ReadyPrep™ Protein Extraction Kit (Membrane I)

Instruction Manual

Catalog #163-2088

For technical service, call your local Bio-Rad office, or in the US, call 1-800-4BIORAD (1-800-424-6723)

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Section 1
Introduction

The ReadyPrep protein extraction kit (membrane I) is a simple, rapid and reproducible method to prepare cellular protein fractions highly enriched in membrane and hydrophobic proteins. This kit is part of a group of related kits from Bio-Rad developed to reduce sample complexity in order to improve the chances of identifying low-abundance proteins, such as membrane proteins, and to simplify proteomic studies. The fractionation protocol can be applied to a wide variety of biological samples and the isolated proteins are suitable for 2-D gel analysis or other applications such as SDS-PAGE and western blotting. Unlike many procedures used to isolate membrane proteins, this kit does not require the use of ultracentrifugation nor the preparation of density gradients.

The kit is based on the separation of membrane proteins by temperature-dependent phase partitioning using Triton X-114 detergent (Bordier 1981, Santoni et al. 2000). The sample is first mixed and homogenized in the membrane protein extraction buffers. After a brief incubation at 37°C, the sample is centrifuged, producing an upper aqueous phase and a lower detergent-rich phase. Proteins that were anchored to the membrane or proteins containing one or two transmembrane domains are efficiently partitioned to

the detergent-rich phase. Cytoplasmic proteins partition into the aqueous phase. An insoluble pellet is also formed and is a source of more complex membrane proteins.

Proteins extracted into the two phases are treated using the ReadyPrep 2-D cleanup kit (provided) to remove agents in the extraction process that interfere with IEF. Finally, the hydrophilic and hydrophobic protein pellets, as well as the insoluble pellet, are efficiently solubilized using the strongly chaotropic extraction buffer provided in the kit. The solubilized proteins can then be immediately used in 2-D gel electrophoretic applications.

Section 2
Kit Specifications

Each ReadyPrep protein extraction kit (membrane I) provides sufficient reagents to perform 50 extractions of 25–50 mg of cells or tissue. The procedure can easily be scaled up to accommodate larger amounts of cells or tissue.

Items Supplied With Kit

One bottle containing 50 ml of membrane protein extraction buffer 1 (M1)
One bottle containing 50 ml of membrane protein extraction buffer 2 (M2)
One bottle containing 25 g of protein solubilization buffer (PSB)
One bottle containing 30 ml of PSB diluent
One ReadyPrep 2-D cleanup kit

Items Required But Not Provided

- 1.5 ml microcentrifuge tubes
- Microcentrifuge capable of spinning at 12–16,000 x g at 4°C.
- Vortex mixer
- Sonicator
- Heat block/bath set at 37°C

- Protease inhibitor(s)
- Reducing agent (for example, DTT, TBP, or TCEP)
- Carrier ampholytes (for example, Bio-Lyte® 3/10 ampholyte)
- *RC DC*™ Protein Assay (Bio-Rad catalog #500-0121 or #500-0122)

Section 3
Storage Conditions

The unopened kit can be stored at room temperature. Buffer M1, buffer M2, and PSB diluent should be stored at 2–8°C after opening. Use aseptic technique when handling buffer M1, buffer M2, and PSB diluent to prevent contamination. For storage of the ReadyPrep 2-D cleanup kit, follow the instructions provided in the kit.

Section 4
Instructions for Use

4.1 Membrane Extraction Protocol

Note: Chill buffers M1 and M2 on ice for at least 15 min before beginning (invert bottles several times during the incubation).

1. In a microcentrifuge tube, add 0.5 ml of buffer M1 per 25–50 mg of animal tissue or 0.05 ml of wet cell pellet from sources such as cell culture, yeast, or bacteria. For plant tissue add 0.5 ml of buffer M1 per 0.25 g. The sample-to-buffer volume ratio indicated above is only a guide and may be adjusted depending upon the desired scale of the preparation.

   Notes: Protease inhibitors may be added to buffer M1 immediately prior to use to prevent proteolysis during extraction.

   Insufficient volume of buffer M1 may result in poor cell lysis, low hydrophilic protein yield, and contamination of the membrane protein fraction with cytoplasmic proteins.

   Plant tissue should be ground to a fine powder using a mortar and pestle in liquid nitrogen before addition of buffer M1.

2. Sonicate the suspension on ice with an ultrasonic probe to break open the cells and fragment the genomic DNA. During sonication, care must be

exercised to prevent heating of the sample. Sonicate the sample using 30–40 sec bursts until lysis is complete (typically 3–4 times). Chill the suspension on ice for 1 min between each ultrasonic treatment.

Note: Disruption of cells by sonication is dependent on the cell type. For example, *E. coli* requires longer sonication times than animal cells and tissues. Yeast cell disruption requires even more vigorous sonication, and the addition of glass beads or use of a Bead Beater (BioSpec Products) can greatly improve cell lysis.

3. Add an equal volume of chilled buffer M2 into the cell extract. Vortex the suspension 4–5 times, 60 sec each. Maintain the tube in an ice-water bath for 30–60 sec between each vortexing step. At the end of the vortexing procedure, incubate the tube in an ice-water bath for 10 min.

4. Transfer the tube to a 37°C heating block or water bath and incubate for 30 min. Mix the suspension for 30 sec periodically (3 to 4 times).

5. Centrifuge the tube at maximum speed in a microcentrifuge (~16,000 x g) for 5 min at room temperature.

6. Examine the tube carefully. You should notice two phases. Remove and transfer the top layer, containing the hydrophilic proteins, to a clean tube.

7. Add 0.5 ml of prechilled buffer M2 (the same volume as used in step 3) to the tube containing the bottom phase. Vortex and incubate as described in step 3. Repeat steps 4 through 6. Again remove the top layer and pool with the top layer that was collected earlier. Label this tube of pooled top layers Hydrophilic Protein Fraction (Refer to the note in step 9 for further instructions on storing this protein fraction).

8. If there is any debris at the interface, either remove it and set aside for later analysis or discard.

9. Collect the bottom phase and transfer to a new tube. Label this tube Hydrophobic Membrane Protein Fraction (Refer to the note below for further instructions on storing this protein fraction). The sediment, which is an additional source of membrane proteins, can be saved and stored at -70°C for later analysis (step 11).

   Note: If no further downstream applications are to be immediately performed, aliquot and quick-freeze the hydrophilic and hydrophobic membrane protein fractions on dryice and then store at -70°C. Also, to avoid repeated freeze-thawing of the sample, it is often convenient at this stage to set aside a 5–10 µl aliquot of each protein fraction before freezing for protein quantitation. This amount is sufficient for the *RC DC* protein assay (catalog #500-0121 and #500-0122).

4.2 Processing Hydrophilic and Hydrophobic Membrane Protein Fractions for IEF/2-D Analysis

10. Determine the protein concentration of the hydrophilic and hydrophobic membrane protein fractions. The *RC DC* Protein Assay is recommended. This assay allows accurate protein quantitation in the presence of detergents, reducing agents, and other substances that typically interfere with other protein assays.

11. Process an appropriate amount of the hydrophobic membrane protein fraction, and if desired, hydrophilic protein fraction through the ReadyPrep 2-D cleanup kit. To determine an appropriate amount, refer to the guidelines in Section 5.1. Follow the ReadyPrep 2-D cleanup kit protocol provided. At the end of the 2-D cleanup protocol, resuspend the protein pellet in complete PSB (See Section 5.2 for preparation instructions) and then perform IEF/2-D analysis.

Section 5
Appendix

5.1 2-D Rehydration/Sample Buffer Volume

In the final step for this kit, all samples are resuspended in a 2-D rehydration/sample buffer (Section 5.2). To best determine the volume of 2-D rehydration/sample buffer to use, consider the questions listed below. To assist with these calculations, the table that follows indicates appropriate volumes of 2-D rehydration/sample buffer needed to rehydrate IPG strips of specific lengths and the approximate amounts of protein required for detection using silver stain or Coomassie Blue G-250 stain. An example illustrates how to calculate the volume of 2-D rehydration/sample buffer required.

1. What is the quantity of protein precipitated in the tube?
2. For 2-D electrophoresis experiments using IPG strips, what length strip will be used?
3. What is the pH range of the IPG strip to be used?
4. How complex is the protein sample?
5. What staining or detection method will be used? (for example, Bio-Safe™ Coomassie stain, silver stain, etc.)

IPG strip length	7 cm	11 cm	17 cm	18 cm	24 cm
Rehydration volume per strip	125 µl	185 µl	300 µl	315 µl	410 µl
Protein load–Silver stain	5–20 µg	20–50 µg	50–80 µg	50–80 µg	80–150 µg
Protein load–Coomassie G-250	50–100 µg	100–200 µg	200–400 µg	200–400 µg	400–800 µg

Sample calculation: If you precipitate 100 µg of protein and are going to run 7 cm pH 3–10NL IPG strips (125 µl per strip) and silver stain the 2-D gels, then you may want to solubilize the protein pellet in ~900 µl of rehydration/sample buffer, which is enough to rehydrate about seven 7 cm IPG strips (~14 µg/strip). However, if you are planning to use a 24 cm pH 3–10NL IPG strip, then you may want to solubilize the protein pellet in 410 µl of rehydration/sample buffer, which is enough to rehydrate one 24 cm IPG strip (100 µg/strip). In this simple example, sample complexity and IPG strip pH range were not addressed. As a general rule, increased protein loads may be required for micro-range IPG strips and for samples of higher protein complexity.

5.2 Preparation of 2-D Rehydration/Sample Buffers

The PSB and PSB diluent are provided with this kit to solubilize the protein pellet after using the ReadyPrep 2-D cleanup kit. PSB is a proprietary, strongly chaotropic 2-D rehydration/sample buffer that will solubililze both hydrophilic as well as hydrophobic proteins. Other strongly chaotropic 2-D rehydration/sample buffers (Section 5.2.2) may be substituted for PSB or used for diluting an appropriate amount of sample for IEF/2-D analysis.

5.2.1. Complete Protein Solubilization Buffer (PSB)

o make 2 ml of complete 2-D rehydration/sample buffer, add 1.1 ml of PSB diluent to each 1 g of PSB powder.

Note: Before weighing out the PSB powder, shake the bottle vigorously 10-15 seconds to break up any clumps and to ensure a uniform blend of the different components.

Mix the solution until the powder is completely dissolved (the tube can be warmed to speed dissolution of the solids, but do not allow the temperature to exceed 30°C). Add DTT, Bio-Lyte ampholyte, and Bromophenol Blue according to the table below to complete the preparation of the buffer.

Component	Final Concentration	Amount to Make 2 ml
DTT* (FW 154.3)	50 mM	15.4 mg
100X Bio-Lyte 3/10 ampholyte**	0.2% (w/v)	20 µl
Bromophenol Blue	0.002% (w/v)	4 µl of a 1% (w/v) solution

*If TBP or TCEP is substituted for DTT as the reducing agent, use at a concentration of 2 mM.

**Use an ampholyte buffer that corresponds to the pH range of the IEF separation to be performed. For example, ReadyStrip™ micro-range buffers with ReadyStrip micro-range IPG strips and ReadyStrip 7-10 buffer with ReadyStrip pH 7-10 IPG strips. Bio-Lyte 3/10 ampholyte can be used with all other ReadyStrip IPG strip pH ranges.

5.2.2. Strongly Chaotropic 2-D Rehydration/Sample Buffer

(7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte, 0.002% Bromophenol Blue).

Component	**Final Concentration**	**Amount to make 2 ml**
Urea (FW 60.06)	7 M	0.84 g
Thiourea (FW 76.12)	2 M	0.304 g
CHAPS*	4% (w/v)	0.08 g
DTT (FW 154.3)	50 mM	15.4 mg
100X Bio-Lyte 3/10 ampholyte**	0.2% (w/v)	20 µl
Bromophenol Blue	0.002% (w/v)	4 µl of a 1% (w/v) solution
Proteomic grade water		1.1 ml

*Other neutral or zwitterionic detergents can also be used at concentrations of 1% to 2% (w/v) to improve solubilization of membrane and hydrophobic proteins. Examples are n-octyl-β-D-glucopyranoside, SB3-10 (N-decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate) and ASB14 (tetradecanoylamido-propyl-dimethylammonio-propane-sulfonate).

**Use an ampholyte buffer that corresponds to the pH range of the IEF separation to be performed. For example, ReadyStrip™ micro-range buffers with ReadyStrip micro-range IPG strips and ReadyStrip 7-10 buffer with ReadyStrip pH 7-10 IPG strips. Bio-Lyte 3/10 ampholyte can be used with all other ReadyStrip IPG strip pH ranges.

Section 6
References

1. Bordier C, Phase separation of integral membrane proteins in Triton X-114, J Biol Chem 256, 1604–1607 (1981).

2. Santoni V et al., Membrane proteomics: Use of additive main effects with multiplicative interaction model to classify plasma membrane proteins according to their solubility and electrophoresis properties, Electrophoresis 21, 3329–3344 (2000) .

Section 7
Product Information

Catalog #	Description
Sample Preparation Kits	
163-2130	ReadyPrep 2-D Cleanup Kit, 50 preps
163-2089	ReadyPrep Protein Extraction Kit (Cytoplasmic/Nuclear), 50 preps
163-2088	ReadyPrep Protein Extraction Kit (Membrane I), 50 preps
163-2087	ReadyPrep Protein Extraction Kit (Signal), 50 preps
163-2090	ReadyPrep Reduction Alkylation Kit
163-2100	ReadyPrep Sequential Extraction Kit, 5–15 preps
Protein Quantitation Kits (also see bulletin 2610)	
500-0121	*RC DC* Protein Assay Kit I, 500 standard assays, bovine γ-globulin standard
500-0122	*RC DC* Protein Assay Kit II, 500 standard assays, bovine serum albumin standard

Buffer Components

161-0611	Dithiothreitol (DTT), 5 g
163-2101	Tributylphophine (TBP), 200 mM, 0.6 ml
161-0460	CHAPS, 1 g
161-0731	Urea, 1 kg
161-0716	Tris, 500 g
161-0302	Sodium Dodecyl Sulfate (SDS), 1 kg
163-2094	100X Bio-Lyte 3/10 Ampholyte, 1 ml
163-2091	ReadyPrep Proteomic Grade Water

Rehydration/Sample Buffers

163-2106	ReadyPrep 2-D Starter Kit Rehydration/Sample Buffer, 10 ml, containing 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte, Bromophenol Blue