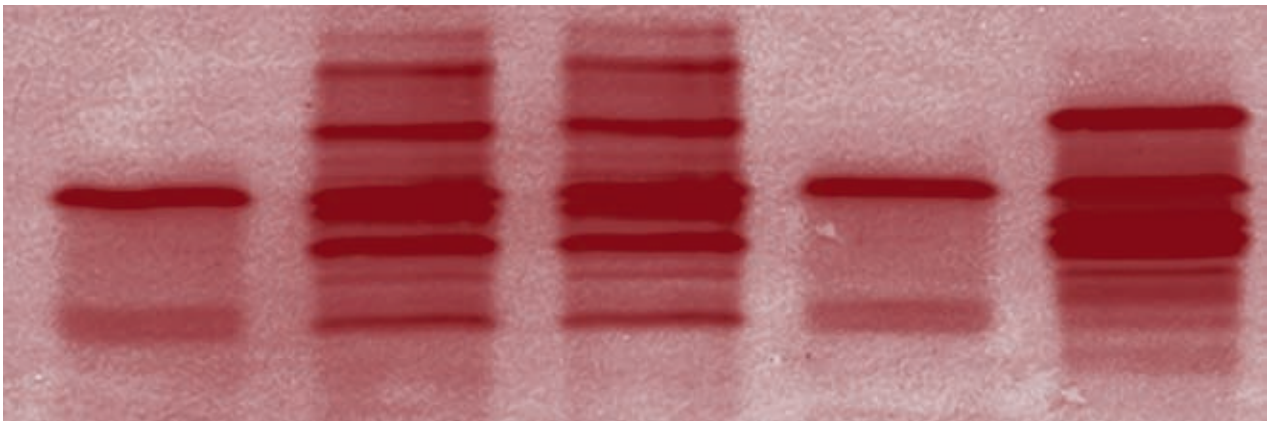

In This Section

Section XIII: Protein Separation 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 Da.

Advantages

- Separate high-molecular-weight proteins (>600,000 Da)
- 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 Lonza Agaroses that are recommended for protein electrophoresis. For performing routine separations, we recommend a standard melting temperature agarose such as MetaPhor® Agarose or SeaKem® Gold Agarose. When proteins are to be recovered for further analysis, use a low melting temperature agarose such as SeaPlaque® GTG® or NuSieve® GTG® Agarose.

Protein Size Range (kDa)	Agarose	Concentration
20 - 200	MetaPhor® or NuSieve® GTG®	5%
150 - 300	MetaPhor® or NuSieve® GTG®	3%
300 - 600	MetaPhor® or NuSieve® GTG®	2%
600 - 1,000	SeaKem® Gold or SeaPlaque®	1.5%
1,000 - 5,000	SeaKem® Gold or SeaPlaque®	1.0%

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 156).
- 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.

Caution: Materials and methods shown here present hazards to the user and the environment. Refer to the safety information on page 210 before beginning these procedures.

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 Section II).

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 86).

— 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

Section XIII: **Protein Separation in Agarose Gels**

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 Lonza's ProSieve® Color Protein Marker 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

Section XIV: **Protein Separation in Agarose Gels**

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® Brilliant Blue staining (see page 168), using the modifications listed below. For detailed procedures on Silver staining, refer to Staining Proteins with Silver Stain (see page 186 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

Agarose 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.

Proteins Separation in Agarose Gels

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 171). 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.

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.

■ 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

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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