
ReadyPrep™ 2-D Cleanup Kit

Instruction Manual

Catalog #163-2130

For technical service, call your local Bio-Rad office, or
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Table of Contents

Section 1	Introduction.....	1
Section 2	Kit Specifications.....	2–3
Section 3	Storage Conditions.....	3
Section 4	Instructions for Use.....	4–9
Section 5	Appendix.....	10–15
Section 6	Product Information.....	16–17

Section 1

Introduction

The ReadyPrep 2-D cleanup kit facilitates the preparation of low conductivity samples suitable for isoelectric focusing (IEF) and 2-D gel electrophoresis. Additionally, the kit concentrates proteins from samples that are too dilute, allowing for higher protein loads that can improve spot detection. The ReadyPrep 2-D cleanup kit can be used with virtually any protein sample to generate improved 2-D results by reducing streaking, background staining, and other gel artifacts associated with substances contaminating 2D/IEF samples. The entire process can be completed in approximately one hour and does not produce alterations in the isoelectric point of proteins. The procedure works by quantitatively precipitating and concentrating proteins in a sample while leaving behind and washing away substances like ionic detergents, salts, nucleic acids, lipids, and plant-derived phenolic compounds, all of which are known to interfere with IEF. The precipitation is not affected by the presence of detergents, chaotropes or other common buffers, salts and reagents used to extract or treat proteins. After precipitation, the proteins are washed and then resuspended in the IEF/2D-compatible rehydration/sample buffer of choice, ready for isoelectric focusing or Laemmli sample buffer, ready for SDS-PAGE.

Section 2

Kit Specifications

Each ReadyPrep 2-D cleanup kit provides sufficient reagent to purify 50 samples of 100 μ l, each sample containing up to 500 μ g of protein. The kit contains the components listed below. Certificates of analysis or MSDS forms are available upon request.

Items Supplied With Kit

One bottle containing 15 ml of precipitation agent 1*

One bottle containing 15 ml of precipitation agent 2

One tube containing 2 ml of wash reagent 1

One bottle containing 50 ml of wash reagent 2*

One tube containing 0.25 ml of wash 2 additive

One instruction manual

*Care should be exercised when handling precipitation agent 1 and wash reagent 2.

Precipitation agent 1 is corrosive!

Wash reagent 2 is flammable and an irritant!

Items Required But Not Provided:

- 1.5 ml microcentrifuge tubes
- Microcentrifuge capable of spinning at 12-16,000 x g at 4°C.
- Rehydration/sample buffer (see Section 5 for recipes and Section 6 for premixed, ready-to-use rehydration/sample buffers)
- Vortex mixer
- ReadyPrep proteomic grade water or other ultrapure water
- *RC DC*TM Protein Assay (Bio-Rad catalog #500-0121 or #500-0122)

Section 3

Storage Conditions

Store the kit at room temperature. Wash reagent 2 must be stored at -20°C for at least 1 hr prior to use. For convenience, store wash reagent 2 in a -20°C freezer.

Section 4

Instructions for Use

4.1 Protocol for Sample Volumes up to 100 μ l

Use 1.5 ml microcentrifuge tubes for processing the protein samples. Maintain the sample tubes in an ice bucket during each step of the procedure unless otherwise specified.

Always position the microcentrifuge tubes in the centrifuge in the same orientation (for example: cap hinge facing outward) so that the protein pellet remains on the same side of the tube after each spin. This will minimize loss of protein pellets during centrifugation and washing steps.

For best results, the protein concentration of the sample should be determined before beginning (see step 1). We recommend the Bio-Rad *RC DC* Protein Assay for protein quantitation because the assay is suitable for the widest variety of samples and works in the presence of detergents, reducing agents, and other agents that interfere with other protein assays.

Refer to Section 5.1. for guidelines on the quantity of sample needed with immobilized pH gradient (IPG) strips of different lengths and with different staining(detection) methods. This will help determine what the starting amount of protein should be.

1. Transfer 1–500 μg of protein in a final volume of 100 μl into a 1.5 ml microcentrifuge tube.

Note: Sample quantities > 500 μg of protein may reduce the efficiency of the cleanup leading to poor-quality IEF.

2. Add 300 μl precipitating agent 1 to the protein sample and mix well by vortexing. Incubate on ice for 15 min.

Note: When adding solution, do not touch protein sample with the pipet tip. The protein may precipitate on the tip causing sample loss.

3. Add 300 μl precipitating agent 2 to the mixture of protein and precipitating agent 1. Mix well by vortexing.

Note: When adding solution, do not touch protein sample with the pipet tip. The protein may precipitate on the tip causing sample loss.

4. Centrifuge the tube(s) at maximum speed (> 12,000 x g) for 5 min to form a tight pellet. Remove the tube promptly once the centrifuge stops so that the pellet does not disperse.
5. Without disturbing the pellet, remove and discard the supernatant using a pipet.
6. Position the tube in the centrifuge as before (i.e., cap hinge and protein pellet facing outward) and centrifuge for 15–30 sec to collect any residual liquid at the

bottom of the tube. Use a pipet to carefully remove the remaining supernatant.

7. Add 40 μ l of wash reagent 1 on top of the pellet. Position the tube in the centrifuge as before and centrifuge at maximum speed ($> 12,000 \times g$) for 5 min.

Note: A precipitate may form along the tube wall. In these cases, vortex and/or pipet wash solution over the pellet several times to ensure entire pellet is thoroughly washed.

8. With a pipet, remove and discard the wash.
9. Add 25 μ l of ReadyPrep proteomic grade water or other ultrapure water on top of the pellet. Vortex the tube 10–20 sec. Protein pellets may disperse, but will not dissolve in the water.
10. Add 1 ml of wash reagent 2 (prechilled at -20°C for at least 1 hr) and 5 μ l of wash 2 additive. Vortex the tube for 1 min.

Notes: Protein pellets will not dissolve in wash reagent 2. If wash reagent 2 is not completely chilled, quantitative recovery may be affected.

11. Incubate the tube at -20°C for 30 min. Vortex the tube for 30 sec every 10 min during the incubation period.
12. After the incubation period, centrifuge the tube at top speed for 5 min to form a tight pellet. Remove and

discard the supernatant. Centrifuge the tube briefly (15–30 sec) and remove and discard any remaining wash. The pellet will appear white at this stage. Air-dry the pellet at room temperature for no more than 5 min (the pellet will look translucent once sufficiently dry).

Note: Do not over-dry pellets. Over-dried pellets will be difficult to resuspend.

13. Resuspend each pellet by adding an appropriate volume of 2-D rehydration/sample buffer to the pellet.

Notes: Refer to Section 5.2 for recipes of typical 2-D rehydration/sample buffers or Section 6 for a list of convenient premade 2-D rehydration/sample buffers available from Bio-Rad.

Consult Section 5.1 for a guide to selecting the volume of 2-D rehydration/sample buffer to use to resuspend the protein pellet.

For SDS-PAGE, add an appropriate volume of Laemmli sample buffer to the pellet, vortex the tube until the pellet is dissolved and then heat the sample for 5 min at 95-100°C.

Vortex the tube for at least 30 sec. Incubate the tube at room temperature for 3–5 min. Vortex the tube again for ~1 min or pipet the solution up and down to fully resuspend.

Notes: With some samples (large pellets or over-dry pellets) the pellet may be difficult to resuspend. In these cases, sonication can be used to speed the process.

At the end of the process, some pellet fragments may remain undissolved. When using a urea/thiourea-based 2-D rehydration/sample buffer, all the protein is extracted from the pellet if the above protocol is followed.

14. Centrifuge the tube at maximum speed for 2–5 min at room temperature to clarify the protein sample. The supernatant can be used directly for IEF in IPG strips or in IEF gels. Store any unused or remaining protein sample in a clean tube at -80°C for later analysis.

4.2 Processing Dilute Samples or Samples Greater Than 100 μ l

The above protocol (Section 4.1) can be scaled to almost any volume of original sample. For best results with larger sample volumes, follow the additional recommendations listed below for each step.

Maintain the sample tubes in an ice bucket during each step of the procedure unless otherwise specified.

Always position the tubes in the centrifuge in the same orientation so that the protein pellet remains on the same side of the tube after each spin. This will minimize loss of protein pellets during centrifugation and washing steps.

Use only polypropylene, polyallomer, or glass tubes for processing large volumes as the wash reagent 2 interacts with many plastics. Screwcap tubes are preferred to

reduce possible spilling during vortexing and the incubation at -20°C . A Nalgene Oak Ridge-type tube made of polypropylene copolymer is an example of an appropriate tube. The volume of the tube should be at least 12 times the original volume of the sample.

Step 2. For each volume of sample use 3 volumes of precipitating agent 1.

Step 3. For each volume of sample use 3 volumes of precipitating agent 2.

Step 7. Wash the protein pellet by adding 3–4 times the pellet volume of wash reagent 1.

Step 8. Add enough ReadyPrep proteomic grade water or other ultrapure water to just cover the protein pellet.

Step 9. Add 1 ml of wash reagent 2 (prechilled at -20°C for at least 1 hr) for every 0.1 ml of original sample. Additionally, the volume of wash reagent 2 must be at least 10 times the volume of water used in Step 9. Add 5 μl of wash 2 additive (use only 5 μl of wash 2 additive regardless of the volume of sample).

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 μ l	185 μ l	300 μ l	315 μ l	410 μ l
Protein load– Silver stain	5–20 μ g	20–50 μ g	50–80 μ g	50–80 μ g	80–150 μ g
Protein load– Coomassie G-250	50–100 μ g	100–200 μ g	200–400 μ g	200–400 μ g	400–800 μ g

Sample calculation: If you precipitate 100 μ g of protein and are going to run 7 cm pH 3–10NL IPG strips (125 μ l per strip) and silver stain the 2-D gels, then you may want to solubilize the protein pellet in ~900 μ l of rehydration/sample buffer, which is enough to rehydrate about seven 7 cm IPG strips (~14 μ g/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 μ l of rehydration/sample buffer, which is enough to rehydrate one 24 cm IPG strip (100 μ g/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 2-D rehydration/sample buffer is not a component of this kit, but is necessary for resuspending the final protein pellet. It is not provided in the kit because different protein samples can require different rehydration/sample buffers. For convenience, a selection of 2-D rehydration/sample buffer formulas are provided below. The general-purpose 2-D rehydration/sample buffer is also available preformulated from Bio-Rad (ReadyPrep 2-D starter kit rehydration/sample buffer, 10 ml, catalog #163-2106).

Users of the ReadyPrep protein extraction kit (membrane I), catalog #163-2088 or the ReadyPrep protein extraction kit (cytoplasmic/nuclear), catalog #163-2089 should use the protein solubilization buffer (PSB) and PSB diluent provided with these kits to solubilize the protein pellet. PSB is a proprietary strongly chaotropic 2-D rehydration/sample buffer (refer to Section 5.2.2.2 for preparation instructions).

5.2.1. General-Purpose 2-D Rehydration/Sample Buffer for Most Samples.

(8 M Urea, 2% 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)	8 M	0.96 g
CHAPS	2% (w/v)	0.04 g
DTT (FW 154.3)	50 mM	15.4 mg
100X Bio-Lyte 3/10 ampholyte*	0.2% (w/v)	20 μ l
Bromophenol Blue	0.002% (w/v)	4 μ l of a 1% (w/v) solution
Proteomic grade water		1.25 ml

*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 Buffers for Highly Hydrophobic Proteins.

5.2.2.1. Standard Strongly Chaotropic 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 μ l
Bromophenol Blue	0.002% (w/v)	4 μ l 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 (tetradecanoylamidopropyl-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.

5.2.2.2. Protein Solubilization Buffer (PSB).

Protein solubilization buffer (PSB) and PSB diluent are provided with ReadyPrep protein extraction kit (membrane I) and ReadyPrep protein extraction kit (cytoplasmic/nuclear) 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 solubilize both hydrophilic as well as hydrophobic proteins.

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 for 10-15 sec 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 as directed in Section 5.2.2.1 to complete the preparation of the Buffer.

Section 6

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

Protein Quantitation Kits (also see bulletin 2610)

500-0121	<i>RC DC</i> Protein Assay Kit I, 500 standard assays, bovine γ -globulin standard
500-0122	<i>RC DC</i> Protein Assay Kit II, 500 standard assays, bovine serum albumin standard

Buffer Components

161-0611	Dithiothreitol (DTT), 5 g
163-2101	Tributylphosphine (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

2-D 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
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