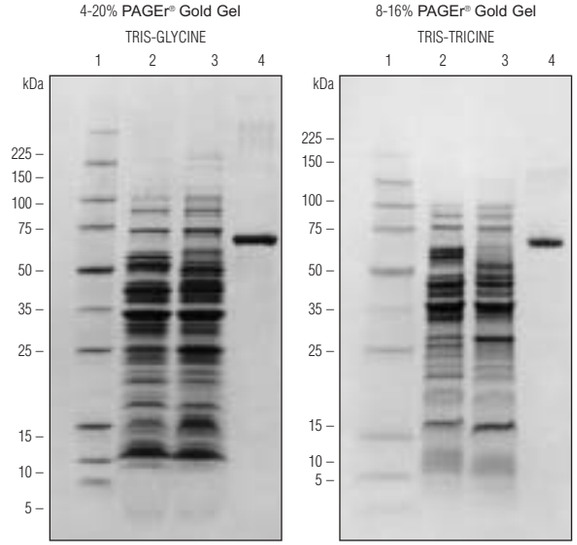
If the gel is adhered to . . .	Then . . .
Large Plate	Run end of comb or a gloved finger behind slot on cassette to push out the lip at the bottom of the gel.
Small Plate	Use the comb or a spatula to loosen one lower corner of the gel.

15. Allow gel to peel away and gently drop into the container.

16. Fix, stain and destain or blot the gel as desired.

PAGEr® Gold Precast Gels can be run at higher voltages to achieve faster run times. If using Tris-glycine running buffer gels can be run at 200 - 250 volts for approximately 40 minutes. If using Tris-Tricine running buffer gels can be run at 150 - 200 volts for approximately 30 minutes.

The photographs below show the separation of protein samples on PAGEr Gold Precast Gels under increased voltage.



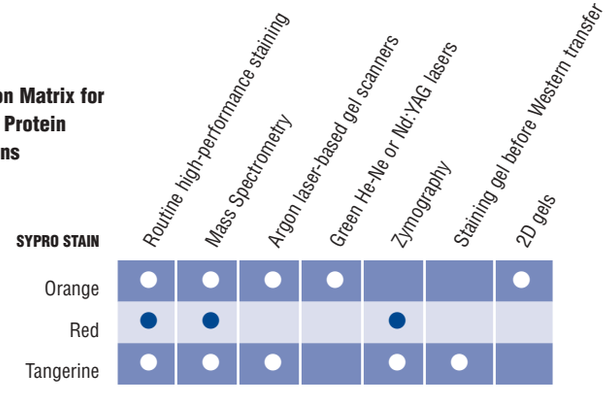
Samples were prepared using Tris-Glycine SDS Sample Buffer and β-Mercaptoethanol as a reducing agent. Lane 1: ProSieve® Protein Markers; Lanes 2&3: *E. coli* extracts; Lane 4: Bovine Serum Albumin (BSA). Gel 1: 4-20% gel in 1X Tris-Glycine SDS Buffer run at 230 volts for 40 minutes. Gel 2: 8-16% gel in 1X Tris-Tricine SDS Buffer run at 185 volts for 30 minutes. Samples were run until the tracking dye reached the base of the gels. Proteins were detected using Coomassie® blue stain.

Detection of Proteins in Polyacrylamide Gels

Detecting proteins with SYPRO® Protein Gel Stains

SYPRO Protein Gel Stains are highly sensitive fluorescent stains for the rapid detection of proteins in polyacrylamide gels. These stains can detect as little as 4 ng - 8 ng of protein per band in 40 - 60 minutes without destaining. Gels stained with SYPRO Protein Gel Stains exhibit low background and minimal protein-to-protein staining variability. Gels can be documented using standard imaging systems.

Selection Matrix for SYPRO® Protein Gel Stains



Tips for staining gels with SYPRO Protein Gel Stains

- The SDS front at the bottom of the gel stains heavily with SYPRO Red, Orange and Tangerine Stains. Unless the protein of interest comigrates with the SDS front we recommend running the SDS front off the end of the gel.
- Colored stains such as Coomassie® blue stains and colored protein markers may interfere with SYPRO staining and quench fluorescence. To stain gels previously stained with Coomassie blue stain, soak the gel in several changes of 7.5% acetic acid to remove the Coomassie stain. Then incubate the gel in 0.05% SDS for 30 minutes and stain with SYPRO Stain as usual.
- Glove powder can leave background markings on gels. Rinse or wash gloves prior to handling gels.
- Clean the surface of the transilluminator after each use with deionized water and a soft cloth. Fluorescent stains (such as SYPRO Stains or ethidium bromide) can accumulate on the transilluminator and may cause high background.
- Handle gels carefully to avoid non-specific staining of areas of the gel that have been squeezed.
- SYPRO Stains may be photobleached after several minutes of exposure to UV light. If a gel becomes photobleached, restain by incubating in the staining solution.
- SYPRO stained gels can be restained with Coomassie® blue or silver stain procedures.
- Plastic wraps and GelBond® Film will autofluoresce when exposed to UV light resulting in very high background. Gels backed with GelBond Film can be photographed by inverting the gel on the transilluminator.

Materials

- Clear polypropylene container (e.g., Rubbermaid® recycling #5 plastics)

Reagents

- 7.5% (v/v) acetic acid
- SYPRO Protein Gel Stain stock solution

General Procedure for staining proteins with SYPRO® Protein Gel Stains

1. Run SDS-polyacrylamide gels according to standard protocols.

   NOTE: To reduce background staining with SYPRO Red and Orange Stains use 0.05% SDS in the running buffer. Gels run in 0.05% SDS show no change in the migration pattern of proteins.

2. Dilute the 10,000X concentrate to a 2X solution, in 7.5% (v/v) acetic acid, in a clear plastic polypropylene container. For example, for every 10 ml acetic acid add 2 µl stock SYPRO Stain solution.
3. Mix well.
4. Place the gel into the staining container and cover with a lid to protect from light.
5. Gently agitate the gel at room temperature.
6. Stain the gel for 40 - 60 minutes.

   NOTE: The optimal staining time depends on the thickness of the gel and the gel concentration. Longer staining times may be required as gel thickness increases.

7. Briefly rinse the gel in 7.5% acetic acid.
8. Remove the gel from the staining container and photograph the gel following the procedure on page 192.

Procedure for staining nondenaturing gels with SYPRO® Red or SYPRO Orange Protein Gel Stains

Options for staining proteins with SYPRO Red and Orange Stains after native gel electrophoresis:

1. Dissolve the stain 1:5,000 in distilled water and follow the general protocol for staining proteins with SYPRO Protein Gel Stains.
   This will be highly protein-selective, and will be less sensitive than staining proteins in SDS gels. To increase the signal, a long film exposure can be used since the background fluorescence is essentially zero.
2. Soak the gel after electrophoresis in 0.05% SDS for 30 minutes, then stain with a 1:5,000 solution of SYPRO Stain diluted in 7.5% acetic acid.
   Proteins will be denatured and fixed after electrophoresis using this treatment.

Procedure for staining proteins with SYPRO Tangerine Protein Gel Stain: Non-fixing protocol

SYPRO Tangerine Protein Gel Stain is an extremely versatile fluorescent stain that does not alter protein structure, interfere with the transfer of proteins to blotting membranes, or use organic solvents. The stain can be diluted into a wide range of buffers with a pH range from 4 - 10 and if fixing is necessary, it can be diluted into 7.5% acetic acid following the General Protocol for Staining Proteins with SYPRO Gel Stains.

If proteins are to be used for subsequent analysis dilute SYPRO Tangerine stock solution into 50 mM phosphate, 150 mM NaCl, pH 7.0 or use one of the buffers listed below prepared as 50 mM - 100 mM solutions containing 150 mM NaCl.

Compatible Buffers

Formate pH 4.0	HEPES pH 7.5
Citrate pH 4.5	Tris acetate pH 8.0
Acetate pH 5.0	Tris-HCl pH 8.5
MES pH 6.0	Tris borate, 20 mM EDTA pH 9.0
Imidazole pH 7.0	Bicarbonate, pH 10.0

Follow this procedure if the gel will be used for subsequent analysis such as zymography or Western blotting. Stained proteins can also be eluted from gels and used for further analysis such as mass spectrometry.

1. Run SDS-polyacrylamide gels according to standard protocols.
   It is not necessary to decrease the amount of SDS present in the running buffer when using SYPRO® Tangerine Gel Stain.
2. Dilute the 10,000X concentrate to a 2X solution, in 7.5% (v/v) acetic acid solution, in a clear plastic polypropylene container. For example, for every 10 ml acetic acid add 2 µl stock SYPRO Stain solution.
3. Mix well.
4. Place the gel into the staining container and cover to protect from light.
5. Gently agitate the gel at room temperature.
6. Stain the gel for 40 - 60 minutes.
   The optimal staining time depends on the thickness of the gel and the gel concentration. Longer staining times may be required as gel thickness increases.
7. Remove the gel from the staining container and immediately photograph the gel following the procedure on page 192.

NOTE: If the gel will be blotted after staining with SYPRO Tangerine Stain, stain the gel according to the procedure above, using 50 mM phosphate, 150 mM NaCl as the stain diluant. Add 0.1% SDS to the Transfer Buffer. This will help in the transfer of some proteins to the membrane.

Materials

- Clear polypropylene container (e.g., Rubbermaid® recycling #5 plastics)

Reagents

- 50 mM Phosphate, 150 mM NaCl, pH 7.0 or suitable buffer

Viewing gels stained with SYPRO® Protein Gel Stains

All SYPRO Protein Gel Stains have two excitation wavelengths; in the UV region at approximately 300 nm and in the blue range of visible spectrum, between 470 and 610 nm. The stains can be visualized using a UV transilluminator or the Dark Reader™ transilluminator.

Photographing gels stained with SYPRO® Protein Gel Stains

- Protein bands stained with SYPRO Protein Gel Stains are best seen by photographing the gel. The integrating effect of a camera or imaging system can detect bands that are not visible to the eye.
- Gels stained with SYPRO Protein Gel Stains can be photographed using Polaroid® cameras, CCD camera systems or laser scanners.
- The highest sensitivity using a Polaroid camera can be obtained using Polaroid 667 black-and-white print film and the SYPRO Protein Gel Stain Photographic Filter. Exposure time will vary with the intensity of the illumination source. Begin with an f-stop of 4.5 and an exposure of 2 - 8 seconds. Use of an ethidium bromide filter is not recommended as it blocks much of the light and leads to lower detection sensitivity.
- The SYPRO Photographic Filter does not work with CCD camera systems. For CCD cameras use the emission and excitation data below and check with the camera manufacturer for the appropriate filter.

Stain	Emission (nm)	Excitation (nm)
SYPRO® Red	630	300 and 550
SYPRO Orange	570	300 and 470
SYPRO Tangerine	640	300 and 490

Detecting proteins with Coomassie® blue stain

Coomassie blue stain binds nonspecifically to most proteins. Proteins are fixed and stained in a Coomassie blue staining solution and subsequently destained to eliminate the blue background from the gel. Gels can be dried, photographed or stored wet.

Coomassie blue stain solution

1X Working Solution	Amount for 1X working Solution*
40% Ethanol	400 ml Ethanol
0.125% Coomassie® blue	1.25 g Coomassie blue R-250
Distilled water	500 ml Distilled water
10% Acetic acid	100 ml Acetic acid

**Add reagents in the order provided*

Coomassie blue destain solution

1X Working Solution	Amount for 1X working Solution*
5% Ethanol	50 ml Ethanol
7.5% Acetic acid	75 ml Acetic acid
	Adjust volume to 1 liter with distilled water

NOTE: These solutions should be prepared under the fume hood

Procedure

1. Prepare enough Coomassie® blue staining solution to cover the surface of the polyacrylamide gel.
2. Prewarm the Coomassie blue stain solution to 55°C.
3. Remove the gel from the glass plates.
4. Submerge the gel in the Coomassie blue stain solution.
5. Gently agitate the gel for 20 minutes at 55°C until the gel becomes blue.
   Alternatively, solutions may be heated to 45°C and incubated at room temperature.
6. Decant stain solution from container.

   NOTE: Coomassie blue staining solutions can be saved and stored to stain multiple gels. As the stain solution reaches its "use limit" gels will appear grainy and will not destain completely and new staining solutions should be prepared.
7. Briefly rinse excess stain from gel in water
8. Transfer the gel into the Destain solution.
9. Gently agitate the gel at 55°C until the gel has destained and bands are visible. (Approximately 1 hour).

   NOTE: The Destain solution may need to be changed occasionally until the background is clear. Do not over destain, which can lead to loss of band intensity. Pieces of paper towel or a Kimwipe® can be placed in the corner of the container to speed up this process. Change the tissues when they are saturated with Coomassie blue stain.

Materials

- Kimwipes or paper towels
- 3 plastic containers with covers
- Shaking incubator set to 55°C

Reagents

- **Stain solution**
  0.125% Coomassie blue, 40% Ethanol, 10% Acetic acid
- **Destain solution**
  5% Ethanol, 7.5% Acetic acid

Materials

- Glass containers
- Orbital shaker

Reagents

- Glass distilled water
- **Destain I** (40% Methanol 7% Acetic acid)
  - 400 ml Methanol
  - 70 ml Acetic acid
  - Adjust volume to 1 liter with distilled water
  - Store at room temperature indefinitely
- **Destain II** (5% Methanol, 7% Acetic acid)
  - 700 ml Acetic acid
  - 500 ml Methanol
  - Adjust volume to 10 liters with distilled water
  - Store at room temperature indefinitely
- **Cross-linking Solution** (10% Glutaraldehyde)
  - 20 ml of 50% glutaraldehyde stock in 100 ml of distilled water.
  - Glutaraldehyde is toxic and must be handled in a fume hood.

continued on page 195

Detecting proteins with Silver stain

Silver staining is based on differential reduction of silver ions bound to sulfhydryl and carboxyl side chains on proteins. After electrophoresis, proteins are fixed, exposed to silver nitrite and developed to form a black precipitate of silver. All silver staining procedures are time consuming. Many good kits are commercially available which make the procedure faster and easier. The BioRad® Silver Stain Plus™ kit is recommended for its versatility and ease of use.

The procedure described below is a modification of Morrisey. This procedure uses dithiothreitol (DTT) to improve reproducibility. An advantage of this version is that development occurs more slowly than many silver staining protocols, giving more control over the final image.

Procedure

Wear gloves and use only glass-distilled water and glass staining containers. All steps can be done at room temperature.

1. Gently agitate the gel in 100 ml Destain I for 30 minutes to overnight.
2. Remove Destain solution I.
3. Gently agitate the gel in 100 ml of Destain II for 30 minutes.
4. Remove Destain II.
5. Gently agitate the gel in 100 ml Cross-linking solution for 30 minutes.

   NOTE: For small peptides, incubate with glutaraldehyde overnight to insure retention of the peptides in the gel.
6. Remove Cross-linking solution.
7. Wash gel with several changes of water over a 2 hour period.

   NOTE: Alternatively the gel can be placed in 2 liters of water and the next morning washed for 30 minutes in fresh water. High background will result if the glutaraldehyde is not completely washed out of the gel.
8. Gently agitate the gel in DTT solution for 30 minutes.
9. Remove DTT solution and drain well, but do not rinse the gel.
10. Gently agitate the gel in 100 ml Silver nitrate solution for 30 minutes.
11. Place the staining tray under running deionized water and swirl for a few seconds.
12. Remove the water.
13. Add 50 ml of Developing solution, swirl briefly, then discard the solution.
14. Repeat step 13.

    NOTE: This reacts with excess silver and prevents nonspecific staining of the gel.
15. Add 100 ml of Developing solution and shake slowly.

    NOTE: Staining occurs slowly at first but then progresses rapidly. Development takes approximately 5 to 10 minutes.
16. When the bands are slightly lighter than the desired staining level, remove developing solution, rinse quickly with water, add Destain II to cover the gel, as the stop solution. Alternatively, add 5 ml of Stop solution to the developer to stop development. In either case, development will not stop immediately but continues for approximately 5 minutes after adding Stop solution.
17. Wash the gel several times in Destain II.
18. Rinse the gel with water.
19. Store in water or dry the gel.

- **Dithiothreitol (DTT) Solution (5 mg/ml)**
  - 5.0 mg DTT in 1 liter of distilled water
- **Silver Nitrate Solution** (0.1% w/v Silver nitrate)
  - 1.0 g Silver nitrate ($AgNO_3$) in 1 liter of distilled water
- **3% Sodium Carbonate** (3% w/v)
  - 60.0 g Sodium carbonate in 2 liters of distilled water
  - Store in glass container
- **Developing Solution** (3% Sodium carbonate, 0.019% Formaldehyde)
  - 200 ml of 3% Sodium carbonate
  - 100 ml of 37% Formaldehyde
  - Prepare just prior to use
- **Stop Solution** (2.3 M Sodium citrate)
  - 67.64 g Sodium citrate, dihydrate (FW 294.1)
  - Adjust volume to 100 ml in distilled water

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Chapter XII

Blotting Proteins from Polyacrylamide Gels

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Blotting Proteins From Polyacrylamide Gels

Introduction

Protein transfer efficiency in blotting applications is dependent upon multiple factors, including gel percentage, gel thickness, protein size, transfer conditions (e.g., buffer and voltage), and type and quality of membrane. To achieve optimal transfer efficiency, transfer conditions must be adjusted to address these varying factors..

Choosing the appropriate membrane

Nitrocellulose	PVDF	Nylon
Hydrophobic binding	Hydrophobic binding	Hydrophobic & electrostatic binding
General purpose membrane	SDS tolerant	Stable if baked
Low background	High background	High background
Low strength	High strength	High strength
Becomes brittle if baked	Suitable for protein sequencing	Least suitable for Western transfer

Transfer solutions

Formula for Towbin transfer solution:

Towbin Transfer Solution

1X WORKING SOLUTION	AMOUNT FOR 1X WORKING SOLUTION
25 mM Tris base	30.3 g Tris base
192 mM Glycine	144.1 g Glycine
0.1% SDS	10.0 g
	Adjust volume to 8 liters with distilled water
	Measure, but do not adjust pH; it should be approximately 8.2 to 8.4
20% Methanol	2 L Methanol
	Adjust volume to 10 liters with distilled water

It may be necessary to lower the concentrations of methanol, SDS or both to obtain the optimal balance of transfer and binding efficiency. The table below outlines the effects that SDS and methanol have on protein transfer.

SDS	Methanol
Improves transfer of proteins >60 kDa	Improves binding efficiency
Decreases binding efficiency	Decreases transfer efficiency
Not compatible with nylon membranes	Do not soak gel in transfer buffer prior to blotting
Include 0.1% - 0.2% in transfer buffer	Include 20% in transfer buffer

Tips for increasing transfer efficiency

- Use the lowest concentration gel that will resolve the protein(s) of interest.
- Avoid using gels ≥1 mm thick. Thick gels may require longer blotting times.
- Decrease the concentration of methanol to optimize transfer efficiency of proteins >150 kDa.
- Small proteins tend to transfer more easily than large proteins. Longer transfer times may be used to ensure complete transfer of large proteins (>60 kDa), proteins from native gels, and thicker gels.
- Use two membranes if transferring for an extended period of time (>1 hour). The second membrane will bind any protein that may transfer through the first. This can be verified by membrane staining.
- Use ProSieve® Color Protein Markers to confirm that transfer has occurred to the membrane and not the filter paper.
- Use a chopping motion when removing the well and foot area. Slicing the gel may cause tearing.
- Gently roll out air bubbles between transfer stack layers using a wet glass rod or pipette.
- If the gel sticks to the filter paper or membrane after transfer, soak for 5 - 15 minutes in water then gently peel the filter paper away.
- If proteins are left in the gel after following recommended transfer conditions, increasing the voltage by no more than 5 volts may be helpful.

Semi-dry Blotting PAGEr® Gold Precast Gels

This protocol is for use with the BioRad Trans-Blot® Semi-Dry cell. If using another manufacturer's blotting apparatus, follow manufacturer's instructions for use.

Materials and Reagents

- Mask
- A piece of GelBond® Film or similar polyester film, the size of the bottom anode, with a rectangular hole the size of the gel cut out of the center. The purpose of the mask is to focus current through the gel stack.
- Whatman® Grade 114 or 54 filter paper
- Extra thick blot paper
- Transfer membrane
- Scalpel or razor blade
- Scissors
- Glass rod or pipette
- 1XTowbin transfer solution at 4°C
- Shallow tray for soaking membranes and filter paper

Tips for Semi-Dry Blotting

- To prevent the stack from drying out, add extra transfer solution (2 ml - 5 ml) to the top layer of filter paper before closing the lid on the blotting apparatus.
- Center the gel on the membrane. Occasionally, the gel will overlap the membrane and stick to the filter paper below. If this occurs, gently break the seal with a scalpel.

Procedure for semi-dry blotting

NOTE: Nitrocellulose or PVDF membranes are recommended for this method and should be wetted in methanol prior to wetting in transfer solution.

1. Electrophorese gel following standard procedure.

2. Carefully trim off stacking gel and bottom foot from the gel.

3. Soak gel for 20 minutes in chilled 1X transfer solution.

4. Cut the filter paper, blot paper and membrane to the size of the gel.

NOTE: Transfer membranes should be handled at the edges with gloves worn.

5. Soak membrane, blotting paper and filter paper for 5 - 10 minutes in 1X transfer solution.

6. To make a semi-dry blotting sandwich, stack in the following order:

6A. Mask

6B. Prewet extra thick blot paper

6C. Prewet nitrocellulose membrane

6D. Polyacrylamide gel.

6E. Prewet Whatman® Grade 114 or 54 filter paper

6F. Prewet extra thick blot paper

6G. Top platen (stainless steel cathode), and safety cover

7. Turn on the power and transfer at 25 volts (constant) at 400 mA for:

60 minutes for 10 kDa - 100 kDa proteins

90 minutes for 100 kDa - 300 kDa proteins

NOTE: Optimized conditions will be required for different proteins or different membranes.

Tank Blotting PAGEr® Gold Precast Gels

Materials
- Whatman® Grade 114 or 54 filter paper
- Extra thick blot paper
- Transfer membrane
- Fiber or foam pad
- Scissors
- Glass rod or pipette
- Scalpel or razor blade
- 1X Towbin transfer solution 1X Tris-Glycine buffer at 4°C
- Shallow tray for soaking membranes and filter paper

For hydrophobic proteins or proteins >100 kDa, tank blotting is preferable to semi-dry blotting because prolonged transfers are possible without gel drying. For more detailed information and protocols concerning tank blotting, consult the blotting apparatus manufacturer's instructions.

Procedure for tank blotting

NOTE: Transfer is performed at 4°C.

NOTE: Nitrocellulose or PVDF membranes are recommended for this method and should be wetted in methanol prior to wetting in transfer solution.

1. Electrophorese gel following standard procedures.

2. Carefully trim off stacking gel and bottom foot from the gel.

3. Soak gel for 20 minutes in chilled 1X Transfer solution.

4. Cut the filter paper, blotting paper and membrane to the size of the gel.

NOTE: Transfer membranes should be handled at the edges with gloves worn.

5. Soak fiber pads, nitrocellulose membrane and blotting paper for 2 minutes in 1X Transfer solution.

6. To make a tank blotting sandwich, stack in the following order:

6A. Cathode unit

6B. 1 prewet fiber pad

6C. 1 sheet prewet extra thick blot paper

6D. 1 sheet prewet Whatman 114 or 54 blotting paper

6E. Polyacrylamide gel

6F. Prewet nitrocellulose membrane

6G. 1 sheet prewet extra thick blot paper

6H. 1 prewet fiber pad

6I. Anode unit

7. Place in tank with nitrocellulose membrane closest to anode (+).

8. Cover with chilled 1X Transfer solution.

9. Turn on the power and transfer by running at 100 volts (constant) at 400 mA for:
90 minutes for 10 kDa–100 kDa proteins
120 minutes for 100 kDa–3,000 kDa proteins

NOTE: Optimized conditions will be required for different proteins or different membranes.

Monitoring protein transfer

Protein transfer can be monitored by staining the gel following transfer (page 188) and/or staining the membrane. Membrane staining should only be performed when duplicate samples have been run on a gel and the membrane can be cut in half, or when a second membrane has been used. Stains commonly used for this purpose include SYPRO® Ruby Protein Blot Stain, India ink stain or colloidal gold stain. Described below is the use of Amersham Bioscience's AuroDye® Forte Kit which is a colloidal gold stain.

Staining membranes with Amersham Bioscience's AuroDye® Forte Kit

This method is compatible with PVDF and nitrocellulose membranes. For more detailed information and protocols, consult the instructions provided with the kit.

1. Place the membrane in a clean glass dish.

2. Add 1X PBS, 0.3% Tween 20.

3. Shake slowly on a shaker for 30 minutes at 37°C.

4. Remove solution and replace with fresh 1X PBS, 0.3% Tween 20.

5. Shake slowly on a shaker for 5 minutes at room temperature.

6. Repeat steps 4 –5 two more times.

7. Briefly rinse the membrane in distilled water.

8. Place the membrane in a thermal seal pouch with 15 ml - 20 ml AuroDye Forte for 2 - 4 hours at room temperature.

9. After fully developed, rinse briefly with distilled water and air dry.

Materials and Reagents
- Previously blotted membrane
- Orbital shaker set to 37°C
- Orbital or rocking platform shaker
- Glass dish
- Thermal seal pouch and sealing unit
- Distilled water
- 1X PBS, 0.3% Tween® 20

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Notes

Chapter XIII

Isoelectric Focusing of Proteins on Agarose Gels

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Isoelectric Focusing of Proteins on Agarose Gels

Overview

Introduction

Separation of proteins in complex mixtures for analytical resolution can be achieved by isoelectric focusing (IEF), in which proteins are separated based on their net charge (isoelectric point, or pI) in the presence of a pH gradient. Analytical focusing is carried out either in polyacrylamide gels—most recently prepared with immobilized pH gradients—or in agarose gels prepared with mobile carrier ampholytes, which form a pH gradient when subjected to electrophoresis. Separation in agarose gels is more rapid, since the pores of the agarose gel are larger than those of polyacrylamide gels.

Advantages

- No toxic monomer solutions are required
- Separation of proteins larger than 2,000 kDa
- Shorter staining times
- Nontacky and compressible (blottable)
- No catalysts to interfere with separation

Applications

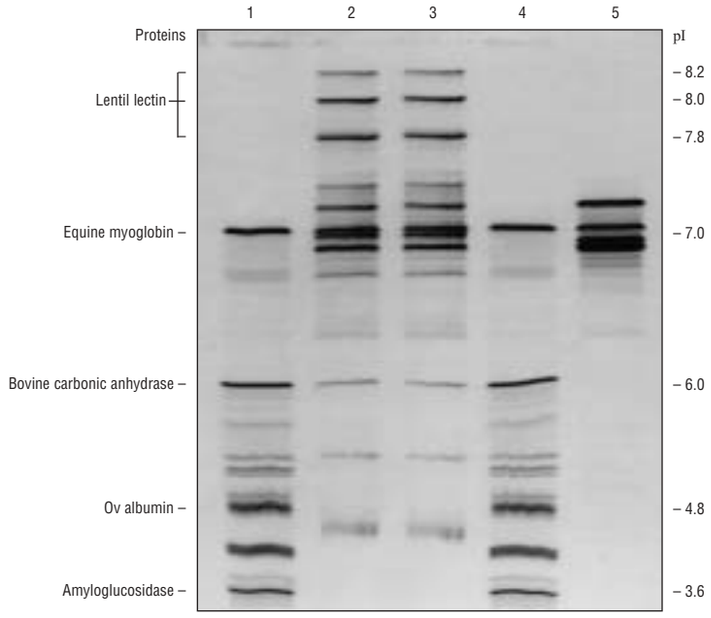
- Immunofixation directly in the gel
- Crossed immunoelectrofocusing
- Direct tissue isoelectric focusing
- Preparative isoelectric focusing

Compatible agaroses

Isoelectric focusing on agarose gels requires the use of an agarose that has no measurable electroendosmosis (EEO). Cambrex has developed two products that can be used for this application, specifically IsoGel® Agarose and IsoGel Agarose IEF Plates.

- IsoGel Agarose is a highly purified agarose that is easy to prepare and produces a less restrictive gel than polyacrylamide, allowing rapid focusing of high molecular weight proteins (>2,000 kDa).
- IsoGel Agarose IEF Plates are ready-to-use 125 mm x 100 mm precast gels that eliminate gel preparation time and provide easy handling throughout IEF processing.

The photograph below demonstrates the separation performance of proteins focused on an IsoGel® Agarose IEF Plate.



Separation of proteins in a IsoGel® Agarose IEF Plate, pH 3-10. Lanes 1 & 4: FMC pI Marker (unavailable). Lanes 2 & 3: Broad Range pI 4.45-9.6 marker (BioRad). Lane 5: Hemoglobin, HB Type AFSC (PE Wallac). 2.5 µl of each sample were loaded on the gel and prefocused at 1 watt for 10 minutes and focused at 2000 volts (max), 25 mA (max), 25 W (max) for 60 minutes on a Pharmacia MultiPhor® II chamber at 10°C. The gel was stained with Crowle's stain.

Tips for Performing IEF in Agarose Gels

Highlighted are some general points for improving protein separation in agarose gels. At the end of the chapter we have included a comprehensive troubleshooting guide which also provides tips to resolution performance.

- Agarose IEF is compatible with carrier ampholytes only.
- Agarose IEF works best between pH 2.5 - 11.
- pK values of the carrier ampholytes and proteins to be analyzed are temperature dependent. Isoelectric focusing should be carried out at constant temperature.
- When selecting anolytes and catholytes, closely bracket the ends of the pH range of the ampholytes.
- To reduce pH gradient drift, place wick strips that have been soaked in the electrolyte solutions (anolyte/catholyte), then blotted of excess fluid under the electrodes.

Preparation of Agarose Isoelectric Focusing Gels

Alternatively, Cambrex offers IsoGel® Agarose IEF Precast Plates.

Suggested anolytes and catholytes

When selecting anolytes and catholytes for any pH gradient, it is important to closely bracket the ends of the pH range of the ampholytes. Avoid creating pH discontinuities between the ends of the ampholyte range and the bracketing electrolytes.

NOTE: pH is dependent on temperature. 25°C pH values are provided for selection of electrolytes. Under running conditions, the pH will be slightly higher.

Anolyte			Catholyte		
	CONCENTRATION	PH (25°C)		CONCENTRATION	PH (25°C)
Phosphoric acid	1 M	1.0	Threonine	50 mM	5.8
Sulfuric acid	0.2 M	1.6	Glycine	50 mM	6.15
Acetic acid	0.5 M	2.6	Hepes	0.4 M	7.3
L-glutamic acid	40 mM	3.2	L-Histidine (free-base)*	40 mM	7.35
Indole acetic acid	3 mM	3.8	Bicine	0.1 M	8.0
L-tyrosine	4 mM	4.5	Sodium hydroxide	1 M	13.0

**Do not substitute Histidine HCl for free-base.*

Preparation of the gel casting assembly

1. Spread a few drops of distilled water or 0.1% nonionic detergent on one glass plate.
2. Lay a sheet of GelBond® Film, cut slightly smaller than the glass plate, onto the plate with the hydrophilic side up.

   NOTE: Water droplets spread on the hydrophilic side but bead up on the hydrophobic side of the film.
3. Cover the GelBond Film with a sheet of blotting paper or the interleaving paper supplied with the film.
4. Firmly roll with a rubber roller or wipe with tissues to squeeze out any air bubbles and excess fluid from behind the GelBond Film.
5. Carefully wipe off any excess liquid at the edges.
6. Place the U-frame spacer on top of the GelBond Film. If a U-frame spacer is unavailable, place two spacers on the GelBond Film along either side and one spacer across the bottom edge.
7. Place the second glass plate over the spacer(s).
8. Clamp the assembly with the stationery clamps, using 2 clamps per side and bottom.
9. Warm the cassette in a 55°C forced hot air oven for 15 minutes.

   NOTE: GelBond Film may warp if the cassette is heated too long or above 75°C.

Continued on page 210

Materials

- **GelBond Film** (110 mm x 125 mm)
- **Blotting paper or interleaving paper supplied with Gelbond Film**
- **Two thick glass plates** (110 mm x 125 mm)
- **A plastic 0.8 mm-thick U-frame spacer or 3 single spacers, 0.8 mm-thick**
- **Six stationery binder clamps**
- **Blotting paper, rubber roller or tissues**
- **Forced hot air oven set to 55°C**

Reagents

- **Distilled water or 0.1% nonionic detergent**

Materials

- Erlenmeyer flask or beaker (50 ml)
- Microwave
- Boiling water bath or hot plate
- Magnetic stir plate
- Magnetic stir bar
- 1-cc tuberculin syringe
- Water bath set to 60°C
- 20-cc syringe
- Prewarmed cassette assembly
- Parafilm® or tape
- Spatula

Reagents

- IsoGel Agarose
- Distilled water
- Ampholytes
- D-sorbitol
- Boiling distilled water
- 60°C distilled water

Preparation and casting of the gel solution

The following procedure is to prepare a 10 ml IsoGel® Agarose gel.

Solution preparation

1. Choose a 50 ml beaker or Erlenmeyer flask.
2. Add 8 ml distilled water and a stir bar to the flask or beaker.
3. Premeasure 0.1 g IsoGel Agarose.
4. Premeasure 1.0 g d-sorbitol.
5. Sprinkle in the premeasured agarose powder, while the solution is rapidly stirring.
6. Using a spatula, break up and disperse any agarose clumps and scrape down any powder adhered to the walls of the flask.
7. Add the d-sorbitol while the solution is rapidly stirring.
8. Remove the stir bar.
9. Follow the procedures on pages 18-21 for dissolving agarose.
10. Cool the solution to approximately 60°C.
11. Add 0.63 ml of ampholyte solution with a 1-cc syringe.
12. Stir the solution well to mix.
13. Maintain the agarose solution at 60°C - 65°C until casting.
14. Correct for evaporation by adding warm distilled water immediately before gel casting.

Gel casting instructions

1. Flush a 20-cc syringe with boiling water to thoroughly heat it.
2. Expel all water from the barrel and needle.
3. Immediately fill the syringe with the warmed agarose solution.
4. Slowly inject the agarose solution into the pre-warmed cassette.

   NOTE: Try to avoid introducing air bubbles into the cassette by injecting the solution in a slow-steady stream.
5. Fill the cassette assembly to the top with agarose solution.
6. Seal the top of the cassette with Parafilm® or tape to prevent evaporation.
7. Allow the casting assembly to cool at room temperature.
8. Place the gel at 4°C for one hour.

Disassembly of casting cassette

1. Remove the tape and clamps.
2. With the cassette lying flat, insert a flat spatula between the glass plates.
3. Twist gently to break the seal.
4. Carefully remove the top plate, leaving the gel and the GelBond® Film attached to the back plate.
5. Remove the spacers.
6. Lift the GelBond Film from the back plate by inserting a flat spatula under the film.
7. Gently lift film and gel away from glass plate.

Sample Preparation

Successful isoelectric focusing, in part, depends upon the condition of the sample. Situations such as insolubility or high salt content, particularly in the case of high sample loading, should be addressed before the sample is loaded onto the gel. Listed below are general guidelines for sample treatment.

High Salt Content: Dialyze the sample against distilled water, 1% glycine, or 0.05 M - 0.1 M ammonium bicarbonate solution.

Dissociation of protein aggregates, subunit assemblies or to unfold peptide chains: Add urea to a final concentration of 4 M - 9 M to both the sample and the gel.

Samples that are hydrophobic or poorly soluble at or near their pI point: Add either nonionic or zwitterionic detergents to the sample and the gel at a final concentration of 0.05% - 1.0%. Detergents should be added to the agarose solution once the agarose has been dissolved and cooled to 60°C.

NONIONIC DETERGENTS	ZWITTERIONIC DETERGENTS
Triton® X-100	CHAPS (available from Sigma Chemical Co., St. Louis, MO)
Nonidet® (NP-40)	Zwittergent® 3-14 (available from Calbiochem/Behring, LaJolla, CA)
Tween® 80	

Urea in IsoGel® Agarose IEF Gels

Caution

Avoid exposing urea to high temperatures. Urea solutions decompose to ammonia and carbon dioxide with excessive heat. Cyanate formation and carbamylation of other gel components, such as ampholytes, may also occur.

Urea is a common additive in the focusing of membrane and aggregated proteins. High concentrations of urea inhibit agarose gelation, and the gels that are formed are much weaker and more fragile than standard IEF gels. Therefore, it is best not to blot an agarose-urea gel. After gelation, gels containing urea should be refrigerated overnight before use.

Agarose-urea gels should be prepared by first dissolving the agarose in a minimum volume of water, then cooling to slightly below 50ºC before stirring in solid urea or a high concentration solution of urea. Once the urea has dissolved, add ampholyte, adjust to the desired total volume with water before casting. Refrigerate high urea concentration gels (7 M - 9 M) overnight. Gels may be quick-chilled by placing them in a freezer for 15 - 20 minutes. Urea crystals that may form may be removed by allowing the gel to sit at room temperature for a few minutes.

Gel strength may be enhanced by increasing the IsoGel Agarose concentration of the gel. If a satisfactory gel cannot be obtained by the above procedure, cast an aqueous IsoGel Agarose gel, then allow it to equilibrate in a urea/ampholyte solution.

Gel Preparation and Focusing

WARNING: Make certain the power supply is turned off before proceeding.

Materials

- Horizontal IEF chamber
- Fine-grained blotting paper
- Razor blade
- Refrigerated circulator bath set to 10°C - 15°C
- Kimwipe® tissues or equivalent
- Electrode wicks
- Scissors
- Forceps
- Glass plate slightly larger than the gel
- Sample applicator mask
- Power supply

Reagents

- Distilled water
- Catholyte solution
- Anolyte solution

Procedure for gel placement

1. Set the refrigerated circulator bath to 10°C - 15ºC.

   NOTE: To prevent condensation on the gel and platen, do not circulate the coolant to the IEF chamber just prior to focusing.

2. Spread 0.2 ml - 0.3 ml of distilled water on the cooling platen of the IEF chamber.
3. Lower the gel (film-side down) onto the wetted area. Avoid trapping air under the GelBond® Film.
4. Wipe excess fluid from the edges of the film.
5. Blot the surface of the gel briefly with a sheet of fine-grained blotting paper.
6. If necessary, trim the edges of the gel parallel to the direction of focusing with a razor blade to ensure the edges are even and free of cracks or small tears.

Procedure for electrode wick application

1. Cut two electrode wicks to the exact width of the gel or slightly shorter.
2. Completely immerse one wick in catholyte solution.
3. Place the wick on blotting paper to remove excess fluid.
4. Place on the negative electrode contact of the gel.
5. Completely immerse a second wick in anolyte solution.
6. Place the wick on blotting paper to remove excess fluid.
7. Place on the positive electrode contact of the gel.

   NOTE: The wicks must lie parallel to each other on the ends of the gel, evenly touching the surface.

8. Place a glass plate on top of the gel and wicks for approximately one minute.

   NOTE: This ensures uniformity of contact between wicks and gel and serves to smooth the wick surface in preparation for electrode contact.

Continued on page 214

Procedure for sample application

1. Place the sample applicator mask across the gel at least 1 cm from either wick (e.g., 3 cm from cathode).
2. Load sample and pI markers into the slots (2 µl - 5 µl maximum; 2 - 10 mg/ml concentration).

   NOTE: In direct tissue isofocusing, tissue samples may be placed directly onto the applicator slots.
3. Ensure electrodes and electrical contacts are clean and there are no breaks in the wire or ribbon.
4. Place the electrodes on the wicks (not the gel surface), aligning them so they lie in parallel upon the wicks.
5. Set the power supply at 1 W constant power.
6. Apply power for 10 minutes.
7. Turn power off.
8. Remove the sample applicator mask.
9. Gently remove any precipitated sample from the gel surface with blotting paper.

IEF power settings and focusing time

1. Start circulation of the coolant to the IEF chamber.
2. Set the voltage, current and power according to the appropriate running conditions listed in the table below.

Running Conditions

AMPHOLYTE pH RANGE	VOLTAGE (LIMITING)	CURRENT	POWER (UPPER LIMITING)	ANOLYTE	CATHOLYTE	FOCUSING TIME (MINUTES)
2.5 - 4.5	250 V	MAX	10 W	*1 M H_3PO_4	0.05 M Threonine	90
3.5 - 9.5	500 V	MAX	10 W	0.5 M HOAc	1 M NaOH	90
3.5 - 9.5	1000 V	MAX	25 W	0.5 M HOAc	1 M NaOH	40
3.5 - 9.5	1500 V	MAX	25 W	0.5 M HOAc	1 M NaOH	30
4 - 6.5	500 V	MAX	10 W	2% solution of pH 2.5 - 4.5 Ampholyte	0.04 M L-Histidine (free-base)	90
5 - 8	500 V	MAX	10 W	2% solution of pH 2.5 - 4.5 Ampholyte	0.1 M NaOH or 0.1 M Bicine	90

**1M H_3PO_4 can be replaced by 0.5 M acetic acid*

3. Once focusing is complete, turn off the power.

   NOTE: The separation progress can be monitored by observing the visible proteins in the pI markers coming into focus and noting the decreasing rate of current flow on the power supply's milliampere indicator. Focusing is attained when the visible pI markers are sharply resolved and the current has stopped decreasing significantly (less than 1 mA in 10 minutes).
4. Discard the wicks.
5. Place the gel in fixative solution.

Staining Proteins in IsoGel® Agarose IEF Gels

Either Coomassie® blue or Crowle's double stain can be used to stain IEF gels. Coomassie stain is used when increased sensitivity is desired, and Crowle's stain produces gels with clear background and sharp resolution.

Materials

- Flask
- Stir bar
- Magnetic stir plate
- Forceps
- Paper towel
- Whatman® 3MM chromatography paper
- 1 kg-2 kg weight
- Glass plate
- Forced hot air oven set to 50°C-55°C
- Staining vessel
- Clamps

Reagents

- Methanol
- Trichloroacetic acid
- Sulfosalicylic acid
- Distilled water
- Coomassie brilliant blue R-250
- Ethanol
- Glacial acetic acid
- Crocein scarlet (C.I. 26905)

Staining proteins with Coomassie blue stain or Crowle's double stain

Preparation of staining solutions

FIXATIVE SOLUTION

180 ml	Methanol
30.0 g	Ttrichloroacetic acid
18.0 g	Sulfosalicylic acid

Adjust volume to 500 ml with distilled water

COOMASSIE STAIN

1.0 g	Coomassie brilliant blue R-250
250 ml	Ethanol
90 ml	Glacial acetic acid

Adjust volume to 1 liter with distilled water

COOMASSIE DESTAINING SOLUTION

250 ml	Ethanol
90 ml	Glacial acetic acid

Adjust volume to 1 liter with distilled water

CROWLE'S DOUBLE STAIN

2.5 g	Crocein scarlet (C.I. 26905)
150.0 mg	Coomassie brilliant blue R-250
50 ml	Glacial acetic acid
30.0 g	Trichloroacetic acid

Adjust volume to 1 liter with distilled water

CROWLE'S DESTAINING SOLUTION

3 ml	Glacial acetic acid

Adjust volume to 1 liter with distilled water

Follow the steps below to stain the gel after electrophoresis using either Coomassie® blue or Crowle's double stain.

1. Place the gel in Fixative solution for 10 minutes.
2. Remove the gel and rinse the surface with distilled water.
3. Drain excess water.
4. Place on a paper towel, gel side up.
5. Pre-wet a piece of Whatman® 3MM chromatography paper with distilled water.
6. Place on gel surface.
7. Overlay the blotting paper with four to six layers of absorbent paper toweling.
8. Place a glass plate on top of the paper toweling.
9. Place a 1 kg - 2 kg weight on top of the toweling for 10 minutes.
10. Remove the weight, the glass plate, and the paper toweling.
11. Rewet the blotting paper thoroughly with distilled water.
12. Gently lift the blotting paper off gel surface.
13. Wash the gel in distilled water for 5 minutes to remove residual fixative and ampholytes.
14. Dry the gel completely in a forced hot air oven (50°C - 55°C).

    NOTE: Drying usually takes less than 30 minutes.
15. Stain with Coomassie or Crowle's double stain solution for 15 - 30 minutes without agitation.

    NOTE: Float gel-face down into the stain, so precipitated stain will not settle on the gel surface.
16. Remove the gel and rinse with distilled water.
17. Place the gel in Destaining solution for 3 minutes.
18. Briefly rinse again in distilled water.
19. Clamp (gel side out), onto a glass plate to prevent curling during drying.
20. Dry the gel in a forced hot air oven (50°C - 55°C) for approximately 15 minutes or dry at room temperature overnight.

    NOTE: Gel may crack if over dried by heating.

Staining Proteins in Agarose IEF Gels with Silver Stain

A modified silver stain procedure has been developed for use with agarose gels cast on GelBond® Film. After electrophoresis, the gels are fixed, press blotted, and completely dried before staining. Perform all fixing and staining steps in acid-cleaned (50% HNO_3) glassware. All washes are done with constant agitation in a volume of at least 250 ml (gel volume:reagent volume = 1:22). Coomassie® brilliant blue stained gels may be silver stained after drying. In this case, proceed directly to step 14.

Materials

- Paper towel
- Whatman® 3MM chromatography paper
- 1 kg-2 kg weight
- Glass plate
- Forceps
- Clamps
- Forced hot air oven set to 50°C-55°C
- Staining containers
- Beakers
- Magnetic stir plate
- Magnetic stir bar
- Acid-cleaned dish
- Orbital or rocking platform shaker

Reagents

- Methanol
- Trichloroacetic acid
- Sulfosalicylic acid
- Distilled water
- Glutaraldehyde
- Dithiothreitol (DTT)
- Anhydrous sodium carbonate
- Ammonium nitrate
- Silver nitrate
- Dodeca-tungstosilicic acid (Gallard-Schlesinger Cat. No. 305453)
- 37% formaldehyde
- Acetic acid

Preparation of staining solutions

FIXATIVE SOLUTION

180 ml	Methanol
30.0 g	Trichloroacetic acid
18.0 g	Sulfosalicylic acid

Adjust volume to 500 ml with distilled water

SILVER STAIN SOLUTION A

50.0 g	Sodium carbonate, anhydrous in 1 liter distilled water

(Stable for 2 - 3 weeks at room temperature)

SILVER STAIN SOLUTION B

In the order given dissolve the following reagents in 1 liter of distilled water, while mixing rapidly.

2.0 g	Ammonium nitrate
2.0 g	Silver nitrate
10.0 g	Dodeca-tungstosilicic acid
and 6.7 ml	37% formaldehyde

(Stable 1 week at room temperature stored in the dark)

STOP SOLUTION

1%	Acetic acid

Follow the steps below to stain the gel after electrophoresis using silver stain

1. Place the gel in Fixative solution for 10 minutes. If gel is prestained with Coomassie® blue and dried, proceed to step 14.
2. Place the gel on a paper towel, gel side up.
3. Pre-wet a sheet of Whatman® 3MM chromatography paper with distilled water.
4. Place on gel surface.
5. Overlay the blotting paper with four to six layers of absorbent paper toweling.
6. Place a glass plate on top of the paper toweling.
7. Place a 1 kg - 2 kg weight on top of the toweling for 10 minutes.
8. Remove the weight, the glass plate, and the paper toweling.
9. Rewet the blotting paper thoroughly with distilled water.
10. Gently lift the blotting paper off the gel surface.
11. Wash the gel in distilled water for 5 minutes to remove residual fixative and ampholytes.
12. Clamp (gel side out), onto a glass plate to prevent curling during drying.
13. Dry the gel in a forced hot air oven (50°C - 55°C) for approximately 15 minutes or until dry.

    NOTE: Gel may crack if over dried by heating.
14. Soak the dried gel in 2% glutaraldehyde for 10 minutes.
15. Wash in distilled water for 10 minutes using mild agitation.
16. Soak the gel for 10 minutes in 0.01% (DTT) dithiothreitol.
17. Wash in distilled water for 10 minutes using mild agitation.
18. Pour an equal volume of Silver stain solution B into vigorously stirring Silver stain solution A (75 ml B and 75 ml A for each gel to be stained).
19. Transfer the solution to an acid-cleaned glass dish containing one gel.
20. Stain the gel for 10 minutes with gentle agitation.

    NOTE: There will be some background.
21. Transfer the gel to a Stop solution and gently agitate for 5 minutes.
22. Rinse the gel in distilled water.
23. Wipe any silver deposits from the back of the film.
24. Allow to air dry.

Press Blot Transfer of Proteins from IsoGel® Agarose

Materials
- Nitrocellulose membrane
- Thick filter paper
- Scissors
- Smooth-tipped forceps
- Container
- Paper towels
- Glass plate

Reagents
- Tris-saline buffer, pH 7.5
  (50.0 g Tris-HCl, 0.94 g Tris-Base, 58.48 g NaCl adjust to 2 liters with distilled water)

Press blot transfer is a quick method of removing proteins from agarose gels. The procedure involves overlaying the gel with a buffer-soaked nitrocellulose membrane covered by a thick filter pad and several layers of dry paper toweling. The assembly is then covered by a glass plate. After just 1½ minutes of press blot, approximately 20% of the proteins are transferred from the gel to the membrane. Up to 85% transfer can be achieved with a 35 - 40 minute blotting time. Transferred proteins can be detected on the membrane and on the gel by standard methods.

1. Prepare Tris-saline buffer, pH 7.5.
2. Cut one piece of nitrocellulose membrane and thick filter paper to the same dimension as the gel.

   NOTE: Wear gloves to prevent contamination by extraneous proteins.
3. Evenly wet the nitrocellulose membrane in the Tris-saline buffer by holding one end of the membrane with smooth-tipped forceps and lowering the other end into the buffer container, dropping the membrane flat on the buffer surface.

   NOTE: The membrane must be completely saturated with buffer.
4. Remove the gel from the focusing chamber.
5. Place on a flat surface, gel side up.
6. Place the buffer-soaked nitrocellulose membrane onto the gel surface.

   NOTE: Avoid trapping air bubbles between the gel and the membrane.
7. Place one piece of buffer soaked filter paper on top of the membrane.
8. Place three layers of dry paper toweling on top of the filter paper.
9. Cover with a glass plate slightly larger than the gel surface. No other weight is required.
10. Press blot for 1½ minutes or longer, as desired.
11. Remove glass plate and discard the paper toweling and filter paper.

Preparative Isoelectric Focusing

Separation of relatively large amounts of biologically active macromolecules is possible by isoelectric focusing in agarose. Typical high-yield recoveries of applied proteins are obtainable with retention of biological activity. This preparative isoelectric focusing procedure is based on the work of Cantarow, *et al*. As much as 120.0 mg of protein can be focused in 9.5 ml (0.75 x 10 x 11.5 cm) of 1% IsoGel® Agarose containing 2.5% ampholytes.

1. Follow steps 1 - 14 for Gel Preparation on page 210. Also see Sample Preparation on page 212.
2. Add the protein sample to the agarose solution once cooled to 60°C.
3. Stir the solution well to mix.
4. Cast the gel and disassemble the casting cassette following the steps previously described on page 211.
5. Place the gel on the 10°C cooling platen for 10 minutes (if gel has not already been chilled after casting).
6. Focus following the steps previously described on page 213.

Procedure for recovering proteins from preparative gel

Materials
- Spatula
- 5-cc plastic syringe with an 18-gauge needle
- Clean centrifuge tube
- Test tube rocker at 4°C
- Centrifuge
- Serum separator
- Parafilm®

Reagent
- Phosphate-buffered saline (PBS)

1. Excise the gel slice containing the protein of interest by using a spatula to strip the agarose from the GelBond® Film in 5 mm-wide slices.
2. Place the agarose strip in a 5-cc plastic syringe fitted with an 18-gauge needle.
3. Macerate the gel slice by expelling it into a clean tube.
4. Add 4 ml of phosphate-buffered saline (PBS) to the macerated gel.
5. Cover the tube securely with Parafilm®.
6. Place on a test tube rocker for 16 hours at 4°C.
7. Centrifuge the tube for one minute at 100 rpm.
8. Separate the supernatant from the gel using a serum separator.

Immunofixation/Immunoperoxidase or Autoradiography

Materials
- **Staining containers**

Reagents
- **Hydrogen peroxide**
- **Distilled water**
- **Periodic acid**
- **Sodium borohydride**
- **0.05 M Tris-saline (pH 7.6), normal serum** (same species as secondary antibody)
- **Anti-serum** (primary antibody)
- **0.15 M PBS** (pH 7.4)
- **Secondary antibody**
- **20 mg/ml avidin solution**
- **Horseradish-peroxidase**
- **0.15 mg/ml diaminobenzidine**
- **0.01 M Tris/ 0.15 M NaCl**

Detection of separate species can be accomplished by protein stains or by overlaying gels with specific antibody solutions coupled to enzymes that will eventually produce a visible end product. The antibody-peroxidase conjugate system or autoradiography with ^{125}I- labeled antibody are frequently used for this purpose. Immunoperoxidase labeling of focused proteins is performed according to Saravis, *et al.* After focusing, fixing, and drying the gel, treat the gel as described below.

Procedure for Immunoperoxidase Staining/Avidin-Biotin Modification

1. Soak the gel in 3% hydrogen peroxide for 10 minutes.
2. Rinse with distilled water.
3. Soak the gel in 2.28% periodic acid for 5 minutes.
4. Rinse with distilled water.
5. Soak the gel in 0.02% sodium borohydride for 2 minutes.
6. Rinse with distilled water.
7. Place the gel in 0.05 M Tris-saline, pH 7.6 for 10 minutes.
8. Incubate the gel with 1:5 normal serum for 10 minutes (same species as secondary antibody).
9. Incubate the gel for 2 - 4 hours at room temperature with Anti-serum(primary antibody).
10. Rinse the gel with 0.15 M PBS, pH 7.4 for 1 hour at room temperature.
11. Incubate the gel with Secondary* antibody (e.g., goat anti-mouse IgG) for 2 - 4 hours at room temperature.

    NOTE: If the avidin/biotin modification is used, proceed with steps 12 and 13 using biotinylated reagents (marked with * in steps 11 and 15).

12. Rinse the gel with 0.15 M PBS for 1 hour at room temperature.
13. Incubate the gel with avidin solution (40 mg/gel) in PBS for 1 hour at room temperature (stock solution of avidin is usually 20 mg/ml).
14. Rinse the gel with 0.15 M PBS for 1 hour at room temperature.
15. Incubate the gel with horseradish-peroxidase* (33 mg/gel) for 2 hours at room temperature.
16. Rinse the gel with 0.15 M PBS for 1 hour at room temperature.
17. Incubate the gel with diaminobenzidine (0.15 mg/ml) and hydrogen peroxide (0.03%) in 0.01 M Tris 0.15 M NaCl for 1 - 17 hours at room temperature.
18. Rinse the gel in PBS and dry.

Direct Tissue Isoelectric Focusing (DTIF)

This method employs the application of tissue or cell pellets directly onto the surface of the IEF gel without concentration of samples, dialysis to remove salts, or the salt extraction of soluble proteins from tissue. DTIF allows more soluble protein per milligram of tissue to enter the gel than is recoverable by extraction procedures and minimizes denaturation of biologically active proteins that can be damaged by extraction.

1. Prepare solutions and cast gels as previously described on page 208.
2. Prepare for focusing as previously described on page 213.
3. Place the sample applicator mask across the gel at least 1 cm from either wick (e.g., 3 cm from cathode).
4. Drape a tissue slice over the open slot of the applicator mask.
5. Ensure the electrodes and the electrical contacts are clean and there are no breaks in the wire or ribbon.
6. Place the electrodes on the wicks (not the gel surface), aligning them so they are in parallel upon the wicks.
7. Set power supply at 1 W (constant power).
8. Prefocus for 10 - 15 minutes to allow sample uptake.
9. Turn power supply off.
10. Remove the tissue slice and the applicator mask.
11. Focusing is continued using standard conditions.
12. After focusing is complete, the gel is fixed, stained and dried following standard procedures.

Resolution Reference Guide

Phenomenon	IEF Band Appearance	Possible Causes	Remedy
Streaks or gaps perpendicular to bands		• Particulates in sample	• Filter or centrifuge sample before applying.
		• Old or denatured sample	• Replace sample.
		• Isoelectric precipitation of sample applied too close to pI point	• Do not prefocus gel before applying sample. • Apply sample in different location.
		• Poorly soluble sample, which precipitates	• Use solubilizing additive, i.e., non-ionic, Zwitterionic • Try different application location.
Fuzzy bands		• Run incomplete	• Use pI markers to monitor run or measure pH gradient before removing gel from platen. • Increase IEF time/power conditions.
		• Focusing time too long	• Decrease focusing time or power/voltage.
		• Insufficient fixation	• Increase fixation time. Be sure to place gel in fixative immediately after IEF run.
Skewed bands		• Electrode not clean	• Rinse electrode with distilled water, then dry.
		• Uneven electrode contact	• Wicks should be evenly wetted and placed on gel parallel to each other. • Place electrodes firmly on wicks but not so fluid is squeezed from wicks.
		• Sample placed too close to edge of gel	• Apply ≥5 mm from edge of gel.
		• Electrode wicks too short	• Wicks should extend to edge of gel.
		• Old or incorrect electrolyte solutions	• Make fresh solutions.
		• Overloading of samples	• Decrease protein load.
		• Excessive salt in sample	• Reduce salt concentration by gel filtration or dialyze against 1% glycine.
Missing or faint bands		• Protein not denatured by fixative	• Establish appropriate denaturing conditions.
		• Sample unstable at pH of site of application	• Use alternative application site.
Wavy bands		• Excessive salt in sample	• Reduce salt concentration by gel filtration or dialyze against 1% glycine.
		• Overloading with sample, distorting pH gradient	• Apply smaller sample volume.
		• Improper anolyte used	• Replace anolyte.
		• Old or incorrect electrolyte solutions	• Make fresh solutions.
		• Dirty electrodes	• Rinse electrode with distilled water, then pat dry.
Arc-shaped bands		• Thin spot in gel	• Reduce evaporation by keeping gel sealed until ready for use. • Keep chamber lid closed except when handling gel.

Phenomenon	IEF Band Appearance	Possible Causes	Remedy
Burning or sparking		• Electrode wicks too dry	• Anodal wick should be wet but not dripping wet; cathodal wick should be damp.
		• Focusing too long	• Reduce focusing time to minimum required to obtain linear pH gradient.
		• Thin spot in gel	• Keep gel sealed until ready for use.
		• Using incorrect electrolyte solutions	• Always use recommended solutions.
		• Excessive power input	• Check power supply settings.
Sparking along edge of gel onto cooling plate		• Excess moisture on the gel, or under gel	• Remove excess moisture by gently blotting gel.
		• Electrode strips overhanging ends of gel	• Cut electrode strips to size of gel.
		• Liquid expelled at sides of electrode due to EEO flow of water to cathode.	• Occasional blotting may be necessary.
		• Excess electrode pressure when placing electrodes on gel	• Use firm but light pressure.
Formation of 'ditch' ultimately causing gel to burn out		• Gradient drift	• Check purity of reagents.
		• High current causing excessive heating early in run.	• Use a low power for first 10-15 minutes of run during sample uptake.
		• Uneven gel thickness due to gel drying out	• Open foil package containing gel just before use.
		• Overblotting of gel, causing gel to dry out	• Initially blot gel only until blotting paper picks up moisture.
Sample smearing or precipitation		• Sample overload	• Decrease sample concentration.
		• Sample aggregation	• Solubilize sample with neutral surfactant or 1% glycine.
		• Sample applied too near pI or at a pH where it is insoluble	• Change application site. Solubilizing additives or detergents may be necessary.
Incomplete Sample Uptake		• Sample application mask not left on long enough	• Allow at least 10 minutes.
		• Insolubles or aggregates in sample	• Filter or centrifuge sample.
		• Gel too wet when sample applied	• Blot gel before sample application.
Sample retained in sample application well		• Sample applied too near pI	• Change application site.
Fluid expression			
• At Cathode		• Unblotted wick	• Blot cathodal wick with filter paper until damp before application.
		• Overfocusing	• Monitor run with pI markers.
• Localized elsewhere		• Salt in sample	• Desalt sample before electrofocusing.
		• Sample overload	• Decrease sample concentration.
		• Uneven electrode contact to wicks	• Check location of electrodes. Gently press electrodes onto wicks.
		• Electrode wicks too wet	• Remove excess moisture. Wicks must be blotted before use.
		• Local hot spot from spillage of electrode solution onto gel.	• Avoid this area for sample application.
• General (over entire gel)		• Insufficient/excessive cooling	• Check coolant flow and temperature.
		• Excessive power applied to gel	• Check power supply settings.
		• Gel not adequately blotted	• Use blotting paper.
		• High ambient humidity	• Use tray with desiccant in chamber.
• Excessive amount along electrode wicks. Persists for duration of run.		• Cathodal drift (pooling of fluid near cathode—over-saturating wick)	• Blot pooled liquid if necessary.
		• Reversed polarity of electrode wicks (lower pH at cathode; higher at anode)	• Check pH of wicks and polarity of plugs into power supply. Reverse polarity if incorrect.
• Spotted over gel		• Localized hot spots caused by bubbles between gel film and chamber platen	• Check for air bubbles—remove if present.

Phenomenon	IEF Band Appearance	Possible Causes	Remedy
Condensation inside chamber		• Inefficient cooling	• Adjust coolant temp. to 10°C.
		• Voltage/power setting too high	• Adjust to 1 W for 1st 10 min., 25 W limiting thereafter (pH 3-10).
		• Salt in sample	• Desalt sample.
		• Humid ambient conditions	• Place humectant cartridge or desiccant tray in chamber.
Gel thinning			
• at electrode		• Improper electrolyte solution	• Check electrolyte solution.
• near point of application		• Sample overload	• Decrease sample concentration.
• generalized		• Gel left out of cassette too long under low humidity conditions	• Leave gel in cassette until ready to use or place in humidity chamber.
Uneven pH gradient/pH gradient different than stated for ampholyte used		• Incorrect calibration of pH electrode	• Check pH electrode
		• Gel overfocused—excessive gradient drift	• Do not exceed time required to form a linear pH gradient.
		• Impure distilled water in sample or electrolyte solutions	• Check water for heavy metals or ionic contaminants.
		• Incorrect electrolyte solution	• Check solutions.
pH gradient is not linear		• Insufficient run time	• Use IsoGel® pI markers to check progress. Measure pH gradient before removing gel.
pH gradient not attaining upper limits desired		• Overfocusing	• Decrease run time.
		• pH measurements may be wrong	• Use surface pH electrode.
pH range shifted toward cathode		• Cathodal drift (entire gradient shifts towards cathode)	• Reduce run time (as much as possible) to attain desired gradient profile.
Current increases with time		• Electrodes applied at the wrong ends of the gel	• Use recommended anolyte/catholyte solutions. • Apply (+) electrode to anode, (−) electrode to cathode. • Reverse polarity.
Diagonal band migration		• Uneven electrode contact	• Apply electrodes evenly on wicks.
		• Too much pressure on electrodes	• Adjust for light but firm contact.
		• Uneven electrode placement	• Ensure electrodes are parallel and perpendicular to the sample migration.
Lateral band migration		• Air pocket(s) trapped beneath mask	• Press gently to expel before sample applied.
		• Too much sample applied to applicator mask	• Reduce sample volume, increase sample concentration.
		• Sample mask left on too long/ not long enough	• Remove mask after 10 minutes at 1 watt.
Sample "focuses" at wrong position		• Run incomplete	• Allow longer focusing time.
		• Sample unstable at pH of application	• Use alternative application site.
		• Removal of ligands during focusing	• Check protein for this possible characteristic.
		• Formation of complex aggregate	• Use urea to treat sample.
A supposedly "pure" sample focuses as multiple bands		• Sample exists in various states of oxidation	• Pretreat sample accordingly.
		• Sample disassociates into subunits	• Natural phenomenon.
		• Sample has become denatured	• Check procedures used to prepare sample.
High background stain		• Residual ampholytes in gel	• Follow recommended press blot procedure. • Extend time of fixing, rinsing and destaining.
		• Concentration of stain too high	• Reduce concentration.
		• Stain is old	• Make fresh stain and be sure all dye is in solution.
		• Gel allowed to settle in bottom of staining dish	• Float gel side down in stain for 15-30 minutes.
Delamination (peeling) of gel from film support on staining after drying.		• Residual fixative in dried gel	• Follow recommended press blot/rinsing procedure.

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Chapter XIV

Protein Separation in Agarose Gels

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Protein Electrophoresis in Agarose Gels

Introduction

Protein electrophoresis in agarose gels is an alternative approach to using polyacrylamide gels and provides several benefits. Gels can be run using a vertical system or a horizontal system and unlike polyacrylamide gels, agarose gels can be used effectively to separate proteins larger than 600,000 kDa.

Advantages

- Separate high-molecular-weight proteins (>600,000 kDa)
- Easy to prepare and handle
- Efficient recovery of proteins
- Excised proteins can be used to immunize animals directly for antibody production
- Non-toxic

Recommended agaroses for protein electrophoresis

The table below is a list of Cambrex Agaroses that are recommended for protein electrophoresis. For performing routine separations, we recommend a standard melting temperature agarose such as MetaPhor® or SeaKem® Gold Agarose. When proteins are to be recovered for further analysis, use a low melting temperature agarose such as SeaPlaque® or NuSieve® GTG® Agarose.

Protein Size Range (kDa)	Agarose	Concentration
20 - 200	MetaPhor or NuSieve GTG Agarose	5%
150 - 300	MetaPhor or NuSieve GTG Agarose	3%
300 - 600	MetaPhor or NuSieve GTG Agarose	2%
600 - 1,000	SeaKem Gold or SeaPlaque Agarose	1.5%
1,000 - 5,000	SeaKem Gold or SeaPlaque Agarose	1.0%

Buffers for Protein Separation in Agarose

The buffer systems used for agarose electrophoresis are similar to those used for polyacrylamide electrophoresis. When performing horizontal electrophoresis, we have found that a Tris-borate gel and running buffer provide greater resolution than using the standard Laemmli buffer system.

Vertical and horizontal gel

1X STACK BUFFER, pH 8.0	g/l FOR 1X STACK BUFFER
125 mM Tris-HCl	19.7 g Tris-HCl in 1 liter distilled water

NOTE: Horizontal gels do not require the use of a stack gel.

1X RESOLVING BUFFER, pH 8.5	g/l FOR 1X RESOLVING BUFFER
500 mM Tris base	60.55 g Tris base
160 mM Boric acid	9.90 g Boric acid
1 M Urea	60.06 g Urea
	Adjust volume to 1 liter with distilled water

1X RUNNING BUFFER, pH 8.5	AMOUNT FOR 1X RUNNING BUFFER
90 mM Tris base	10.90 g Tris base
90 mM Boric acid	5.57 g Boric acid
0.1% SDS	10 ml of 10% SDS
	Adjust volume to 1 liter with distilled water

Laemmli buffer

- Gels can be made with standard Laemmli buffer (see page 173).
- To avoid excess foaming during agarose dissolution; only add SDS to the cathodal buffer and not to the gel buffer. The SDS in the cathodal buffer will migrate faster than the proteins during electrophoresis, maintaining protein denaturation.
- To prevent buffer depletion in vertical systems, use 10X Laemmli buffer without SDS in the anodal buffer.

Tips for buffer preparation

- Add SDS to the cathodal buffer only.
- Do not add SDS to the gel prior to dissolution.
- If other buffer systems are used, the pH should be between pH 5-9.
- Denaturants such as urea and formamide should only be added at low concentrations (4 M-6 M Urea).
- For buffers more alkaline than pH 9, dissolve and cast the agarose in distilled water, allow to gel. Soak horizontal gels in the alkaline gel buffer for 30 minutes prior to electrophoresis. For vertical gel systems, prerun the gel to equilibrate the gel with the alkaline buffer.

Casting Agarose Gels for Protein Separation

The procedures for dissolving and casting agarose gels for protein separation are the same as the procedures used for nucleic acid separation. Refer to Dissolving Agarose and Casting Agarose Gels (see Chapter I).

Tips for casting horizontal agarose gels

- Use of a stacking gel is not necessary for horizontal submarine electrophoresis.
- The resolving gel buffer and running buffer should be the same.
  Dissolve the agarose in running buffer without SDS.
  For denaturing electrophoresis, add SDS to the sample buffer and the running buffer.
- Let the gel set for 20-30 minutes at room temperature.
- For MetaPhor® and SeaPlaque® Agarose, chill the gel at 4°C for 20-30 minutes before removing comb.
- If gels are to be dried, cast the gels onto GelBond® Film.

Tips for casting vertical agarose gels

Stacking gel for vertical gels

- Prepare a 1% SeaKem® Gold Agarose gel in stacking gel buffer.
- For proteins >100 kDa, the use of a stacking gel may be omitted. It will not enhance band resolution.
- After the stacking gel is set, place cassette at 4°C for 30 minutes prior to removing the comb.

Resolving gel for vertical gels

- Dissolve the agarose in running buffer without SDS.
- Refer to Vertical Gel Casting Instructions (see page 24).
- Allow resolving gel to set approximately 3 minutes at room temperature then cast the stacking gel.

To facilitate comb removal from a vertical gel

- The teeth of the comb can be tapered so the width at the bottom is slightly smaller than at the top. A slight rounding of the edges is all that is needed so that the end is U-shaped. Tapering the teeth in this way will not affect the pattern of the protein bands.
- Flood the comb area with running buffer prior to removing the comb.
- If clamps are used, remove the clamps at the top of the gel cassette and gently loosen the comb by moving it forward and back before removal.

Preparation and Loading of Protein Samples

Sample preparation and amount of protein that can be loaded on agarose gels is essentially the same as for polyacrylamide gels and is largely dependent on your application and detection method.

Guidelines

- Suspend protein samples in 2X sample buffer, 1:1 (v:v).
- If denatured proteins are required, incubate at 95°C-100°C for 5 minutes.
- Load the samples into the sample wells.
- The minimal amount of protein detectable by Coomassie® brilliant blue stain is about 1.0 µg; and may vary depending on the protein.
- Larger amounts of protein can be loaded, but band thickness increases accordingly.
- For a 0.8 cm wide well, 25 ml (50 µg total protein) is recommended for a complex mixture, if staining with Coomassie blue, and 1 ml (10 µg total protein) is needed for samples containing one or a few proteins.
- For vertical electrophoresis, load empty wells with sample buffer.

2X Tris-Glycine SDS sample buffer for agarose electrophoresis of proteins

2X concentrate	Amount to add for 10 ml
126 mM Tris-HCl, pH 6.8	2.5 ml of 0.5 M Tris-HCl, pH 6.8
15% Ficoll® Type 400	1.5 g Ficoll Type 400
4% SDS	4 ml of 10% SDS
0.002% Bromophenol Blue	0.2 ml of 0.1% Bromophenol blue
	Adjust volume to 10 ml with distilled water

Before use: add 1 ml β-Mercaptoethanol (βME) to 10 ml of 2X Tris-Glycine SDS sample buffer

Optimal Voltage and Electrophoretic Times

Tips

- Avoid higher power settings as the heat generated may melt the agarose.
- Thick vertical gels (>1 mm) will require proportionally higher current settings to complete the electrophoresis run within the times indicated.
- Electrophorese the gel until the tracking dye travels to the bottom of the resolving gel.
- Prestained molecular weight markers such as Cambrex's ProSieve® Color Protein Markers can be used to monitor electrophoresis. The gels can be electrophoresed longer, but care should be taken that smaller proteins do not travel off the gel.

Gel Type	Horizontal Gel	Vertical Gel	Mini-Vertical
Gel Size	5.3 cm x 8.5 cm x .4 cm	14.5 cm x 16.5 cm x .1 cm	8 cm x 10 cm x .1 cm
Power Setting	100 volts	25 mA (constant) (approximately 80 volts at the start and 200 volts at the end)	20mA (approximately 50 volts at the start and 120 volts at the end)
Time	3 - 4 hours	3 - 3.5 hours	1.5 - 2 hours

Detection of Proteins in Agarose Gels

The procedures for staining agarose gels with Coomassie® blue stain are essentially the same as they are for polyacrylamide gels with some modifications (listed below). For detailed procedures on Coomassie staining refer to Detection of Proteins on Polyacrylamide Gels (see page 193), using the modifications listed below. For detailed procedures on Silver staining refer to Staining Proteins with Silver Stain (see page 218 in Isoelectric Focusing of Proteins on Agarose Gels).

Tips

- Agarose gels require more time to process than polyacrylamide gels of similar dimensions.
- Staining and destaining times will vary depending on the gel concentration, thickness and protein concentration.
- Place container on shaker with gentle motion during staining and destaining procedures.

Staining proteins with Coomassie® brilliant blue stain

COOMASSIE® BLUE STAIN SOLUTION		DESTAIN SOLUTION
40%	Methanol	20% Methanol
10%	Glacial Acetic Acid	5% Glacial Acetic Acid
0.25%	Coomassie brilliant blue R-250	

Room temperature staining

- A 14.5 cm x 16.5 cm, 1 mm thick agarose gel will stain in approximately 1 to 2 hours.
- Destain for 1-4 hours with gentle shaking at room temperature.

Overnight staining

- Use 0.125% Coomassie blue R-250 with the same concentrations of methanol and acetic acid as in the stain solution.
- Destain approximately 4 hours.

Accelerated staining

- Stain gels using standard stain solutions at 50°C.
- A 1 mm thick gel takes approximately 1 hour to stain and 1 hour to destain.
- Change the destaining solution 1 time.
- Agarose gels become softer at 50°C - use a support to transfer between solutions.

Storage

- Do not store agarose gels in destain solution, they may become brittle and fracture.
- Store gels in a 5% glycerol solution or dried.

Gel Drying and Preservation

Agaroses gels can be dried overnight at room temperature, dried in a forced hot-air oven or dried using a standard vacuum gel dryer. When not using a vacuum gel dryer the gel must first have been cast onto GelBond® Film to prevent the gel from shrinking during the drying process. The procedures for drying protein agarose gels are the same as drying DNA agarose gels. For detailed procedures on drying agarose gels by one of the three methods listed, refer to Drying Agarose Gels without a Vacuum Gel Dryer or Drying Agarose Gels with a Vacuum Gel Dryer (see pages 153-155).

Processing Agarose Gels Following Electrophoresis

Autoradiography

After drying, agarose gels can be exposed directly to X-ray film.

Fluorography

Do not immerse agarose gels into any fluorography solution if the gels are attached to GelBond® Film. Solutions containing high concentrations (>50%) of DMSO must not be used, as they will dissolve the agarose. Commercially prepared solutions which precipitate the fluor within the agarose gel matrix (e.g., EN^{3}HANCE® from Perkin Elmer) work best. Follow the manufacturer's instructions. The fluor-impregnated gel can then be dried onto filter paper under vacuum at <50°C in a slab-gel dryer and then exposed directly to X-ray film.

Electroblotting proteins from agarose gels

Proteins can be electroblotted out of agarose gels onto membranes (nitrocellulose, PVDF, etc.) by using the same methods used for polyacrylamide gels. Refer to Blotting Proteins from Polyacrylamide Gels for detailed procedures (see page 198). It is important to note that agarose gels adhered to GelBond Film cannot be electroblotted because GelBond Film is nonporous. The time required for optimal transfer of specific proteins will need to be determined experimentally. In general, proteins transfer 15% faster out of agarose gels than from a polyacrylamide gel.

Recovering Proteins from Agarose Gels

Materials
- Spatula
- 1.5 ml microfuge tube(s)
- Heat block
- -70°C freezer
- Microcentrifuge at 4°C

Reagents
- Extraction buffer (50 mM Tris-HCl, 1 mM EDTA at pH 8.0)
- Ice

Proteins can be readily recovered from agarose gels. When protein is to be recovered the use of a low melting temperature agarose such as NuSieve® GTG®, MetaPhor® or SeaPlaque® Agarose is recommended.

Protein Recovery Tips

- Identifying the protein to be recovered from the gel can be accomplished by several methods. If the gel is fixed and stained with Coomassie® blue as detailed earlier, then 1% SDS should be added to the dilution buffer. Recovery of proteins in the native state requires that they not be fixed prior to recovery. Methods for detecting proteins which do not require fixing include:
  - Using prestained molecular weight markers as a guide for the relative position of the desired protein in the gel.
  - Performing a short (<10 minutes) pressure/capillary blot of the gel so that small (<5%) amounts of the proteins are transferred to a membrane and then gold stained. If prestained molecular weight markers are used, it is possible to place the stained membrane under the gel and identify the region of interest.
- We recommend the use of 50 mM Tris-HCl, 1 mM EDTA at pH 8.0 as the extraction/dilution buffer. The buffer may require modification depending upon the particular protein to be recovered and what further work is planned after it is recovered. The buffer should be one in which the protein of interest will be stable.
- The amount of extraction/dilution buffer added is directly related to the amount of protein that can be recovered: the more the agarose is diluted, the more protein will be recovered. Greater than 90% of a protein can be recovered with a dilution to 0.5% agarose. A second dilute-freeze-spin cycle can be performed to recover additional protein.

Procedure

1. Identify the region of the gel which contains the protein to be recovered.
2. Excise a gel slice containing the protein of interest.
3. Place the gel slice in a 1.5 ml microfuge tube.
4. Determine the volume of the gel slice by weight or size.
5. Add the appropriate amount of extraction buffer so the final concentration of agarose is 0.5%.
6. Melt the gel slice by heating to 70°C for SeaPlaque® or NuSieve® GTG® Agarose or 80°C for MetaPhor® Agarose.
7. Mix thoroughly.

   NOTE: Ensure the gel is completely melted and diluted by the buffer.
8. Incubate on ice for 30 minutes.

   NOTE: It is important that the agarose mixture has gelled as much as possible at this step.
9. Freeze the mixture at -70°C for 1 to 2 hours.
10. Allow the mixture to thaw on ice.
11. Centrifuge the mixture at 13,000 rpm in a microcentrifuge for 10 to 20 minutes at 4°C.
12. Remove the supernatant; this contains the recovered protein.

    NOTE: Each step of this procedure should be carefully followed to obtain quantitative results. Care should be taken to COMPLETELY gel the protein-agarose solution. If the procedure cannot be completed at one time, we recommend keeping the mixture at -70°C, as prolonged freezing will not affect the protein. Protein recovered from MetaPhor Agarose by this technique will co-purify with some residual agarose. The amount of agarose present is between 1% and 3% of the original amount of agarose present in the gel slice. All of the residual agarose will pass through a 0.1 mm pore filter, about 83% will pass through a 100 kDa molecular-weight cutoff filter, and 50% will pass through a 30 kDa molecular-weight cutoff filter. The recovered protein can be separated from the residual agarose by conventional chromatography.

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Chapter XV

Reagents and Solutions for Protein Separations

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Protein Electrophoresis Buffers

Tris-Glycine SDS Running Buffer pH 8.3

10X STOCK SOLUTION	g/l FOR 10X STOCK SOLUTION
250 mM Tris base	30.3 g Tris base
1920 mM Glycine	144.0 g Glycine
1.0% SDS	10.0 g SDS
	Adjust volume to 1 liter with distilled water

1X WORKING SOLUTION

25 mM Tris base
192 mM Glycine
0.1% SDS

Tris-Tricine SDS Running Buffer pH 8.3

10X STOCK SOLUTION	g/l FOR 10X STOCK SOLUTION
1 M Tris base	121.1 g Tris base
1 M Tricine	179.0 g Tricine
1.0% SDS	10.0 g SDS
	Adjust volume to 1 liter with distilled water

1X WORKING SOLUTION

100 mM Tris base
100 mM Tricine
0.1% SDS

Stock Solutions for Polyacrylamide Gel Electrophoresis

30% Acrylamide mix for handcast polyacrylamide gels

29.0 g Acrylamide
1.0 g N,N´-methylenebisacrylamide (bis)
Dissolve in 60 ml distilled water, heating to 37°C to dissolve
Adjust volume to 100 ml with distilled water
Sterilize the solution by filtration through a 0.45 micron Nalgene® filter
The pH of the solution should be ≤pH 7.0
Store solution in dark bottles at room temperature

CAUTION! Acrylamide is a potent neurotoxin and is absorbed through the skin. The effects of acrylamide are cumulative. Wear gloves, lab coat, safety glasses and a mask when handling solutions containing these chemicals. Polymerized gels containing these chemicals are considered to be non-toxic, but should be handled with care because of the possibility that they may contain small amounts of unpolymerized acrylamide.

1 M Tris

121.1 g Tris base to 800 ml distilled water
Adjust the pH by adding concentrated HCl (approximate amounts below)

PH	HCL
7.4	70 ml
7.6	60 ml
8.0	42 ml

The pH of Tris solutions is temperature-dependent so allow the solution to cool to room temperature before making final pH adjustments
Adjust volume to 1 liter with distilled water
Sterilize by autoclaving

1.5 M Tris (pH 8.8)

181.65 g Tris base to 800 ml distilled water
Adjust to pH 8.8 by adding 1 N HCl
The pH of Tris solutions is temperature-dependent so allow the solution to cool to room temperature before making final pH adjustments
Adjust volume to 1 liter with distilled water
Sterilize by autoclaving

Staining Solutions for SDS-PAGE

SYPRO® Red and SYPRO Orange Protein Gel Stains

For SDS-PAGE: 1:5,000 dilution of the stain in 7.5% (v/v) acetic acid in distilled water
or
For native gel staining: 1:5,000 dilution of the stain in distilled water

SYPRO Tangerine Protein Gel Stain

For non-fixing conditions: 1:5,000 dilution of the stain in 50 mM Phosphate, 150 mM NaCl, pH 7.0
or
For fixing conditions: 1:5,000 dilution of the stain in 7.5% (v/v) acetic acid in distilled water

Coomassie® blue staining solutions for SDS-PAGE

Coomassie Blue Stain Solution

1X WORKING SOLUTION	AMOUNT FOR 1X WORKING SOLUTION
40% Ethanol	400 ml Ethanol
0.125% Coomassie blue	1.25 g Coomassie blue R-250
Distilled water	500 ml Distilled water
10% Acetic acid	100 ml Acetic acid

Add materials in the following order: Ethanol, Coomassie blue (mix), 500 ml water, acetic acid.

NOTE: This solution should be prepared under the fume hood

Coomassie blue destain solution

1X WORKING SOLUTION	AMOUNT FOR 1X WORKING SOLUTION
5% Ethanol	50 ml Ethanol
7.5% Acetic acid	75 ml Acetic acid
	Adjust volume to 1 liter with distilled water

NOTE: This solution should be prepared under the fume hood

Silver Stain Solution for SDS-PAGE

Destain II

1X WORKING SOLUTION	AMOUNT FOR 1X WORKING SOLUTION
7% Acetic acid	700 ml Acetic acid
5% Methanol	500 ml Methanol
	Adjust volume to 10 liters with distilled water
	Store at room temperature indefinitely

Cross-linking solution

1X WORKING SOLUTION	AMOUNT FOR 1X WORKING SOLUTION
10% Glutaraldehyde	20 ml of 50% Glutaraldehyde stock
	Adjust volume to 100 ml with distilled water

NOTE: Glutaraldehyde is toxic and must be handled in a fume hood

Dithiothreitol (DTT) solution

1X WORKING SOLUTION	AMOUNT FOR 1X WORKING SOLUTION
5 mg/ml DTT	5.0 mg DTT
	Adjust volume to 1 liter with distilled water

Silver nitrate solution

1X WORKING SOLUTION	AMOUNT FOR 1X WORKING SOLUTION
0.1% Silver nitrate	1.0 g Silver nitrate ($AgNO_3$)
	Adjust volume to 1 liter with distilled water

3% Sodium carbonate

1X WORKING SOLUTION	AMOUNT FOR 1X WORKING SOLUTION
3% Sodium carbonate	60.0 g Sodium carbonate
	Adjust volume to 2 liters with distilled water
	Store in glass container

Developing solution

1X WORKING SOLUTION	AMOUNT FOR 1X WORKING SOLUTION
3% Sodium carbonate	200 ml of 3% Sodium carbonate
0.019% Formaldehyde	100 ml of 37% Formaldehyde
Prepare just prior to use	

Stop solution

1X WORKING SOLUTION	AMOUNT FOR 1X WORKING SOLUTION
2.3 M Sodium citrate	67.64 g Sodium citrate, dihydrate (FW: 294.1)
	Adjust volume to 100 ml with distilled water

Staining Solutions for IsoGel® Agarose IEF Gels

Coomassie® blue staining solutions for IsoGel Agarose IEF gels

Fixative solution
1X WORKING SOLUTION
180 ml Methanol
30.0 g Trichloroacetic acid
18.0 g Sulfosalicylic acid
Adjust volume to 500 ml with distilled water

Coomassie blue stain solution
1X WORKING SOLUTION
1.0 g Coomassie blue R-250
250 ml Ethanol
90 ml Glacial acetic acid
Adjust volume to 1 liter with distilled water

Coomassie blue destain solution
1X WORKING SOLUTION
250 ml Ethanol
90 ml Glacial acetic acid
Adjust volume to 1 liter with distilled water

Crowle's double stain solution
1X WORKING SOLUTION
2.5 g Crocein scarlet
150.0 mg Coomassie blue R-250
50 ml Glacial acetic acid
30.0 g Trichloroacetic acid
Adjust volume to 1 liter with distilled water

Crowle's destain solution
1X WORKING SOLUTION
3 ml Glacial acetic acid
Adjust volume to 1 liter with distilled water

Silver stain solutions for IsoGel® Agarose IEF gels

Fixative solution
1X WORKING SOLUTION
180 ml Methanol
30.0 g Trichloroacetic acid
18.0 g Sulfosalicylic acid
Adjust volume to 500 ml with distilled water

Pretreatment solution 1
1X WORKING SOLUTION
2% Glutaraldehyde in distilled water

Pretreatment solution 2
1X WORKING SOLUTION
0.01% Dithiothreitol (DTT) in distilled water

Silver stain solution A
1X WORKING SOLUTION
50.0 g Sodium carbonate, anhydrous
Add to 1 liter distilled water
Stable for 2 - 3 weeks at room temperature

Silver stain solution B
1X WORKING SOLUTION
Combine the following solutions **in the order given**
into 1 liter of distilled water, while mixing rapidly.
2.0 g Ammonium nitrate
3.0 g Silver nitrate
10.0 g Dodeca-tungstosilicic acid (Gallard-Schlesinger Cat. No.305453)
6.7 ml 37% Formaldehyde.
Stable for 1 week at room temperature stored in the dark

Stop bath
1X WORKING SOLUTION
1% acetic acid (v/v in distilled water)

Staining Solutions for Proteins in Agarose Gels

Coomassie® blue stain solution

1X WORKING SOLUTION	AMOUNT FOR 1X WORKING SOLUTION
40% Ethanol	400 ml Ethanol
0.25% Coomassie blue	2.5 g Coomassie blue R-250
Distilled water	500 ml Distilled water
10% Acetic acid	100 ml Acetic acid

Add materials in the following order: Ethanol, Coomassie blue (mix)
500 ml water, acetic acid.

NOTE: This solution should be prepared under the fume hood

Coomassie blue destain solution

1X WORKING SOLUTION	AMOUNT FOR 1X WORKING SOLUTION
20% Methanol	200 ml Methanol
5% Glacial Acetic Acid	50 ml Glacial acetic acid

Adjust volume to 1 liter with distilled water

NOTE: This solution should be prepared under the fume hood

Anolyte and Catholyte Solutions for Isoelectric Focusing in IsoGel® Agarose IEF Gels

Anolyte solutions (pH at 25°C)

1 M PHOSPHORIC ACID, pH 1.0

Slowly add 68 ml H_3PO_4 to 800 ml distilled water
Adjust volume to 1 liter with distilled water

0.2 M SULFURIC ACID, pH 1.6

Slowly add 11.2 ml H_2SO_4 to 800 ml distilled water
Adjust volume to 1 liter with distilled water

0.5 M ACETIC ACID, pH 2.6

Slowly add 28.6 ml CH_3COOH to 800 ml distilled water
Adjust volume to 1 liter with distilled water

40 MM L-GLUTAMIC ACID, pH 3.2

5.88 g Glutamic acid in 1 liter distilled water

3 MM INDOLE ACETIC ACID, pH 3.8

0.53 g Indole acetic acid in 1 liter distilled water

4 MM L-TYROSINE, pH 4.5

0.73 g L-tyrosine in 1 liter distilled water

Catholyte solutions (pH at 25°C)

50 MM THREONINE, pH 5.8

5.95 g Threonine in 1 liter distilled water

50 MM GLYCINE, pH 6.15

3.75 g Glycine in 1 liter distilled water

0.4 M HEPES, pH 7.3

95.32 g Hepes in 1 liter distilled water

40 MM L-HISTIDINE, pH 7.35

6.21 g L-Histidine in 1 liter distilled water

0.1 M BICINE, pH 8.0

16.32 g Bicine in 1 liter distilled water

1 M SODIUM HYDROXIDE, pH 13.0

40.0 g NaOH in 1 liter distilled water

Loading Buffers for Protein Electrophoresis

2X Tris-Glycine SDS sample buffer

2X CONCENTRATE	AMOUNT TO ADD FOR 2X CONCENTRATE
126 mM Tris-HCl, pH 6.8	2.5 ml of 0.5 M Tris-HCl, pH 6.8
20% Glycerol	2 ml Glycerol
4% SDS	4 ml of 10% SDS solution
0.005% Bromophenol blue	0.5 ml of 0.1% Bromophenol blue
	Adjust volume to 10 ml with distilled water

1X CONCENTRATE

63 mM Tris-HCl, pH 6.8
10% Glycerol
2% SDS solution
0.0025% Bromophenol blue
2.5% βME

Before use add 0.5 ml β-Mercaptoethanol (βME) to 10 ml of 2X Tris-Glycine SDS sample buffer
Add 1 part 2X sample buffer to 1 part sample and mix well
Heat the sample at 95°C for 4 minutes prior to loading

2X Tris-Glycine SDS sample buffer for agarose electrophoresis of proteins

2X CONCENTRATE	AMOUNT TO ADD FOR 2X CONCENTRATE
126 mM Tris-HCl, pH 6.8	2.5 ml of 0.5M Tris-HCl, pH 6.8
15 % Ficoll® Type 400	1.5 g Ficoll Type 400
4% SDS	4 ml of 10% SDS
0.002% Bromophenol blue	0.2 ml of 0.1% Bromophenol blue

Adjust volume to 10 ml with distilled water

1X CONCENTRATE

63 mM Tris-HCl, pH 6.8
7.5% Ficoll® Type 400
2% SDS
0.001% Bromophenol blue
5% βME

Before use add 1 ml β-Mercaptoethanol (βME) to 10 ml of 2X Tris-Glycine SDS sample buffer. Add 1 part 2X Sample buffer to 1 part protein sample and mix well

Electroblotting Solutions for SDS-PAGE

Tris-Glycine Transfer Buffer + 20% Methanol (10 liters)

1X WORKING SOLUTION	AMOUNT FOR 1X WORKING SOLUTION
25 mM Tris base	30.3 g Tris base
192 mM Glycine	144.1 g Glycine
	Adjust volume to 8 liters with distilled water
	Measure, but do not adjust pH; it should be approximately 8.2 to 8.4
20% Methanol	2 L Methanol

Adjust volume to 10 liters with distilled water

Buffers for Electrophoresis of Proteins in Vertical Agarose Gels

1X STACK BUFFER pH 8.0	g/l FOR 1X STACK BUFFER
125 mM Tris HCl	19.7 g/l Tris HCl
1X RESOLVING BUFFER pH 8.5	**g/l FOR 1X RESOLVING BUFFER**
500 mM Tris base	60.55 g Tris base
160 mM Boric acid	9.90 g Boric acid
1 M Urea	60.06 g Urea
	Adjust volume to 1 liter with distilled water
1X RUNNING BUFFER pH 8.5	**g/l FOR 1X RUNNING BUFFER**
90 mM Tris base	10.90 g Tris base
90 mM Boric acid	5.57 g Boric acid
0.1% SDS	10 ml of 10% SDS solution
	Adjust volume to 1 liter with distilled water

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Notes

Appendix A

Electrophoretic Theory

IN THIS CHAPTER

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Constant Electrophoretic Parameters 255

Voltage, Current and Power: Interactive Effects on Gel Electrophoresis

Introduction

Electrophoresis is defined as the movement of ions and charged macromolecules through a medium when an electric current is applied. Agarose and polyacrylamide are the primary stabilizing media used in the electrophoresis of macromolecules.

Macromolecules are separated through the matrix based on size, charge distribution and structure. Proteins will separate through the matrix based on size, structure and charge. In general, nucleic acids migrate through a gel based on size, with little influence from base composition or sequence.

Two equations are relevant to the use of power supplies for electrophoresis of macromolecules: Ohm's Law and the Second Law of electrophoresis. These two laws and the interactions of these parameters (watts, volts, current) are critical to understanding electrophoresis.

Ohm's Law

Current (I)=Voltage (V)/Resistance (R)

Ohm's Law states that current is directly proportional to the voltage and is inversely proportional to the resistance. Resistance of the system is determined by the buffers used, the type and configurations of the gels being run, and the total volume of all the gels being run.

Second Law

Watts (W)=Current (I) x Voltage (V)

The Second Law states that power or watts (a measure of the heat produced) is equal to the product of the current and voltage. Since V=I x R, this can also be written as Watts=I^2 x R.

Electrophoresis

During electrophoresis one of the parameters is held constant and the other two are allowed to vary as the resistance of the electrophoretic system changes. In vertical systems, the resistance of the gel increases as highly conductive ions like Cl are electrophoresed out of the gel. As these ions are removed from the gel, the current is carried by less conductive ions like glycine, borate, acetate, etc. Under normal conditions in horizontal systems, there is little change in resistance. However, with high voltage or extended runs in horizontal systems, resistance can decrease.

Introduction

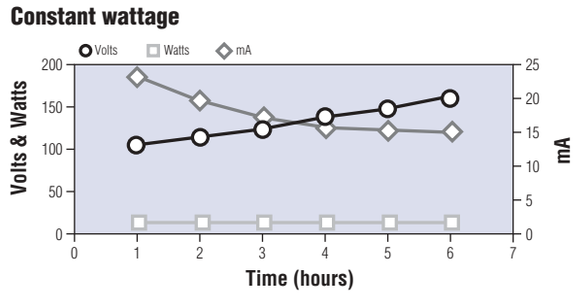
There are advantages and disadvantages for setting each of the critical parameters as the limiting factor in electrophoresis. Sequencing gels are usually run at constant wattage to maintain a uniform temperature. Agarose and acrylamide gels for protein and DNA separation are run at constant voltage or constant current.

Constant wattage

In a vertical system when wattage is held constant, the velocity of the samples will decrease because the current, which is in part carried by the DNA, decreases to compensate for the increase in voltage. The generation of heat will remain uniform.

If the current should decrease disproportionately (from a buffer problem, a buffer leak or a hardware problem), the power supply will increase the voltage to compensate.

Since voltage and current vary over time at a constant wattage, it is not possible to predict mobility of samples from the calculation of watt-hours.

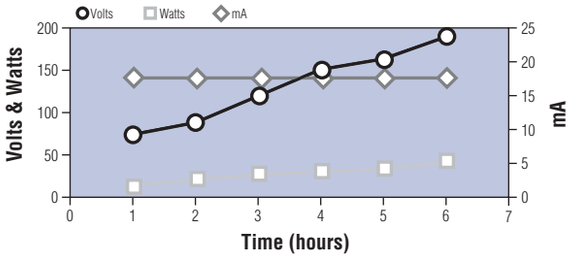


Constant current

When the current is held constant, the samples will migrate at a constant rate. voltage and wattage will increase as the resistance increases, resulting in an increase in heat generation during the run.

If a break occurs in the system such as a damaged lead or electrode or a buffer leak, the resistance of the gel will be greatly increased. This will cause a large increase in wattage and voltage resulting in the generation of excessive heat. It is even possible for the system to get hot enough to boil, or start the apparatus to scorch or burn.

Constant current

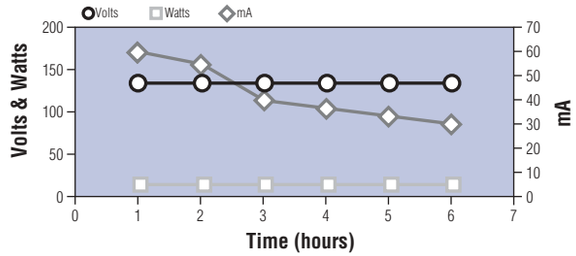


Constant voltage

When voltage is set constant, current and wattage will decrease as the resistance increases, resulting in a decrease of heat and DNA migration.

Since the heat generated will decrease, the margin of safety will increase over the length of the run. If a problem develops and the resistance increases dramatically, the current and wattage will fall since the voltage cannot increase. Even if the apparatus fails, the worst that is likely to happen is that the resistance will increase so much that the power supply will not be able to compensate, and it will shut off.

Constant voltage



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Appendix B

Agarose Physical Chemistry

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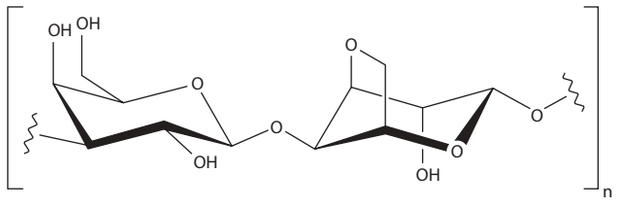
Introduction

Source of agarose

Agarose is derived from a series of naturally occurring derivatives from seaweed. Most agar comes from various species of *Gelidium* and *Gracilaria*. All species contain ester sulfates and some, except *Gracilaria*, contain varying amounts of pyruvates. *Gracilaria* agarose contains methyl ethers, the position of which is variable according to the species.

Structure of agarose

Agarose consists of 1,3-linked ß-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose. This basic agarobiose repeat unit forms long chains with an average molecular mass of 120,000 daltons, representing about 400 agarobiose units. There are also charged groups present on the polysaccharide, most notably pyruvates and sulfates.



AGAROBIOSE —
BASIC REPEATING UNIT OF AGAROSE

Advantages of agarose

- Agarose forms a macroporous matrix which allows rapid diffusion of high molecular weight (10^6 dalton range) macromolecules without significant restriction by the gel.
- Agarose gels have a high gel strength, allowing the use of concentrations of 1% or less, while retaining sieving and anticonvective properties.
- Agarose is nontoxic and, unlike polyacrylamide, contains no potentially damaging polymerization by-products. There is no free radical polymerization involved in agarose gelation.
- Rapid staining and destaining can be performed with minimal background.
- Agarose gels are thermoreversible. Low gelling and melting temperature agaroses permit easy recovery of samples, including sensitive heat-labile materials.
- Agarose gels may be air dried.

Properties of Agarose

Background

The charged groups present on the polysaccharide, pyruvates and sulfates are responsible for many of the agarose properties. By careful selection of raw materials, these properties can be controlled to meet specific needs.

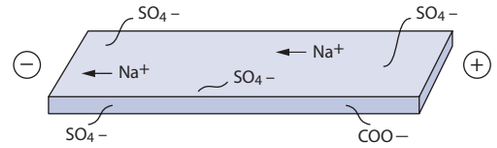
Properties of agarose

A few of the properties for agarose are listed below:

- Electroendosmosis (EEO)
- Melting and gelling temperatures
- Gelation mechanism
- Gel strength

Electroendosmosis (EEO)

Electroendosmosis (EEO) is a functional measure of the number of sulfate and pyruvate residues present on the agarose polysaccharide. This phenomenon occurs during electrophoresis when the anticonvective medium (the agarose in this case) has a fixed negative charge. In an electric field, the hydrated positive ions associated with the fixed anionic groups in the agarose gel migrate toward the cathode. Water is thus pulled along with the positive ions, and migration of negative molecules such as DNA is retarded.



How EEO is measured

Electroendosmosis is quantitated by subjecting a mixture of dextran and albumin to electrophoresis, then visualizing them and measuring their respective distances from the origin. The amount of EEO expressed in terms of relative mobility ($-m_r$) is calculated by dividing the migration distance of the neutral dextran (O_D) by the sum of the migration distances of the dextran and the albumin ($O_D + O_A$): $-m_r = O_D/(O_D + O_A)$.

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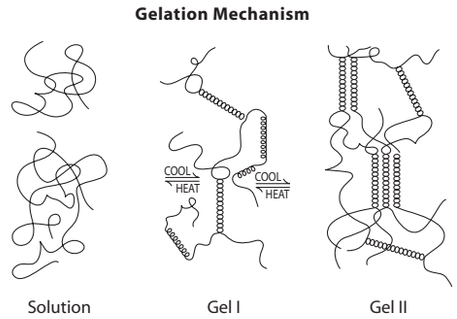
Gelation

The mechanism for gelation of agarose was first suggested by Rees and later demonstrated by Arnott. It involves a shift from a random coil in solution to a double helix in the initial stages of gelation, and then to bundles of double helices in the final stage.

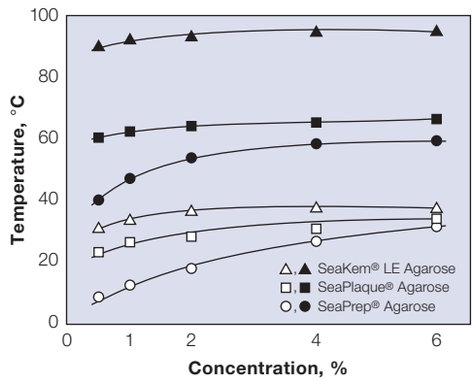
The average pore size varies with concentration and type of agarose, but is typically 100 to 300 nm.



Melting and gelling temperature

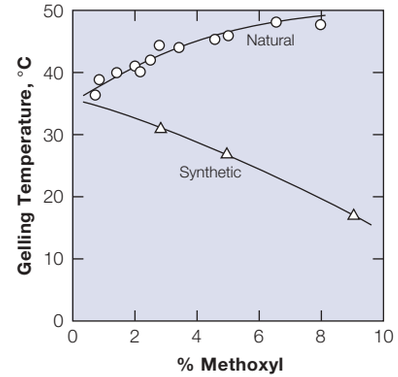
The energy needed to melt an agarose gel increases as the gel concentration increases. The gelling temperature of an agarose gel is also influenced by the gel concentration.

For this reason, gelling or remelting temperatures are expressed at a given agarose concentration. This property is of practical value since it is possible to vary gelling and melting parameters by using lower or higher concentrations of agarose. The dependence of gelling and melting temperatures on concentration is most pronounced at concentrations less than 1%.



Methylation of agarose

The agarose polysaccharide also contains uncharged methyl groups. The extent of natural methylation is directly proportional to the gelling temperature. Unexpectedly, synthetically methylated agaroses have lower, rather than higher, gelling temperatures, and the degree of synthetic methylation is inversely proportional to the gelling temperature.



Gel strength

One of the most important factors contributing to the success of agarose as an anticonvection medium is its ability to exhibit high gel strength at low concentrations (<6%). Gel strength is defined as the force, expressed in g/cm^2, that must be applied to fracture an agarose gel of a standard concentration. As there are several test methods used to measure gel strength, a direct comparison of gel strength values between different manufacturers is sometimes difficult. The gel strength of a specific lot of agarose will decrease over time because of the spontaneous hydrolysis of the agarose polysaccharide chains. This loss of gel strength can be particularly noticeable after 5 years from the manufacturing date.

References

Adamson, A.W., *Physical Chemistry of Surfaces*, John Wiley & Sons, 1976.
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Appendix C

Polyacrylamide Gel Electrophoresis

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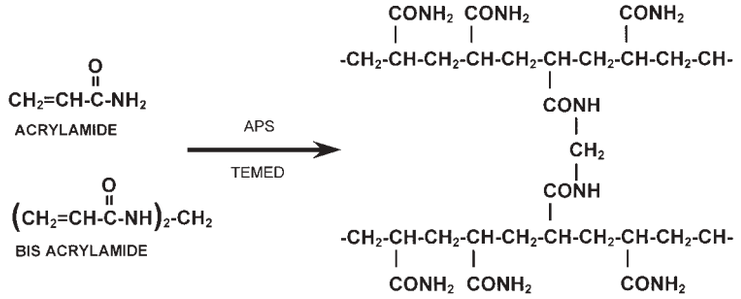
Introduction

Polyacrylamide gel electrophoresis (PAGE) has become a popular technique to separate proteins and nucleic acids because of its' high resolution, ease of use, and flexibility. PAGE can be performed using native (non-denatured) or denatured proteins. Sodium dodecyl sulfate PAGE or SDS-PAGE is widely used as a system for separating denatured proteins. Although there are many variations in formulations for polyacrylamide gels, the core chemistry has remained consistent over time. By understanding the basic concepts of polyacrylamide gel formation, one can adjust conditions to best optimize a given system or protocol.

Acrylamide polymerization

Polyacrylamide gels form by the co-polymerization of acrylamide and a crosslinking agent. The crosslinker most commonly used is bis-acrylamide (N,N´-methylene-bis-acrylamide). The polymerization reaction proceeds via a vinyl addition initiated by the production of free radicals. The system most commonly used for the production of free radicals involves the use of ammonium persulfate (APS) and tetramethylethylenediamine (TEMED). In this system, free radicals are formed from the persulfate. TEMED acts to accelerate the formation of the free radicals from the persulfate. This produces a chain reaction in which the polymer chains elongate and are randomly crosslinked by the crosslinker (Figure 1). This results in polymer, which acts as a sieve with defined pore size. The pore size of the resulting gel will depend upon the initial concentration of acrylamide, the amount of crosslinker used, the concentrations of APS and TEMED used, and the ambient conditions under which the polymerization occurred.

Figure 1. Gel Polymerization



Tips to ensure consistent polymerization

- Use chemicals (acrylamide, APS, etc.) that are electrophoresis grade or purer. The presence of impurities will result in inconsistent polymerization and poor quality gels.
- Do not use old chemicals. Use acrylamide, bis-acrylamide, and APS approximately one year old or less. Use TEMED approximately six months old or less.
- Avoid variations in temperature. Since the polymerization reaction is exothermic, temperature has a strong effect upon how polymerization proceeds. Carrying out polymerization at between 23°C and 25°C will help to ensure strong, consistent gels.
- If using buffers or acrylamide stock solutions stored at 4°C, warm to room temperature prior to use.
- Acrylamide monomer solutions should be degassed prior to use. Oxygen is a potent inhibitor of the polymerization reaction. Monomer solutions should be placed under vacuum (at least 125 torr) for 15 - 30 minutes at room temperature.
- Polymerization can be slowed by low pH. If polymerization is slowing, check the solutions pH to ensure a neutral or slightly basic pH. Adjust pH as needed using 1 N NaOH.
- Gels made with APS/TEMED should be allowed to polymerize for at least two hours in order to ensure complete polymerization and increase reproducibility.

Relationship between Percentage Crosslinker and Pore Size

The nature of the acrylamide polymer formed by the reaction discussed previously is commonly expressed in terms of monomer and crosslinker concentrations. %T is the amount of acrylamide monomer (g) plus the amount of crosslinker (g) divided by the total volume (ml). Thus a 20%T gel has 20% w/v acrylamide plus crosslinker. %C is the amount of crosslinker (g) to the amount of total monomer (acrylamide plus crosslinker). For example, a 5%C gel would have 5% of the total monomer weight made up by crosslinker. Figure 2 shows %T and %C as equations.

%T and %C have a direct relation to pore size of the polymerized gel. As the %T increases, the pore size decreases. Alternatively, for any given %T, 5%C creates the smallest pore size. Pore size progressively decreases from 2 - 5%C and progressively increases from 5 - 50%C. It is important to remember that these calculations assume complete conversion of the monomer to polymer. Factors such as the purity of the reagents used and ambient conditions during the polymerization reaction will affect the accuracy of these calculations.

Figure 2

$$\%T = \frac{\text{acrylamide (g) + crosslinker (g)}}{\text{100 ml}} \text{ X 100}$$

$$\%C = \frac{\text{crosslinker (g) v}}{\text{acrylamide (g) + crosslinker (g)}} \text{ X 100}$$

Alternative crosslinkers

In some cases, it may be desirable to use crosslinkers other than bis-acrylamide. Examples of alternative crosslinkers include DHEBA (dihydroxy ethylene bis acrylamide), DATD N,N′-Diallyl L-tartardianide, BAC N,N′-bis (acrybylcystamine), and PDA (piperzine di-acrylamide). DHEBA, DATD, and BAC are used to improve the gel's solubility for the recovery of proteins and nucleic acids subsequent to electrophoresis. PDA is used to increase the resolution and strength of gels. Gels crosslinked with PDA also display reduced background when silver stained.

All of these alternative crosslinker will vary from bis-acrylamide in their efficiency of crosslinking. Adjustments in concentration need to be made accordingly.

References

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Gelfi, C. and Righetti, P.G., *Electrophoresis*, **2:** 220, 1981.
Chen, B. and Chrambach, A., *J. Biochem Biophys. Methods*, **1:** 105, 1979.

Product Listing by Catalog Number

Cambrex Agarose for Nucleic Acid Separations

CAT. NO.	DESCRIPTION	SIZE
50000	SeaKem® LE Agarose	125 g
50001	SeaKem LE Agarose	25 g
50004	SeaKem LE Agarose	500 g
50100	SeaPlaque® Agarose	125 g
50101	SeaPlaque Agarose	25 g
50070	SeaKem GTG® Agarose	125 g
50071	SeaKem GTG Agarose	25 g
50074	SeaKem GTG Agarose	500 g
50110	SeaPlaque GTG Agarose	125 g
50111	SeaPlaque GTG Agarose	25 g
50090	NuSieve® 3:1 Agarose	125 g
50091	NuSieve 3:1 Agarose	25 g
50080	NuSieve GTG Agarose	125 g
50081	NuSieve GTG Agarose	25 g
50084	NuSieve GTG Agarose	500 g
50150	SeaKem Gold Agarose	125 g
50152	SeaKem Gold Agarose	25 g
50180	MetaPhor® Agarose	125 g
50181	MetaPhor Agarose	25 g
50184	MetaPhor Agarose	500 g

Stains, Markers and Buffers for Nucleic Acid Separations

50320	20 bp DNA Ladder	100 Applications
50321	100 bp DNA Ladder	100 Applications
50322	100 bp Extended Range DNA Ladder	100 Applications
50323	500 bp DNA Ladder	100 Applications
50461	DNA Marker 50 - 1,000 bp 50 applications	250 µl
50471	DNA Marker 1 to 10 kb 100 - 200 applications	2 x 250 µl
50631	DNA Marker 50 to 2,500 bp 50 applications	250 µl
50575	RNA Marker 0.5 to 9 kb	250 µl
50512	SYBR® Green I Nucleic Acid Gel Stain	2 X 500 µl
50513	SYBR Green I Nucleic Acid Gel Stain	10 X 50 µl
50522	SYBR Green II Nucleic Acid Gel Stain	2 X 500 µl
50523	SYBR Green II Nucleic Acid Gel Stain	10 X 50 µl
50530	SYBR Green Gel Stain Photographic Filter	3" square
50535	GelStar® Nucleic Acid Gel Stain	2 X 250 µl
50536	GelStar Gel Stain Photographic Filter	3" square
50560	Glyoxal Sample Buffer	1.7 ml

Stains, Markers and Buffers for Protein Electrophoresis

CAT. NO.	DESCRIPTION	SIZE
50540	SYPRO® Protein Gel Stain Photographic Filter	3" square
50542	SYPRO Red Protein Gel Stain	10 X 50 µl
50543	SYPRO Red Protein Gel Stain	500 µl
50544	SYPRO Red Starter Kit	2 X 50 µl
50547	ProSieve® Protein Markers	500 µl
50550	ProSieve Color Protein Markers	500 µl
50554	SYPRO Orange Protein Gel Stain	10 X 50 µl
50556	SYPRO Tangerine Protein Gel Stain	500 µl

DNA Sequencing Accessories

50640	Gel Slick Solution	250 ml

AccuGENE® Electrophoresis Buffers

50835	AccuGENE 5X TBE Buffer	10 L
50836	AccuGENE 5X TBE Buffer	20 L
50837	AccuGENE 10X TBE Buffer	10 L
50838	AccuGENE 10X TBE Buffer	20 L
50839	AccuGENE 5X TBE Buffer	4 L
50840	AccuGENE 10X TBE Buffer	4 L
50841	AccuGENE 10X TAE Buffer	4 L
50843	AccuGENE 10X TBE Buffer	1 L
50844	AccuGENE 10X TAE Buffer	1 L
50876	AccuGENE 10X MOPS Buffer	1 L
50879	AccuGENE 10X Tris-Glycine Buffer	1 L
50880	AccuGENE 10X Tris-Glycine SDS Buffer	1 L
51216	AccuGENE 50X TAE Buffer	1 L

AccuGENE Molecular Biology Buffers

51200	AccuGENE Molecular Biology Water	1 L
51201	AccuGENE 0.5 M EDTA Solution	100 ml
51202	AccuGENE 5 M Sodium Chloride	1 L
51203	AccuGENE 3 M Sodium Acetate pH 5.2	500 ml
51205	AccuGENE 20X SSC Buffer	1 L
51206	AccuGENE 10% SDS	100 ml
51213	AccuGENE 10% SDS	500 ml
51214	AccuGENE 20X SSPE Buffer	1 L
51217	AccuGENE LB Broth (Luria Bertani Medium)	500 ml
51219	AccuGENE Super Broth (Terrific Broth)	500 ml
51221	AccuGENE Super Broth (Terrific Broth)	1 L
51223	AccuGENE Molecular Biology Water	10 L
51224	AccuGENE Molecular Biology Water	20 L
51225	AccuGENE 1X PBS	1 L

Continued on page 270

AccuGENE® Molecular Biology Buffers (continued)

CAT. NO.	DESCRIPTION	SIZE
51226	AccuGENE 10X PBS	1 L
51227	AccuGENE Denaturing Solution	1 L
51228	AccuGENE Denaturing Solution	10 L
51229	AccuGENE Neutralization Solution	1 L
51230	AccuGENE Neutralization Solution	10 L
51232	AccuGENE 20X SSC Buffer	20 L
51233	AccuGENE 20X SSC Buffer	10 L
51234	AccuGENE 0.5 M EDTA Solution	1 L
51235	AccuGENE 1X TE Buffer	500 ml
51236	AccuGENE 1 M Tris HCl Buffer, pH 7.2	1 L
51237	AccuGENE 1 M Tris HCl Buffer, pH 7.4	1 L
51238	AccuGENE 1 M Tris HCl Buffer, pH 8.0	1 L

GelBond® Film

53734	GelBond Film Sheets 85 X 100 mm	100 per pack
53745	GelBond Film Sheets 110 X 125 mm	100 per pack
53746	GelBond Film Sheets 100 X 150 mm	100 per pack
53748	GelBond Film Sheets 110 X 205 mm	100 per pack
53749	GelBond Film Sheets 160 X 180 mm	100 per pack
53759	GelBond Film Sheets 125 X 245 mm	100 per pack
53761	GelBond Film Sheets 124 X 258 mm	100 per pack
53740	GelBond Film 102 mm X 0.2 mm	16.5 m roll
53750	GelBond Film 127 mm X 0.2 mm	16.5 m roll
53760	GelBond Film 152 mm X 0.2 mm	16.5 m roll
53780	GelBond Film 203 mm X 0.2 mm	16.5 m roll
54711	GelBond PAG Film Sheets 138 mm X 158 mm	50 sheets per pack
54723	GelBond PAG Film Sheets 160 mm X 180 mm	50 sheets per pack
54727	GelBond PAG Film Sheets 124 mm X 258 mm	50 sheets per pack
54729	GelBond PAG Film Sheets 220 mm X 165 mm	50 sheets per pack
54731	GelBond PAG Film Sheets 199 mm X 264 mm	50 sheets per pack
54733	GelBond PAG Film Sheets 203 mm X 260 mm	50 sheets per pack
54735	GelBond PAG Film Sheets 195 mm X 370 mm	50 sheets per pack
54746	GelBond PAG Film Sheets 350 mm X 430 mm	10 sheets per pack

Reliant® Precast Agarose Gels

54801	8 well, 1X TAE Buffer, 1% SeaKem® Gold w/o ethidium bromide	20 Gels
54803	8 well, 1X TAE Buffer, 1% SeaKem Gold with ethidium bromide	20 Gels
54813	2 x 12 well, 1X TBE Buffer, 2%SeaKem Gold with ethidium bromide	20 Gels
54820	1 x 12 well, 1X TBE Buffer, 1% SeaKem Gold with ethidium bromide	20 Gels
54821	1 x 12 well, 1X TAE Buffer, 1% SeaKem Gold with ethidium bromide	20 Gels
54822	1 x 12 well, 1X TAE Buffer, 1% SeaKem Gold w/o ethidium bromide	20 Gels
54823	1 x 12 well, 1X TBE Buffer, 4% NuSieve® Plus with ethidium bromide	20 Gels
54825	1 x 12 well, 1X TBE Buffer, 2% SeaKem Gold with ethidium bromide	20 Gels
54901	8 well, 1X TBE Buffer, 1% SeaKem Gold w/o ethidium bromide	20 Gels
54903	8 well, 1X TBE Buffer, 1% SeaKem Gold with ethidium bromide	20 Gels
54905	2 x 12 well, 1X TBE Buffer, 1% SeaKem Gold with ethidium bromide	20 Gels

Reliant® Precast Agarose Gels (continued)

CAT. NO.	DESCRIPTION	SIZE
54907	20 well, 1X TBE Buffer, 1% SeaKem® Gold with ethidium bromide	20 Gels
54922	8 well, 1X MOPS Buffer, 1.25% SeaKem Gold for RNA	20 Gels
54924	8 well, 1X TAE Buffer, 4% NuSieve® 3:1 Plus w/o ethidium bromide	20 Gels
54925	8 well, 1X TAE Buffer, 4% NuSieve 3:1 Plus with ethidium bromide	20 Gels
54926	8 well, 1X TBE Buffer, 4% NuSieve 3:1 w/o ethidium bromide	20 Gels
54927	8 well, 1X TBE Buffer, 4% NuSieve 3:1 Plus with ethidium bromide	20 Gels
54928	20 well, 1X TBE Buffer, 4% NuSieve 3:1 Plus with ethidium bromide	20 Gels
54929	2 x 12 well, 1X TBE Buffer, 4% NuSieve 3:1 Plus with ethidium bromide	20 Gels
54936	2 x 12 well, 1X TBE Buffer, 1% SeaKem Gold w/o ethidium bromide	20 Gels
54937	2 x 12 well, 1X TBE Buffer, 4% NuSieve 3:1 Plus w/o ethidium bromide	20 Gels
54938	20 well, 1X TBE Buffer, 2% SeaKem Gold w/o ethidium bromide	20 Gels
54939	20 well, 1X TBE Buffer, 2% SeaKem Gold with ethidium bromide	20 Gels
54940	2 x 12 well, 1X TBE Buffer, 2% SeaKem Gold w/o ethidium bromide	20 Gels
54943	20 well, 1X TBE Buffer, 1% SeaKem Gold w/o ethidium bromide	20 Gels
54944	20 well, 1X TBE Buffer, 4% NuSieve 3:1 Plus w/o ethidium bromide	20 Gels
54948	20 well, 1X MOPS Buffer, 1.25% SeaKem Gold for RNA	20 Gels

Reliant HS Precast Agarose Gels

54950	1 x 12 well, 1% SeaKem Gold Agarose	20 Gels
54956	1 x 12 well, 2% SeaKem Gold Agarose	20 Gels
54960	1 x 12 well 3.6% NuSieve Hi-Res Agarose	20 Gels
54954	2 x 12 well, 1% SeaKem Gold Agarose	20 Gels
54958	2 x 12 well, 2% SeaKem Gold Agarose	20 Gels
54962	2 x 12 well, 3.6% NuSieve Hi-Res Agarose	20 Gels
54955	8 well, 1% SeaKem Gold Agarose	20 Gels
54959	8 well, 2% SeaKem Gold Agarose	20 Gels
54963	8 well, 3.6% NuSieve Hi-Res Agarose	20 Gels
54953	20 well, 1% SeaKem Gold Agarose	20 Gels
54957	20 well, 2% SeaKem Gold Agarose	20 Gels
54961	20 well, 3.6% NuSieve Hi-Res Agarose	20 Gels
54945	UV Transparent Tray for Reliant Gels-Landscape	Each
54946	UV Transparent Tray for Reliant Gels-Portrait	Each

Latitude® Precast Agarose Gels + Ethidium Bromide

56990	Latitude Midigel Chamber	Each
57200	1 x 20 well, 1X TAE Buffer, 1% SeaKem LE Plus	8 Midigels
57201	1 x 20 well, 1X TAE Buffer, 2% SeaKem LE Plus	8 Midigels
57202	1 x 20 well, 1X TAE Buffer, 3.6 % NuSieve Hi-Res	8 Midigels
57210	2 x 20 well, 1X TAE Buffer, 1% SeaKem LE Plus	8 Midigels
57211	2 x 20 well, 1X TAE Buffer, 2% SeaKem LE Plus	8 Midigels
57212	2 x 20 well, 1X TAE Buffer, 3.6% NuSieve Hi-Res	8 Midigels
57263	6 x 18 well, 1X TAE Buffer, 1% SeaKem LE Plus	8 Midigels
57265	6 x 18 well, 1X TAE Buffer, 2% SeaKem LE Plus	8 Midigels
57267	6 x 18 well, 1X TAE Buffer, 3.6% NuSieve Hi-Res	8 Midigels
57220	1 x 20 well, 1X TBE Buffer, 1% SeaKem LE Plus	8 Midigels
57221	1 x 20 well, 1X TBE Buffer, 2% SeaKem LE Plus	8 Midigels

Continued on page 272

Latitude® Precast Agarose Gels + Ethidium Bromide (continued)

CAT. NO.	DESCRIPTION	SIZE
57222	1 x 20 well, 1X TBE Buffer, 4% NuSieve® 3:1 Plus	8 Midigels
57230	2 x 20 well, 1X TBE Buffer, 1% SeaKem® LE Plus	8 Midigels
57231	2 x 20 well, 1X TBE Buffer, 2% SeaKem LE Plus	8 Midigels
57232	2 x 20 well, 1X TBE Buffer, 4% NuSieve 3:1 Plus	8 Midigels
57264	6 x 18 well, 1X TBE Buffer, 1% SeaKem LE Plus	8 Midigels
57266	6 x 18 well, 1X TBE Buffer, 2% SeaKem LE Plus	8 Midigels
57268	6 x 18 well, 1X TBE Buffer, 4% NuSieve 3:1 Plus	8 Midigels
57237	2 x 20 well, with 1X MOPS Buffer for RNA, 1.25% SeaKem Gold	8 Midigels
57238	1 X 20 well, with 1X MOPS Buffer for RNA, 1.25% SeaKem Gold	8 Midigels
56988	TruBand™ Anchor for 10 x 15 cm gels, Latitude Midigel Chamber	Each
56989	TruBand Anchor for 10 x 15 cm gels, standard chambers	Each

Latitude HT Precast Agarose Gels + Ethidium Bromide

57204	2 x 50 well, 1X TAE Buffer, 1% SeaKem LE Plus	5 HT Gels
57205	2 x 50 well, 1X TAE Buffer, 3.6% NuSieve Hi-Res	5 HT Gels
57206	2 x 50 well, 1X TAE Buffer, 2% SeaKem LE Plus	5 HT Gels
57214	4 x 50 well, 1X TAE Buffer, 1% SeaKem LE Plus	5 HT Gels
57215	4 x 50 well, 1X TAE Buffer, 3.6% NuSieve Hi-Res	5 HT Gels
57216	4 x 50 well, 1X TAE Buffer, 2% SeaKem LE Plus	5 HT Gels
57245	4 x 25 well, 1X TAE Buffer, 2% SeaKem LE Plus	5 HT Gels
57249	4 x 25 well, 1X TAE Buffer, 1% SeaKem LE Plus	5 HT Gels
57253	4 x 25 well, 1X TAE Buffer, 3.6% NuSieve Hi-Res	5 HT Gels
57224	2 x 50 well, 1X TBE Buffer, 1% SeaKem LE Plus	5 HT Gels
57225	2 x 50 well, 1X TBE Buffer, 4% NuSieve 3:1 Plus	5 HT Gels
57226	2 x 50 well, 1X TBE Buffer, 2% SeaKem LE Plus	5 HT Gels
57234	4 x 50 well, 1X TBE Buffer, 1% SeaKem LE Plus	5 HT Gels
57235	4 x 50 well, 1X TBE Buffer, 4% NuSieve 3:1 Plus	5 HT Gels
57236	4 x 50 well, 1X TBE Buffer, 2% SeaKem LE Plus	5 HT Gels
57246	4 x 25 well, 1X TBE Buffer, 2% SeaKem LE Plus	5 HT Gels
57250	4 x 25 well, 1X TBE Buffer, 1% SeaKem LE Plus	5 HT Gels
57255	4 x 25 well, 1X TBE Buffer, 4% NuSieve 3:1 Plus	5 HT Gels
57269	6 x 50 well, 1X TAE Buffer, 1% SeaKem LE Plus	5 HT Gels
57271	6 x 50 well, 1X TAE Buffer, 2% SeaKem LE Plus	5 HT Gels
57273	6 x 50 well, 1X TAE Buffer, 3.6% NuSieve Hi-Res	5 HT Gels
57270	6 x 50 well, 1X TBE Buffer, 1% SeaKem LE Plus	5 HT Gels
57272	6 x 50 well, 1X TBE Buffer, 2% SeaKem LE Plus	5 HT Gels
57274	6 x 50 well, 1X TBE Buffer, 4% NuSieve 3:1 Plus	5 HT Gels
56991	TruBand Anchor for 24 x 14 cm gels,	Each
	Owl Millipede; Shelton JSB-96; Fisher SB-2318	
56992	TruBand Anchor for 24 x 20 cm gels, Other standard chambers	Each
56993	TruBand Anchor for 24 x 14 cm gels, Other standard chambers	Each
56998	TruBand Anchor for 24 x 20 cm gels, Owl Millipede®	Each

Latitude® HS Precast Agarose Gels

CAT. NO.	DESCRIPTION	SIZE
57280	2 x 50 well, 1% SeaKem® LE Plus	5 Gels per box
57281	2 x 50 well, 2% SeaKem LE Plus	5 Gels per box
57282	2 x 50 well, 3.6% NuSieve® Hi-Res	5 Gels per box
57283	4 x 50 well, 1% SeaKem LE Plus	5 Gels per box
57284	4 x 50 well, 2% SeaKem LE Plus	5 Gels per box
57285	4 x 50 well, 3.6% NuSieve Hi-Res	5 Gels per box
50890	10X HS Electrophoresis Buffer	500 ml
50891	10X HS Electrophoresis Buffer	1 L
50639	Tri-Dye Loading Buffer (6X)	1 ml
50649	Tri-Dye Loading Buffer (6X)	5 x 1 ml

PAGEr® Gold Precast Gels

58100	PAGEr Gold Scouting Kit 9 x 10 cm	6 Gels
58501	12 well, 7.5% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58502	12 well, 10% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58503	12 well, 12% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58504	12 well, 15% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58505	12 well, 4 - 20% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58506	12 well, 10 - 20% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58507	10 well, 7.5% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58508	10 well, 10% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58509	10 well, 12% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58510	10 well, 15% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58511	10 well, 4 - 20% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58512	10 well, 10 - 20% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58513	16 well, 7.5% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58514	16 well, 10% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58515	16 well, 12% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58516	16 well, 15% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58517	16 well, 4 - 20% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58518	16 well, 10 - 20% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58519	10 well, 8 - 16% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58520	10 well, 4 - 12% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58521	12 well, 8 - 16% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58522	12 well, 4 - 12% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58523	16 well, 8 - 16% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58524	16 well, 4 - 12% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58525	10 well, 6% 9 x 10 cm TBE	1 Box/10 Gels
58526	10 well, 10% 9 x 10 cm TBE	1 Box/10 Gels
58527	10 well, 4 - 20% gradient 9 x 10 cm TBE	1 Box/10 Gels
58528	12 well, 6% 9 x 10 cm TBE	1 Box/10 Gels
58529	12 well, 10% 9 x 10 cm TBE	1 Box/10 Gels
58530	12 well, 4 - 20% gradient 9 x 10 cm TBE	1 Box/10 Gels
58531	16 well, 6% 9 x 10 cm TBE	1 Box/10 Gels
58532	16 well, 10% 9 x 10 cm TBE	1 Box/10 Gels

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PAGEr® Gold Precast Gels (continued)

CAT. NO.	DESCRIPTION	SIZE
58533	16 well, 4 - 20% gradient 9 x 10 cm TBE	1 Box/10 Gels
58542	17 well, 10% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58544	17 well, 15% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58545	17 well, 4 - 20% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58548	8 + 1 well, 10% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58550	8 + 1 well, 15% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58551	8 + 1 well, 4 - 20% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58554	2D well, 10% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58556	2D well, 15% 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58557	2D well, 4 - 20% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58560	17 well, 8 - 16% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58562	8 + 1 well, 8 - 16% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
58564	2D well, 8 - 16% gradient 9 x 10 cm Tris-Glycine	1 Box/10 Gels
59100	PAGEr Gold Scouting Kit 10 x 10 cm	6 Gels
59501	12 well, 7.5% 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59502	12 well, 10% 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59503	12 well, 12% 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59504	12 well, 15% 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59505	12 well, 4 - 20% gradient 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59506	12 well, 10 - 20% gradient 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59507	10 well, 7.5% 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59508	10 well, 10% 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59509	10 well, 12% 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59510	10 well, 15% 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59511	10 well, 4 - 20% gradient 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59512	10 well, 10 - 20% gradient 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59513	16 well, 7.5% 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59514	16 well, 10% 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59515	16 well, 12% 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59516	16 well, 15% 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59517	16 well, 4 - 20% gradient 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59518	16 well, 10 - 20% gradient 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59519	10 well, 8 - 16% gradient 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59520	10 well, 4 - 12% gradient 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59521	12 well, 8 - 16% gradient 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59522	12 well, 4 - 12% gradient 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59523	16 well, 8 - 16% gradient 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59524	16 well, 4 - 12% gradient 10 x 10 cm Tris-Glycine	1 Box/10 Gels
59525	10 well, 6% 10 x 10 cm TBE	1 Box/10 Gels
59526	10 well, 10% 10 x 10 cm TBE	1 Box/10 Gels
59527	10 well, 4 - 20% gradient 10 x 10 cm TBE	1 Box/10 Gels
58528	12 well, 6% 10 x 10 cm TBE	1 Box/10 Gels
59529	12 well, 10% 10 x 10 cm TBE	1 Box/10 Gels
59530	12 well, 4 - 20% gradient 10 x 10 cm TBE	1 Box/10 Gels
59531	16 well, 6% 10 x 10 cm TBE	1 Box/10 Gels

PAGEr® Gold Precast Gels (continued)

CAT. NO.	DESCRIPTION	SIZE
59532	16 well, 10% 10 x 10 cm TBE	1 Box/10 Gels
59533	16 well, 4 - 20% gradient 10 x 10 cm TBE	1 Box/10 Gels

Specialty Products

50302	SeaPrep Agarose	25 g
58001	ß-Agarase	100 Units
58005	ß-Agarase	500 Units
50202	IsoGel Agarose	25 g
56010	IsoGel® Agarose IEF Plate Accessory Bulk Pack	100 Each
56014	IsoGel Agarose IEF Plate Accessory Pack	Sufficient for 6 Plates
56015	IsoGel Agarose IEF Plates pH range 3 - 10	6 Plates
56018	IsoGel Agarose IEF Plates pH range 6 - 10.5	6 Plates

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