AE-1435 EzApply 2D Kit

1. Safety warnings and precautions

Correct operations are necessary for safe use of this product. The complete instructions should be read and fully understood before attempting to use the product.
The Procedure described in the instruction manual applies only to the use for the intended purpose. Using the product for any purpose other than the intended use or in any manner other than that described in the manual is forbidden.
User shall be liable for all safety measures needed for any use other than specified in the manual.
This kit contains acrylamide-based reagents. When using these reagents, protect yourself using rubber gloves, etc.

2. Introduction

EzApply 2D Kit is designed to extract proteins from mammalian tissues / cells and prepare samples through the reduction and alkylation of proteins, for 2- dimensional electrophoresis.

3. Package

Wash buffer 30mL・・・2 bottles
Solution 1 20mL ・・・1 bottle
Solution 2 10mL ・・・1 bottle
Solution 1-2 2mL ・・・1 bottle
Solution 2-2 2mL ・・・1 bottle
Stratified Solution 1 1mL ・・・1 bottle
Stratified Solution 2 1mL ・・・1 bottle
DTT tube for Solution 1 ・・・1 bottle
DTT tube for Solution 2 ・・・1 bottle

4. Components

Solution 1	Major components
Solution 2	Tris, Ampholine, Urea, Thoreau, Detergent, DTT
Solution 1-2	BPB, acrylamide, glycerin
Solution	BPB, acrylamide
Stratified Solution 1	Glycerin
Stratified Solution 2	Urea

5. Disposal

Follow the procedure specified at your laboratory.

6. Storage

· The kit should be stored at -20°C, avoiding direct light. Unopened reagent is stable until mentioned expiration date.
· DTT is very susceptible to oxidation. Ensure that the tube bottle containing DTT is closed tightly.
· Solution 1 and Solution 2, after mixed with DTT, should be divided into single-use aliquots (1 mL for Solution 1, 0.5 mL of Solution 2) and stored at -20°C. Use the aliquots within 2 weeks.

7. Additional equipments

Different equipments / apparatus are required depending on sample to be analyzed. The following equipments and apparatus are used for tissue or cell samples.
· Homogenizer
· Ultrasound homogenizer
· Refrigerated microcentrifuge
· Voltex mixer

8. Procedure

1. A series of operations should be carried out in a cold room or on ice. Refrigerated centrifuge should be powered on prior to use.
2. Thaw frozen Solution 1 and Solution 2 at room temperature. Urea or detergent may be crystallized during thawing. Dissolve these crystalline completely.
2. Add each 1 mL of Solution 1 and Solution 2 to the DTT tubes, respectively, to dissolve DTT. Then, return the aliquots of Solution 1 and Solution 2 containing DTT, respectively, to the bottles of Solution 1 and Solution 2. If DTT is not dissolved completely, repeat this operation.
4. Add a protease inhibitor to Solution 1 and Solution 2, respectively. The protease inhibitor should be prepared just before use.
We recommend Complete, Mini, EDTA-free (Roche), as a protease inhibitor. Dissolve one tablet in 300 μL of distilled water in a microtube, and add 30μL of the diluted protease inhibitor per 1 mL of Solution 1-2. For detailed information about the protease inhibitor, contact the manufacturer.

8. Procedure

Mammalian tissues

Cut 100 mg of tissue sample into pieces (less than 2-mm block) and transfer them to a tube.

↓

Add 1 mL of Wash Buffer. After voltex-mixing, centrifuge the tissue (4000 rpm, 5 seconds). Repeat this wash step for 3 times.

↓

Transfer the washed tissue into a suitable container for homogenization, and add 1 mL of Solution 1.

↓

Homogenize the tissue sample on ice. Homogenization conditions should be optimized. Different samples require different methods to achieve complete disruption.

↓

Centrifuge at 13000 - 17000 g for 20 - 40 minutes at 4°C. Centrifugation conditions should be optimized for samples.

↓

Transfer a 0.5 - 0.7 mL aliquot from the middle layer of the supernatant to new tube.

↓

Add a one-fifth volume (100 - 140 µL) of Solution 1-2 to the aliquot, and incubate the mixture at room temperature for 10 minutes.

↓

Apply the sample to the first dimensional agarose gel and overlay it with 10 µL of Stratified Solution 1, and then begin the first dimensional electrophoresis.

<Hydrophilic fraction>

↓

Add 0.5 mL of Solution 2 to the homogenized tissue sample.

↓

Sonicate the homogenized tissue on ice (10 - 20 times at 2-second intervals).

↓

Centrifuge at 13000 - 17000 g for 20 minutes at 4°C

↓

Transfer the supernatant to new tube and mix with a one-fifth volume of Solution 2-2. Incubate the mixture at room temperature for 10 minutes.

↓

Apply the sample to the first dimensional agarose gel and overlay it with 10 µL of Stratified Solution 2, and then begin the first dimensional electrophoresis.

<Hydrophobic fraction>

Cells / Bacterial cells

Harvest cells from culture medium / bacterial suspension by centrifugation.

↓

Suspend approximately 100 mg of cell pellet / bacterial cells in 1 mL of Wash Buffer by pipetting. Transfer the suspension to a microtube. After voltex-mixing, centrifuge at 4000 rpm for 5 seconds. Repeat this wash step for 3 times.

↓

Transfer the washed cells into a suitable container for homogenization, and add 1 mL of Solution 1.

↓

Homogenize the washed cells on ice. Homogenization conditions should be optimized. Different samples require different methods to achieve complete disruption.

↓

Centrifuge at 13000 - 17000 g for 20 - 40 minutes at 4°C. Centrifugation conditions should be optimized for samples.

↓

Transfer a 0.5 - 0.7 mL aliquot from the middle layer of the supernatant to new tube.

↓

Add a one-fifth volume (100 - 140 µL) of Solution 1-2 to the above aliquot, and incubate the mixture at room temperature for 10 minutes.

↓

Apply the sample to the first dimensional agarose gel and overlay it with 10 µL of Stratified Solution 1, and then begin the first dimensional electrophoresis.

<Hydrophilic fraction>

↓

Add 0.5 mL of Solution 2 to the homogenized cells.

↓

Sonicate the homogenized cells on ice (10 - 20 times at 2-second intervals).

↓

Centrifuge at 13000 - 17000 g for 20 minutes at 4°C

↓

Transfer the supernatant to new tube and mix with a one-fifth volume of Solution 2-2. Incubate the mixture at room temperature for 10 minutes.

↓

Apply the sample to the first dimensional agarose gel and overlay it with 10 µL of Stratified Solution 2, and then begin the first dimensional electrophoresis.

<Hydrophobic fraction>

8. Procedure

Liquid samples (*1 Serum, urine , liquid medium, etc.)

Perform desalting, delipidation, or removal of culture medium-derived proteins or albumin, as necessary.
A method for the desalting and delipidation of serum samples using trichloroacetate (TCA) is described below. For detailed information, refer to literatures.

↓

Add 0.9 mL of Solution 1 to 100 µL of serum sample.

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(*2) Add 100 µL of 100% TCA.

↓

Incubate the mixture on ice for 30 minutes.

↓

Centrifuge it at 13000 - 17000 g for 10 minutes at 4°C.

↓

Remove all the supernatant. Mix the precipitates with 1 mL of chilled acetone (-20°C)

↓

Centrifuge it at 13000 - 17000 g for 10 minutes at 4°C. Repeat twice this wash step with acetone.

↓

Remove acetone and dry up the precipitates.

↓

Add 1 mL of Solution 2 to the precipitates and sonicate them on ice (10 - 20 times at 2-second intervals).

↓

Mix the precipitates with a one-fifth volume of Solution 2-2. Incubate the mixture at room temperature for 10 minutes.

↓

Apply the sample to the first dimensional agarose gel and overlay it with 10 µL of Stratified Solution 2, and then begin the first dimensional electrophoresis.

*1 It is based on the premise that all proteins contained in the liquid sample are hydrophilic. Hydrophilic proteins cannot be separated from hydrophobic proteins.
*2 One gram of TCA is dissolved in distilled water to give a final volume of 1 mL.

Plants

Cut 20 - 40 mg of plant tissue into pieces (less than 2 mm-block) and transfer them to an appropriate container.

↓

*1 After adding 1 mL of Solution 1, homogenize the tissue. Homogenization conditions should be optimized.

↓

Centrifuge at 13000 - 17000 g for 20 - 40 minutes at 4°C. Centrifugation conditions should be optimized for the tissue. Different samples require different methods to achieve complete disruption.

↓

*2 Transfer the supernatant to new tube and mix it with a one-tenth volume of 100% TCA.

↓

Incubate the mixture on ice for 30 - 60 minutes and centrifuge it at 13000 - 17000 g for 10 minutes at 4°C.

↓

Remove all the supernatant and mix the precipitates with 1 mL of chilled acetone (-20°C).

↓

Centrifuge at 13000 - 17000 g for 10 minutes at 4°C. Repeat twice this wash step with acetone.

↓

Remove acetone and dry up the precipitates.

↓

Add a 3- to 5-fold volume of Solution 2 to the precipitates, and sonicate them on ice (10 - 20 times at 2-second intervals).

↓

Add a one-fifth volume of Solution 2-2 to the precipitates, and incubate them at room temperature for 10 minutes

↓

Apply the sample to the first dimensional agarose gel and overlay it with 10 µL of Stratified Solution 2, and then begin the first dimensional electrophoresis.

*1 Solution 1 included in this kit contains DTT, but not any polyphenol scavenger or antioxidant. Select appropriate reagents for the sample.
*2 This kit is not suitable for the fractional extraction of plant samples.

Troubleshooting Guide

Spots after the second dimension electrophoresis appear spread, not sharp.

1) Protein reduction is insufficient

DTT, once dissolved, degenerates with time and its reducing ability decreases, even when stored -20°C.

2) Protein thiol modification is insufficient

Thiol modification with acrylamide occurs at pH 8.8 - 9.3. The pH of the solution may vary depending on the properties (status) of samples.

3) Contamination by lipid or contaminants

A tissue sample may be contaminated by lipid, if washing is insufficient. For plants or other samples which are easily contaminated by contaminants, extract with Solution 1 and filter the supernatant (using a 0.45 μm filter unit, etc.)

4) Protein concentration exceeds the separation limit

Apply the specified concentration of protein sample to the first dimensional agarose gel.

5) Incomplete contact between first dimensional gel and second dimensional gel

The occurrence of a gap between first and second dimensional gels results in the diffusion of proteins. Make the first and second gels contact each other completely, taking care to avoid air bubbles being tracked between them.

No spot is detected.

1) Sample quantity (protein concentrations are too low)

When the sample produces no visible spots though the band of a molecular mass marker appears, protein amount containing in the sample may be too small. Measure protein concentration in the sample using a protein assay kit for two-dimensional electrophoresis samples, etc. When protein concentration cannot be measured, prepare a dilution series of the sample and subject the serial dilutions to SDS-PAGE, preliminarily. When a sample extracted using EzApply 2D Kit is subjected to SDS-PAGE, it should be diluted 10 times with AE-1430 EzApply (sample buffer for SDS-PAGE) and heated before SDS-PAGE.

2) Failure in first dimensional electrophoresis

Contamination by materials that interfere with isoelectric focusing electrophoresis may occur. High concentrations of lipid components, materials carrying electrical charge (except proteins) and high concentrations of salts may interfere with isoelectric focusing electrophoresis.

3) Decomposition / degeneration of proteins

Decomposition / degeneration of proteins should be prevented by preparing samples in a cold room or on ice and adding protease inhibitors. Prepared sample solutions should be stored at -80°C and used within 2 weeks, avoiding repeated freezing / thawing cycle.

TCA precipitate is hardly soluble.

1) Remaining of TCA

When TCA remains, the solubility of the precipitate may be reduced. Remove TCA completely and wash the precipitate thoroughly with acetone.

Tips for dissolution of TCA precipitate

1) Washing with acetone

After adding acetone, break the precipitate into smaller pieces using a 100-μL tip. Do not do pipetting during this, otherwise the tip would be blocked with protein pieces.

2) Change of pH

Remaining TCA causes low pH, which reduces the solubility of the precipitate. Addition of 0.5 - 1 M Tris may facilitate easy dissolution of the precipitate

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