ReadyPrep™ Sequential Extraction Kit

Instruction Manual

Catalog # 163-2100

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Section 1. Introduction

The ReadyPrep sequential extraction kit provides the reagents necessary to extract proteins of differing solubility from cell lysates in a form suitable for two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). It is based on the work of Molloy *et al.* [1] and Herbert *et al.* [2]. Each of the three solutions in this kit solubilizes a different set of proteins. Reagent 1 extracts only the most soluble proteins, such as cytosolic proteins. Reagent 2 is used to extract proteins of intermediate solubility, while Reagent 3 extracts proteins otherwise insoluble in Reagents 1 and 2. Some proteins partition into two or more fractions and others remain insoluble in the reagents used in this kit.

The extraction solutions can be used independently or sequentially. Sequential extraction provides a third dimension of separation based on solubility. It results in manageable 2-D PAGE protein patterns for simplified protein identification and gel matching. In addition, the high protein loads obtainable with the individual fractions enable visualization of low-abundance proteins.

The three solutions of the ReadyPrep sequential extraction kit differ in their detergent and chaotrope concentrations [3]. The insoluble residues from each extraction step are further extracted with reagents of increasingly stronger solubilizing power. The reducing agent used in the extractions, tributyl phosphine (TBP), is more efficient than the commonly used dithiothreitol, especially in 2-D PAGE applications [4, 5].

Sequential extraction can be carried out in a single microcentrifuge tube, minimizing protein loss. The kit is particularly well suited for use with 2-D PAGE systems employing immobilized pH gradients for the first-dimension IEF.

Section 2. Kit Components

Reagent 1. One vial. Lyophilized. Each vial of rehydrated Reagent 1 contains 50 ml of 40 mM Tris base.

Reagent 2. Three vials. Lyophilized. Each vial of rehydrated Reagent 2 contains 10 ml of 8 M urea, 4% (w/v) CHAPS, 40 mM Tris, and 0.2% (w/v) Bio-Lyte 3/10 ampholyte.

Reagent 3. Two vials. Lyophilized. Each vial of rehydrated Reagent 3 contains 10 ml of 5 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (w/v) SB 3-10, 40 mM Tris, and 0.2% (w/v) Bio-Lyte 3/10 ampholyte.

Reducing Agent TBP. One ampoule containing 0.6 ml of 200 mM tributyl phosphine (TBP) in 1-methyl-2-pyrrolidinone (NMP) sealed under nitrogen gas.

Empty Vial. One storage vial for reducing agent TBP.

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Note: CHAPS is 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate, a zwitterionic detergent.

SB 3-10 is N-decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, or caprylyl sulfobetaine, a zwitterionic detergent.

Bio-Lyte 3/10 is a mixture of carrier ampholytes, pH 3–10.

Section 3. Storage

Store the lyophilized contents of the kit, Reagents 1, 2, and 3, unopened at room temperature.

Store the sealed ampoule of reducing agent TBP unopened at room temperature.

Section 4. Reagent Preparation

Reagent 1: Add 50 ml of deionized water to the vial of Reagent 1. Swirl the vial gently at intervals until the contents are completely dissolved. Reconstituted Reagent 1 consists of 50 ml of 40 mM Tris base per vial.

Reagent 2: Add 6 ml of deionized water to each vial of Reagent 2. Swirl the vials gently at intervals until the contents are completely dissolved. It will take some time for the contents of the vial to dissolve. The vial will chill as the urea in the solids dissolves. Rehydrated Reagent 2 consists of 10 ml of 8 M urea, 4% (w/v) CHAPS, 40 mM Tris, and 0.2% (w/v) Bio-Lyte 3/10 per vial.

Reagent 3: Add 6.3 ml of deionized water to each vial of Reagent 3. Swirl the vials gently at intervals until the contents are completely dissolved. It will take some time for the contents of the vials to dissolve. The vial will be chilled as the urea and thiourea in the solids dissolve. The SB 3-10 detergent will not dissolve completely until the solution has been warmed slightly. It is often sufficient to roll a vial of Reagent 3 between the hands for a short period of time to dissolve the SB 3-10. In some situations, it might be

necessary to hold the vial in a bath of warm (tap) water for more efficient warming. Rehydrated Reagent 3 consists of 10 ml of 5 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (w/v) SB 3-10, 40 mM Tris, and 0.2% (w/v) Bio-Lyte 3/10 per vial.

Storage of rehydrated solution 1, 2, 3. Following rehydration, aliquot the solutions as convenient and store the aliquots at -20 °C or lower. A convenient aliquot size is 1 ml, each placed into a microcentrifuge tube for storage. Thaw the frozen aliquots prior to use and discard unused portions of each aliquot. It may be necessary to warm the solution of Reagent 3 to disperse the SB 3-10 detergent.

Reducing Agent TBP. Tributyl phosphine (TBP) has an unpleasant odor and is very volatile. Work with it in a fume hood. Wear a laboratory coat and gloves when handling the ampoule of tributyl phosphine. Change gloves often. 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 screw-top vial provided. Screw the cap of the vial down tightly and store the vial at -20 °C or lower to prevent evaporation of the TBP.

Extraction Solutions. Before carrying out an extraction, mix **10 µl** of TBP solution with every **1 ml** of **Reagent 2** and (or) **Reagent 3** to be used in the extraction (1:100 dilution). TBP is not used with Reagent 1. Use only polypropylene or glass pipettes when distributing the reducing agent TBP concentrate. Most standard pipette tips, such as those sold by Bio-Rad, are made of polypropylene. Do not use polystyrene pipettes, since they will dissolve in the NMP solvent.

Section 5. Instructions for Use

General Guidelines. The goal of the first step is to lyse the cells of interest directly in Reagent 1 using a physical lysis procedure. The method of lysis will vary greatly depending on cell type. Follow standard procedures for cell growth and tissue harvesting and standard methods for physical cell lysis. For example, bacterial cultures can be harvested by centrifugation then washed in Reagent 1 to remove excess medium. The cells can then be suspended in Reagent 1 and lysed by sonication or by liquid shearing, as in a French press. Animal tissues can be harvested and then homogenized or sonicated directly in Reagent 1. Plant tissues can be ground in liquid nitrogen and the powder suspended in Reagent 1 followed by homogenization if necessary. Tissue culture cells can be harvested by centrifugation, washed in a medium-free, isotonic buffer and repelleted. It is important to remove all of the wash buffer from the pellet prior to the addition of Reagent 1. Sonication or homogenization can be used to achieve complete lysis. For best 2-D PAGE results, it is generally worthwhile to treat the homogenates with nucleases. For this, add a mixture of DNase I and RNase A to final concentrations of 20–100 µg/ml and 5–25 µg/ml, respectively. Alternatively, nonspecific endonuclease at 150 units/ml can be used to degrade nucleic acids present in the mixture.

Use relative amounts of sample cells or tissue and lysis buffer estimated to yield about 50 mg/ml of protein solution. The amount of protein in a cell sample can be reasonably estimated as its dried weight. For example, sonication of a

suspension of 0.5 g (wet weight — equivalent to about 100 mg dry weight) of *Escherichia coli* in 2 ml of Reagent 1 results in effective lysis and fractionation.

Lysis conditions are very important to the success of a sequential extraction. Proper conditions for thorough lysis should be determined empirically. For example, overly aggressive sonication can denature some proteins, while insufficient sonication can leave some of the cells intact. Several texts and Internet sites can be consulted for details of cell culturing and growth and for methods to lyse a wide variety of cell types. For example, the ExPASy (http://www.expasy.ch) and Biobase (http://biobase.dk/cgi-bin/celis) web sites with their associated links contain much information on sample preparation. Several texts, such as References 6–9, are valuable sources of information.

Sequential Extraction. Refer to the schematic illustration for a flow chart of the sequential extraction procedure.

Extraction 1

1 Place the desired starting mass of cells or tissue in a suitable tube and add Reagent 1 in a ratio estimated to yield at least 50 mg/ml of protein upon lysis.

2 Lyse the cells by sonication or any other suitable physical means.

3 Transfer the suspension to centrifuge tubes and centrifuge the sample until firm pellets form. For example, centrifuge *E. coli* lysates at top speed in a benchtop microcentrifuge at room temperature for 10 min.

4 Recover the supernatant and determine its protein content

with the Bio-Rad protein assay (Bradford), catalog # 500-0001, or the modified assay shown below.

5 Wash the insoluble pellet from Step 3 twice with the same volume of Reagent 1 used in Step 1. It is valuable to determine the protein concentrations in the washes. Discard the washes.

6 Store the supernatant frozen until it is used in 2-D PAGE.

7 Dilute the supernatant from Step 6 to standard protein loads for 2-D PAGE (roughly 1 µg/µl) with extraction solution 2 [Reagent 2 containing a 1:100 dilution (2 mM) of reducing agent TBP].

8 Because of the ionic nature of the extracts, it is beneficial to begin the isoelectric focusing run at low voltage. For example, limit the voltage to 250 V for 1 hr then 500 V for 1 hr before beginning a ramp up to the final focusing voltage. Use paper wicks under the electrodes to capture impurities in the samples.

Extraction 2

1 Prepare extraction solution 2 by making a 1:100 dilution of reducing agent TBP (to 2 mM) into a quantity of Reagent 2. Mix 10 µl of reducing agent TBP with each 1 ml of Reagent 2 that is used.

2 Use extraction solution 2 to solubilize proteins in the pellet from Extraction 1 (Extraction 1, Step 5). Use a volume of extraction solution 2 that is about half the volume of Reagent 1 used in the first extraction. The best volume to use should be determined empirically.

3 Vortex the mixture for 5 min. For some samples, it may be necessary to also sonicate the suspension or to aspirate it through a fine-gauge needle to solubilize the protein.

4 Centrifuge the mixture to give a firm pellet and a clear supernatant.

5 Recover the supernatant and determine its protein concentration. The modified Bio-Rad protein assay procedure shown below is recommended for determining protein concentrations in extraction solution 2. Store the supernatant frozen until it is used in 2-D PAGE.

6 Wash the pellet from Step 4 twice with extraction solution 2, determine the protein concentrations in the washes and discard them.

7 Dilute the supernatant from Step 5 to standard protein loads for 2-D PAGE (roughly 1 µg/µl) with extraction solution 2.

8 Because of the ionic nature of the extracts, it is beneficial to begin the isoelectric focusing run at low voltage. For example, limit the voltage to 250 V for 1 hr then 500 V for 1 hr before beginning a ramp up to the final focusing voltage. Use paper wicks under the electrodes to capture impurities in the samples.

9 Discard unused extraction solution 2.

Extraction 3

1 Prepare extraction solution 3 by making a 1:100 dilution of reducing agent TBP (to 2 mM) into a quantity of Reagent 3. Mix 10 µl of reducing agent TBP with each 1 ml of Reagent 3 that is used.

2 Use extraction solution 3 to solubilize proteins in the pellet from Extraction 2. Use approximately the same volume of extraction solution 3 as was used of extraction solution 2. Empirically determine the best volume for the third extraction.

3 Vortex and centrifuge as described above.

4 Recover the supernatant and determine its protein content. The modified Bio-Rad protein assay procedure shown below is recommended for determining the protein content in extraction solution 3. Store the supernatant frozen until it is used in 2-D PAGE. It may be informative to wash the pellet and determine the protein concentration in the wash solution.

5 The pellet can be extracted with SDS to yield highly insoluble proteins as described in [1].

6 Dilute the supernatant from Step 4 to standard protein loads for 2-D PAGE (roughly 1 µg/µl) with extraction solution 3.

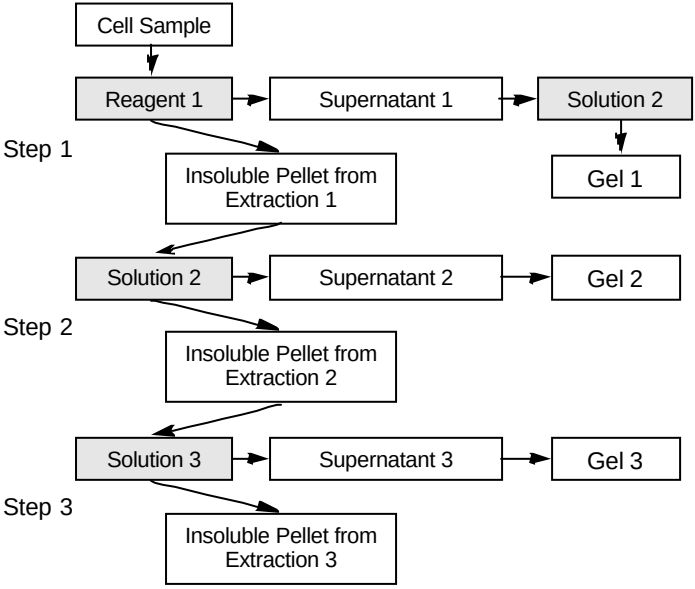
7 Because of the ionic nature of the extracts, it is beneficial to begin the isoelectric focusing run at low voltage. For example, limit the voltage to 250 V for 1 hr then 500 V for 1 hr before beginning a ramp to the final focusing voltage. Use paper wicks under the electrodes to capture impurities in the samples.

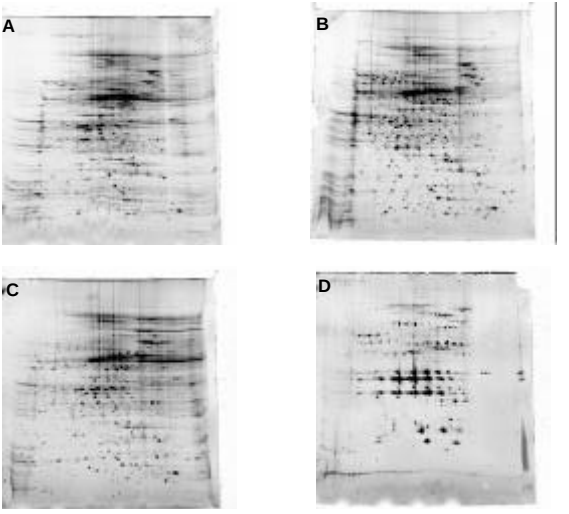
8 Discard unused extraction solution 3.

The three extracts can have different conductivity. With some isoelectric focusing chambers, best reproducibility is achieved by focusing the three extracts separately.

An example of the sequential extraction of an *E. coli* preparation is shown in the figure.

Schematic Illustration of the 3-Step Sequential Extraction Protocol





Two-dimensional gel analysis of extracts from *E. coli.*
E. coli W3110 was collected by centrifugation. The cell pellet was suspended in Reagent 1 and the cells were lysed by sonication. One portion of the sonicated cell suspension, containing 200 µg of protein, was diluted in extraction solution 3. The proteins soluble in solution 3 were separated by 2-D PAGE as a "whole cell extract" (A). Another portion of the sonicated cell suspension was subjected to the sequential extraction protocol. (B) 2-D PAGE separation of 200 µg of protein from Extract 1. (C) 2-D PAGE separation of 200 µg of protein from Extract 2. (D) 2-D PAGE separation of 200 µg of protein from Extract 3. First-dimension separation was by isoelectric focusing using ReadyStrip IPG strips pH 4 to pH 7. The second-dimension separation was by SDS-PAGE in an 8%T–16%T polyacrylamide gradient gel.

Modified Bio-Rad Protein Assay

This modification to the standard Bio-Rad protein assay procedure (catalog # 500-0001) is recommended to determine the protein content of Extracts 2 and 3 (modified from [10] and [11]). It is not necessary with Extracts 1, but it can be used with Extracts 1, the content of the samples, not the procedures. The high concentrations of detergents and chaotropes in Reagents 2 and 3 interfere with many other types of assays.

1 Prepare bovine gamma globulin (BgG) standards at about 14 mg/ml.

- Rehydrate protein standard I with 2 ml of deionized water.

  Disregard the instructions with the standard that call for rehydrating this protein with 20 ml of water. The protein concentration of standard, when rehydrated with 2 ml of water, will be 10 times the concentration shown on the bottle.

2 Prepare a 1:4 dilution of dye reagent concentrate.

- Mix 1 part of the dye reagent with 3 parts of water and filter the diluted dye through Whatman Number 1 filter paper, or equivalent.

  3.5 ml of diluted dye reagent are used in each standard and assay tube. Prepare excess dye.

3 Prepare 0.12 N HCl (nominal) by diluting concentrated HCl 1:100 with water.

- Add 10 µl of concentrated HCl to 1 ml of water.
- 80 µl are used in each assay tube. Prepare excess.

4 Prepare a standard curve covering the range of 2–280 µg.

- Dilute the BgG standard with extraction solution in a two-fold (or other) dilution series from 14 to 0.1 mg/ml. TBP can be omitted from the standard-curve dilutions.

  The standard curves generated with gamma globulin in the three extraction solutions are similar. For approximating concentrations, it is acceptable to use a single standard curve generated with the protein dissolved in only one of the extraction solutions (or in water). For more accurate determinations, prepare the dilution series in the individual extraction solutions. The high-concentration standard can be in water.

- Place 80 µl of 0.12 N HCl (nominal) in each assay tube.
- Mix 20 µl of each dilution of BgG standard with the 80 µl of 0.12 N HCl (nominal) in each separate assay tube. Mix gently.
- Add 3.5 ml of diluted dye reagent to each tube. Vortex gently.
- Measure the absorbances at 595 nm (A_{595}) after 5 minutes.
- Draw a curve of A_{595} versus the amount of protein (µg) for the dilutions of BgG.

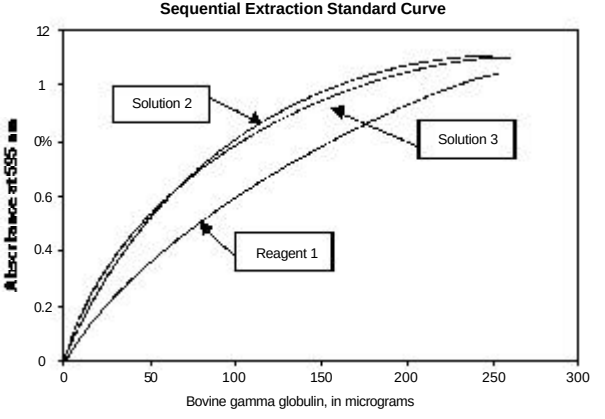
  The curve is nonlinear.

5 Assay the extracts in a manner analogous to the preparation of the standard curve.

- Place 80 µl of 0.12 N HCl (nominal) in each assay tube.
- Mix 20 µl of each extract with the 80 µl of 0.12 N HCl (nominal) in each assay tube.

Very high-concentration extracts, with protein levels above the upper limit of the standard curve, require that lesser volumes of them be assayed. In such cases, increase the amount of 0.12 N HCl (nominal) used so that the total volume is 100 µl.

- Add 3.5 ml of diluted dye reagent to each tube. Vortex gently.
- Measure the absorbances at 595 nm (A_{595}) after 5 minutes.
- Use the standard curve generated in Step 4 to estimate the concentration of the sample proteins from the A_{595} measurements.



Representative Standard Curves. Standard curves were generated as described above with serial dilutions of bovine gamma globulin in the three extraction solutions

Section 6. References

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9 Link, AJ, Ed., *2-D Proteome Analysis Protocols. Methods, Mol Biol.* **112**. (1999).

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Section 7. Product Information

Catalog #	Product Description
163-2100	**ReadyPrep Sequential Extraction Kit**
163-2101	**Reducing Agent TBP, 200 mM,** 0.6 ml
163-2102	**ReadyPrep Reagent 1,** 1 vial
163-2103	**ReadyPrep Reagent 2,** 1 vial
163-2104	**ReadyPrep Reagent 3,** 1 vial
500-0001	**Bio-Rad Protein Assay Kit I with Bovine Gamma Globulin Standard**
500-0005	**Protein Standard I, bovine gamma globulin**
500-0006	**Bio-Rad Protein Assay Dye Reagent Concentrate,** 450 ml

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

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