ReadyPrep™ Protein Extraction Kit (Membrane II)

Instruction Manual

Catalog #163-2084

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 II) is designed as a simple, rapid and reproducible method to isolate cellular membranes and then to extract the associated integral membrane proteins into a solution compatible with two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). The ReadyPrep protein extraction kit (membrane II) joins two existing ReadyPrep kits for isolation of membrane proteins; it is optimized to effectively isolate more complex proteins that contain more than two transmembrane domains, or are resistant to extraction by the ReadyPrep membrane I kit. The signal kit is specific for isolation of proteins involved in intracellular membrane trafficking and signaling pathways. All three of these membrane kits reduce sample complexity using cellular location as a way to fractionate. The procedure for the ReadyPrep protein extraction kit (membrane II) is based on the original work of Fujiki et al.[1] for the isolation of cellular membrane fractions from rat liver after carbonate extraction and the later adaption of the technique by Molloy et al.[2] for Proteomic analysis of *E. coli* outer membrane proteins.

The kit contains all the reagents necessary for membrane isolation and membrane-protein extraction and the protocol

can be applied to a wide variety of biological samples, from animal cells and tissues to yeast, bacteria and plant tissues. After the cells or tissue are disrupted using an ultrasonic probe, the extract is incubated in a Membrane Protein Concentrating Reagent. This high pH buffer strips away proteins weakly-associated with membranes while leaving membrane fragments with their associated integral proteins intact. Ultracentrifugation is used to concentrate the membranes into a pellet, and the proteins are solubilized from the washed pellet using an isoelectric focusing-compatible 2-D rehydration/sample buffer. The 2-D Rehydration/Sample Buffer 1 utilized in the kit is a strongly chaotropic buffer containing the zwitterionic detergent ASB-14, making this solution one of the most powerful solubilizing reagents available for 2-D gel electrophoresis. The kit also contains the reducing agent tributylphosphine (TBP), which has been shown to be more efficient than dithiothreitol in keeping proteins reduced in 2-D PAGE applications.[3]

Section 2
Kit Specifications

Each ReadyPrep protein extraction kit (membrane II) provides sufficient reagents to perform 10 extractions each starting with 100–200 mg of cells or tissue. The procedure can easily be scaled up to accommodate larger amounts of cells or tissue. The entire procedure can be completed in less than 3 hr.

Items Supplied With Kit

- **2-D Rehydration/Sample Buffer 1.** 2 vials. Lyophilized. After reconstituting, each vial contains 10 ml of 7 M urea, 2 M thiourea, 1 % (w/v) ASB-14 detergent, 40 mM Tris base, and 0.001% Bromophenol Blue.
- **Lysis Buffer.** 5 pouches. Each pouch reconstitutes to make 50 ml of Lysis Buffer.
- **Membrane Protein Concentrating Reagent.** 5 pouches. Each pouch reconstitutes to make 125 ml of membrane protein concentrating reagent.
- **TBP Reducing Agent.** 1 ampoule containing 0.6 ml of 200 mM tributylphosphine (TBP) in 1-methyl-2-pyrrolidinone sealed under nitrogen gas.
- **Empty Vial.** 1 storage vial for TBP reducing agent.
- **Instruction Manual,** 1.

Items Required But Not Provided

- 2.0 ml microcentrifuge tubes (for example, VWR catalog #20170-237).
- Disposable round-bottom tubes for plant sample extraction (for example, VWR catalog # 60818-725).
- ReadyPrep 2-D cleanup kit (Bio-Rad catalog #163-2130) for plant sample extraction. When working with plant leaf tissue, removal of interfering compounds, such as phenolics, is essential for high quality 2-D gels with minimal horizontal streaking.
- 50 ml conical centrifuge tube(s), disposable, (for example, VWR catalog #20171-030).
- 2 clean 125–150 ml beakers or bottles plus stirring bars.
- Ultracentrifuge, rotor and tubes capable of spinning at 100,000 x g. Typically, tubes should have ~30 ml capacity.
- Microcentrifuge capable of spinning at 12–16,000 x g at 4°C.
- Sonicator
- Benzonase (endonuclease) (for example, Sigma catalog #E1014 or #E8263)
- 1 M $MgCl_2$ solution
- Protease inhibitor(s) (optional)
- Carrier ampholytes (for example, Bio-Lyte® 3/10 ampholyte, Bio-Rad catalog #163-2094)

- ReadyPrep proteomic grade water (Bio-Rad catalog #163-2091)
- *RC DC*™ Protein Assay (Bio-Rad catalog #500-0121 or #500-0122)

Section 3
Storage Conditions

Store the unopened kit at room temperature. After opening, unused reconstituted ReadyPrep 2-D Rehydration/Sample Buffer 1 should be aliquoted in 1 to 2 ml volumes and stored frozen at –80°C. After opening, transfer the ReadyPrep TBP reducing agent to the empty glass vial provided and store the vial at –20°C to prevent evaporation of the TBP. Aliquot and store unused Lysis Buffer and Membrane Protein Concentrating Reagent at –20°C.

Section 4
Reagent Preparation

ReadyPrep 2-D Rehydration/Sample Buffer 1: Add 5.6 ml of ReadyPrep proteomic grade water or similar quality water to one bottle. Swirl the vial gently until the contents are completely dissolved. The solution can be warmed slightly in the palm of the hand or in a water bath to speed the dissolution process. DO NOT heat the solution above 25 to 30°C to avoid the formation of cyanates. Cyanates can react with and modify the proteins in the sample.

ReadyPrep TBP Reducing Agent. Tributylphosphine (TBP) has an unpleasant odor and is very volatile. Work with TBP in a fume hood. Wear a laboratory coat and gloves when handling the ampoule of TBP reducing agent. Wipe up spills with wet towels. Open the ampoule by snapping the top off at the scored neck. Transfer the entire contents of the ampoule to the empty screw-cap storage vial provided. Screw the cap of the vial down tightly and store the vial at –20°C to prevent evaporation of the TBP. While using, keep the vial of TBP reducing agent on ice.

Lysis Buffer. Empty the entire contents of one pouch of Lysis Buffer into a 50 ml disposable tube. Add 50 ml of ReadyPrep proteomic grade water or similar quality water to the tube and mix until the solids are completely dissolved. Rinse the pouch with a small portion of this buffer, and return the solution to the 50 ml tube. If desired, protease inhibitors can be added to a portion of this buffer just before use.

Membrane Protein Concentrating Reagent. Empty the entire contents of one pouch of Membrane Protein Concentrating Reagent into a beaker or bottle containing 125 ml of ReadyPrep proteomic grade water or similar quality water. To speed up dissolving the solid, add the contents of the pouch while the water is stirring. Mix until all the solids are completely dissolved. Rinse the pouch with a small amount of the solution (10–15 ml) and then return it to the container. Protease inhibitors can be added to a portion of this buffer just before use if desired.

Section 5
Instructions for Use

Extraction of 100–200 mg of sample.

1. Immediately before performing an extraction, prepare Lysis Buffer, Membrane Protein Concentrating Reagent and ReadyPrep 2-D rehydration/sample buffer 1 per the instructions in **Section 4.** For a standard extraction you will need approximately 15 ml of Lysis Buffer, 60 ml of Membrane Protein Concentrating Reagent and 1 ml of 2-D Rehydration/Sample Buffer 1. Chill both the Lysis Buffer and the Membrane Protein Concentrating Reagent on ice for 10–15 min before proceeding.

2. In a 2.0 ml microcentrifuge tube (on ice), add 1 ml of Lysis Buffer per 100–200 mg of animal tissue or wet cell pellet from sources such as cell culture, yeast, or bacteria. For plant tissue add 2–3 ml of Lysis Buffer per each gram of tissue in, for example, a disposable 14 ml round-bottom tube. The sample-to-buffer volume ratio indicated above is only a guide and may be adjusted depending upon the desired scale of the preparation and type of sample used.

   Insufficient volume of Lysis Buffer may result in poor cell lysis and incomplete solubilization of proteins.

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

3. With the sample on ice, sonicate the suspension with an ultrasonic probe to disrupt the cells and fragment the genomic DNA. Sonicate the sample using 30 sec bursts, typically 3–4 times, or until lysis is complete. Chill the suspension on ice briefly between each ultrasonic treatment. If necessary, transfer the extract to a microcentrifuge tube when this step is complete.

   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 The addition of glass beads or use of a Bead Beater (BioSpec Products) can greatly improve cell lysis of these sample types.

3a. **Optional**. Nucleic acid can be degraded by adding ~150 U of Benzonase and 2 µl/ml of 1 M $MgCl_2$ to the extract and incubating the sample at 4–8°C for 20 min before proceeding to step 4.

4. Centrifuge the tube for 10 min at ~3,000 x g in a microcentrifuge at 4°C to pellet insoluble material and unbroken cells.

5. Remove and dilute the supernatant directly into a beaker or bottle containing 60 ml of the ice-cold Membrane Protein Concentrating Reagent. Stir the suspension slowly on ice for 60 min.

6. Transfer the sample to ultracentrifuge tubes and centrifuge at 100,000 x g for 60 min at 4°C to pellet the membranes and membrane proteins.

7. Remove the tubes from the rotor and mark the position of the pellet. Carefully decant and discard the supernatant.

8. Wash each pellet briefly with 3 ml of cold Lysis Buffer. Do not disturb the pellet when adding the buffer. Allow the buffer to sit on ice, covering the pellet, for 1–2 min before decanting (longer incubation times may be required for unusually large pellets). Typically, the wash will easily decant without dislodging the pellet. However, if any of the pellet dislodges or is seen floating, then the sample should be re-centrifuged at >20,000 x g for 20–30 min at 4°C.

9. Repeat this wash step using 3 ml of Lysis Buffer per pellet.

10. Let the tube stand on ice for 1 min and remove any liquid collected at the bottom of the tube by using a pipet.

11. Before performing the extraction of the pellet, complete the preparation of the 2-D Rehydration/ Sample Buffer 1. Prepare only enough complete 2-D rehydration/sample buffer 1 for the number of extractions being performed. Each extraction will require 1.0 ml of the solution. The solution is prepared by adding 10 µl of ReadyPrep TBP reducing agent and the appropriate ampholyte to 0.2 % (w/v) to every 1 ml of reconstituted 2-D Rehydration/Sample Buffer 1. The ampholyte is chosen to match the pH range of the IPG strip to be used for 2D analysis, though for most applications, Bio-Rad's Bio-Lyte 3/10 ampholyte (catalog #163-2094) can be used. If desired, other additions, such as protease inhibitors can also be made at this time.

12. Resuspend the membrane-protein pellet(s) from each extraction with a total of 1.0–2.0 ml of complete 2-D rehydration/sample buffer 1. Alternatively, the pellets can be stored at –80°C for later analysis.

13. Vortex and/or sonicate the sample to completely solubilize the proteins in the pellet.

    Note: If using sonication, care must be exercised to prevent heating of the sample. The temperature of the sample should not be allowed to rise above 30°C. Similarly, if the sample becomes too cold, precipitation of the urea and thiourea can occur. If this happens, gently warm the sample until the precipitate dissolves before proceeding further.

14. Centrifuge the tube at maximum speed in a microcentrifuge (~16,000 x g) for 10–20 min at 18–20°C to pellet any remaining cell debris.

15. Remove and transfer the supernatant (Membrane Protein fraction) to a clean tube and discard any residual pellet.

16. Determine the protein concentration of the membrane protein sample. The Bio-Rad *RC DC* Protein Assay (catalog #500-0121 or #500-0122) 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.

17. An appropriate dilution of the membrane protein fraction may be necessary before IEF/2-D gel analysis. Refer to **Section 6** for guidelines on selecting the appropriate protein load and volume of sample needed for a selected IPG strip. For 2-D separation of the membrane proteins, it is recommended that 2-D Rehydration/Sample Buffer 1 containing TBP reducing agent and ampholytes be used for all dilutions. Using other rehydration/sample buffers that do not contain ASB-14 detergent may result in some proteins precipitating out before or during isoelectric focusing.

Additional ReadyPrep 2-D rehydration/sample buffer 1 is available and sold separately for this purpose (see Section 8).

Note: For some applications (for example using basic pH range IPG strips), the membrane protein sample may require reduction and alkylation treatment before IEF in order to achieve optimal 2-D separation. The ReadyPrep Reduction-Alkylation Kit can be purchased separately for this purpose (catalog #163-2090).

18. The samples are now ready to be loaded onto IPG strips. Unused membrane protein extracts should be stored in aliquots at –80°C.

Note when working with plant leaf samples: for best 2-D separation results treat the sample using the ReadyPrep 2-D Cleanup Kit (Bio-Rad catalog #163-2130) prior to performing IEF.

Section 6
Appendix

2-D Rehydration/Sample Buffer Volume

Before IEF and 2D gel electrophoresis, the protein sample may need to be diluted to achieve the desired protein load for the chosen stain. To best determine the volume of diluent to use, consider the IPG strip length, the pH gradient of the IPG strip, and the staining or detection method. 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.

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

Section 7
References

1. Fujiki Y et al., Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. J. Cell Biology 93, 97–102 (1982).

2. Molloy MP et al., Proteomic analysis of the Eschericia coli outer membrane. Eur. J. Biochem. 267, 2871–2881 (2000).

3. Herbert BR et al., Improved protein solubility in two-dimensional electrophoresis using tributyl phosphine as reducing agent. Electrophoresis. 19(5), 845–851 (1998).

Section 8
Product Information

Catalog #	Description

Sample Preparation Kits

163-2086	ReadyPrep Protein Extraction Kit (Total Protein), 20 preps
163-2085	ReadyPrep Protein Extraction Kit (Soluble/Insoluble), 20 preps
163-2084	ReadyPrep Protein Extraction Kit (Membrane II), 10 preps
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, 50 preps
163-2100	ReadyPrep Sequential Extraction Kit, 5–15 preps

Rehydration/Sample Buffers

163-2083	ReadyPrep 2-D Rehydration/Sample Buffer 1, 10 ml, containing 7 M urea, 2M thiourea, 1% ASB-14, 40 mM Tris base, and 0.001% Bromophenol Blue

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

163-2101	Tributylphosphine (TBP), 200 mM, 0.6 ml
163-2094	100X Bio-Lyte 3/10 Ampholyte, 1 ml
163-2091	ReadyPrep Proteomic Grade Water

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