ReadyPrep™ Protein Extraction Kit (Signal)

Instruction Manual

Catalog #163-2087

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Section 1
Introduction

The ReadyPrep protein extraction kit (signal) is designed as a simple, rapid and reproducible method to prepare a cellular protein fraction that is enriched in membrane-associated signaling 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 and particular classes of proteins, such as signaling 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.

The plasma membrane of most cell types contains organized microdomain structures that are rich in sphingolipids and cholesterol and have distinct protein compositions. Proteins associated with these lipid rafts are involved in intracellular membrane trafficking and signaling pathways, and include glycosylphosphatidylinositol (GPI)-anchored proteins, caveolin, caveolae(caveolin)-associated proteins, acylated tyrosine kinases, heterotrimeric G-proteins and some proteins with transmembrane domains (Simons and Ikonen 1997, Brown and Rose 1992,

Parton and Simons 1995, Anderson et al. 1992). Due to the lipid composition of these membrane subdomains, these proteins become resistant to solubilization at 4°C with many nonionic detergents such as Triton X-100 and can be selectively recovered from the detergent-insoluble membrane fraction. Based upon the solubility properties of these signaling proteins, the ReadyPrep protein extraction kit (signal) utilizes a proprietary mixture of nonionic detergents to extract and remove hydrophilic and soluble proteins, leaving behind the signal proteins in a detergent-insoluble fraction. After the fractionation procedure has been completed, the signal proteins are solubilized in the protein solubilization buffer (PSB) supplied with 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 (signal) 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 signal protein extraction buffer 1 (S1)
One bottle containing 50 ml of signal protein extraction buffer 2 (S2)
One bottle containing 25 g of protein solubilization buffer (PSB)
One bottle containing 30 ml of PSB diluent

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
- 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 S1, buffer S2, and PSB diluent should be stored at 2–8°C after opening. Use aseptic technique when handling buffer S1, buffer S2 and PSB diluent to prevent contamination.

Section 4
Instructions for Use

Note: Chill buffers S1 and S2 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 S1 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 S1 per 0.25 g tissue. 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 S1 immediately prior to use to prevent proteolysis during extraction.

   Insufficient volume of buffer S1 may result in poor cell lysis and contamination of the signal 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 S1.

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 S2 into the cell extract.

4. 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 15 min.

5. Centrifuge the tube at maximum speed in a microcentrifuge (~16,000 x g) for 20 min at 4°C. Remove and discard the supernatant.

6. Keeping the tube cold at all times, suspend the pellet in one-third the volume used initially (see step 1) of chilled buffer S1. Vortex to resuspend. Add an equal volume of chilled buffer S2 (i.e. one-third the original volume used in step 3). Repeat steps 4 and 5.

7. The final pellet contains the signal proteins. Resuspend the pellet in 0.2–0.3 ml of complete PSB. Vortex the tube 4 to 5 times for 60 seconds each. Incubate the tube at room temperature for 10–15 min vortexing periodically to ensure the pellet is completely dissolved. Centrifuge the sample at maximum speed in a microcentrifuge for 10 minutes at room temperature and collect the clarified supernatant. To improve recovery of the signal proteins, reextract any residual pellet with about one-third the original volume of complete PSB and combine the supernatant with that obtained previously. See Section 5.2 for instructions on preparing the complete PSB.

8. Determine the protein concentration of the signal proteins. The Bio-Rad *RC DC* Protein Assay is recommended for this measurement. This assay allows for accurate protein quantitation in the presence of detergents, reducing agents, and other substances that typically interfere with other protein assays. Make an appropriate dilution of the resuspended signal proteins into complete PSB or other strongly chaotropic 2-D rehydration/sample buffer before IEF/2-D gel analysis. Refer to Section 5.1 for guidelines on selecting the appropriate volume of buffer to use.

Section 5
Appendix

5.1 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 final pellet containing the signal proteins. 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)

To 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. Simons K and Ikonen E, Functional rafts in cell membranes, Nature 387, 569–572 (1997).

2. Brown D and Rose J, Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface, Cell 68, 533–544 (1992).

3. Parton RG and Simons K, Digging into caveolae, Science 269, 1398–1399 (1995).

4. Anderson RG et al., Potocytosis: sequestration and transport of small molecules by caveolae, Science 255, 410–411 (1992).

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