ReadyPrep™ Reduction-Alkylation Kit

Instruction Manual

Catalog #163-2090

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

BIO-RAD

Bio-Rad Laboratories, Inc.
2000 Alfred Nobel Dr.
Hercules, CA 94547 USA
(510) 741-1000

1-800-424-6723

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Section 1
Introduction

The ReadyPrep reduction-alkylation kit offers an easy and effective way of improving spot resolution during 2-D electrophoresis. This general-purpose kit minimizes streaking and aberrant spot migration by reducing and irreversibly blocking the formation of inter- and intramolecular disulfide bonds prior to isoelectric focusing (IEF). Disulfide bond formation is particularly problematic for basic proteins because of the increased rate of their formation in an alkaline environment. Moreover, many reducing agents become negatively charged during isoelectric focusing and migrate off the IPG strip, thus allowing disulfide bonds to re-form (Görg 2000, Hoving 2002). The ReadyPrep reduction-alkylation kit eliminates the potential for disulfide bond formation during IEF by first reducing the disulfide bonds with tributylphosphine (TBP), a powerful nonionic reducing agent, and then alkylating the sulfhydryl groups with iodoacetamide (Herbert et al. 2001). This treatment produces a spot pattern with more spots, fewer streaks, and greater reproducibility. The entire process can be completed directly in the rehydration/sample buffer in <2 hr with very little hands-on time. This kit is compatible with other ReadyPrep sample preparation kits.

Section 2
Kit Specifications

The components provided in the ReadyPrep reduction-alkylation kit are listed below. Each kit contains sufficient reagent to perform a reduction-alkylation reaction on 100 samples of 300 µl each containing up to 10 mg/ml of protein.

Items Supplied With Kit

3 ampoules TBP (200 mM), 0.6 ml
3 empty glass vials for storage of TBP after opening the ampoules
5 amber vials iodoacetamide, 56 mg
1 tube alkylation buffer, 2 ml

Caution!

Care should be exercised when handling the components.

TBP should be used only in areas with good ventilation. Avoid breathing vapor. Avoid contact with skin. In case of contact with eyes, flush with water and contact a physician.

MSDS forms are available upon request.

Items Required But Not Provided:

- 1.5 ml microcentrifuge tubes
- Microcentrifuge capable of spinning at 12,000–16,000 x g
- Rehydration/sample buffer

Section 3
Storage Conditions

Store the unopened kit at room temperature. Once opened, follow the storage instructions provided on each component.

Once an ampoule of TBP has been opened, transfer the TBP to one of the empty glass vials provided and store at -20°C.

Iodoacetamide powder should be stored in the dark and is unstable once in solution. Discard unused iodoacetamide solution.

Section 4
Instructions for Use

Per 500 µl sample volume:

Note: This protocol can be scaled for smaller and larger protein sample volumes by adjusting the amounts of alkylation buffer, TBP, and iodoacetamide solution accordingly.

1. Prepare the iodoacetamide solution immediately before use by dissolving the contents of one vial in 0.6 ml of ultrapure water. Bio-Rad ReadyPrep proteomic grade water (catalog #163-2091) is recommended. If not prepared previously, open an ampoule of TBP and transfer the solution into one of the empty glass vials provided. Keep both solutions on ice.

   Note: The iodoacetamide is unstable in solution and therefore requires preparation immediately before use.

2. Prepare a protein sample in the desired rehydration/sample buffer (refer to **Section 5.1** for recommendations on buffer recipes and volumes). The reduction-alkylation reaction can be performed on a concentrated protein sample (up to 10 mg/ml) and diluted later with additional rehydration/sample buffer for loading onto IPG strips.

For best results, the protein concentration of the sample should be determined. We recommend the Bio-Rad *RC DC*™ Protein Assay for protein quatitation 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.

Note: The protein sample must be free of reducing agent (i.e., TBP, DTT, or TCEP) before the reduction-alkylation reaction is performed. This ensures the necessary molar excess of iodoacetamide to reducing agent during the reaction. Additionally, the sample must have a low salt concentration (<40 mM) after the reduction-alkylation reaction is performed to maintain low conductivity during IEF. The reduction-alkylation reaction itself contributes salt to the sample (30 mM). Therefore, it may be necessary to remove excess salt prior to IEF. (Alternatively, the reduction-alkylation reaction can be performed in a higher protein and salt concentration and subsequently diluted for IEF.) The ReadyPrep 2-D cleanup kit (catalog #163-2130) can be used to remove excess reducing agent, salt, and detergent from the sample, and to concentrate the protein in the sample, if desired.

3. Adjust the pH of the sample to between 8.0 and 9.0 by adding 15 µl of the alkylation buffer. Mix well by vortexing.

4. Add 12.5 µl of TBP for a final concentration of 5 mM. Mix well by vortexing. Incubate at room temperature for 30 min.

5. Add 15 µl of 0.5 M iodoacetamide for a final concentration of 15 mM. Mix well by vortexing. Incubate at room temperature for 1 hr.

   Note: Longer incubation times may cause spurious reactions between the iodoacetamide and the protein that could create artifacts in mass spectrometry analysis.

6. Add an additional 12.5 µl of TBP to quench any unreacted iodoacetamide. Mix well by vortexing. Incubate at room temperature for 15 min.

7. Centrifuge at 12,000–16,000 x g for 5 min at room temperature to pellet any insoluble material.

8. Dilute the protein sample to the appropriate final volume in rehydration/sample buffer and load onto the IPG strip. Refer to **Section 5.1** to determine the volumes of rehydration/sample buffer and amounts of protein needed for different strip lengths and staining methods. For further improvement of spot resolution, especially for basic strips (pH 7–10), cup loading is required. For information on cup loading, see the Cup Loading Tray instruction manual (4006216) and bulletin 2812.

Section 5
Appendix

5.1 2-D Rehydration/Sample Buffer Volume

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. When loading the protein onto the IPG strips by cup loading, refer to the Cup Loading Tray instruction manual (4006216) for detailed instructions. When loading the protein onto the IPG strips by passive/active rehyrdration, refer to the ReadyStrip™ IPG Strip instruction manual (4006166) for detailed instructions.

IPG strip length	7 cm	11 cm	17 cm	18 cm	24 cm
Rehydration volume-cup loading	135 µl	200 µl	330 µl	345 µl	450 µl
Rehydration volume–passive/ active rehyrdration	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

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 performing 2-D electrophoresis. 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.

Do not add reducing agent (i.e. TBP, DTT or TCEP) to the rehydration/sample buffer before performing the reduction-alkylation reaction. A molar excess of alkylating agent to reducing agent is required to ensure efficient alkylation.

Users of the ReadyPrep protein extraction kit (membrane I), catalog #163-2088, or the ReadyPrep protein extraction kit (cytoplasmic/nuclear), catalog #163-2089, or the ReadyPrep protein extraction kit (signal), catalog #163-2087, should use the protein solubilization buffer (PSB) and PSB diluent provided with these kits to solubilize the protein samples. 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 (no reducing agent)

(8 M urea, 2% CHAPS, 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
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		1.25 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 (no reducing agent)

(7 M urea, 2 M thiourea, 4% CHAPS, 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	2 M	0.304 g
CHAPS*	4% (w/v)	0.08 g
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.

5.2.2.2. Protein Solubilization Buffer (PSB)

PSB and PSB diluent are provided with the ReadyPrep protein extraction kit (membrane I), the ReadyPrep protein extraction kit (cytoplasmic/nuclear), and the ReadyPrep protein extraction kit (signal) to solubilize the protein sample. PSB is a proprietary, strongly chaotropic 2-D rehydration/sample buffer that will solubilize both hydrophilic and 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 Bio-Lyte ampholyte and Bromophenol Blue as directed in **Section 5.2.2.1** to complete the preparation of the buffer. **Do not add reducing agent.**

Section 6
References

Görg A et al., The current state of two-dimensional electrophoresis with immobilized pH gradients, Electrophoresis 21, 1037–1053 (2000)

Hoving S et al., Preparative two-dimensional gel electrophoresis at alkaline pH using narrow range immobilized pH gradients, Proteomics 2, 127 (2002)

Herbert B et al., Reduction and alkylation of proteins in preparation of two-dimensional map analysis: Why, when and how? Electrophoresis 22, 2046–2057 (2001)

Section 7
Product Information

Catalog #	Description
Buffer Components	
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
163-2093	100X Bio-Lyte 7/10 Ampholyte, 1 ml
163-2091	ReadyPrep Proteomic Grade Water
163-2101	Tributylphosphine (TBP), 200 mM, 0.6 ml
163-2109	Iodoacetamide, 30 g
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
163-2100	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

Cup Loading Accessories (also see Cup Loading Tray instruction manual, 4006216, and bulletin 2812)

165-4050	Cup Loading Tray, includes 1 pair moveable electrodes, 1 pack each of large and small replacement cups
165-4055	Cup Loading Tray with Forceps
165-4051	Large Replacement Cups, 1 pack (120 count)
165-4052	Small Replacement Cups, 1 pack (120 count)