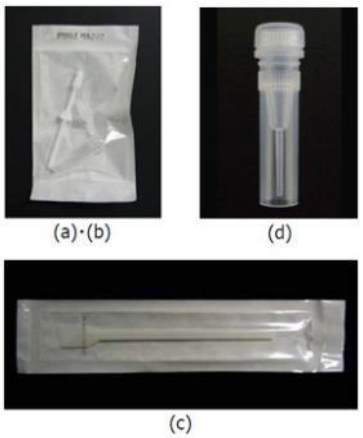
Preparation of Cell Lysate for Reverse Phase Protein Array (RPPA)

Reagents * (Storage Conditions)

- Lysis Buffer (4 °C)
- 100x Protease Inhibitor (-20 °C) **
- 100x Phosphatase Inhibitor (4 °C) **
- Bradford protein assay reagent (4 °C)
- BSA (-20 °C)



Materials *

- Microtubes for homogenization (a)
- Stirrer sticks for homogenization (b)
- Cell scraper (c)
- Microtubes for cell lysate collection (d)

* Note that the quantity of reagent and materials provided depends on the size of the experiment. To determine the amount of cell lysates to prepare, please refer to the section 2.2 below. In the service application form, please let us know the number and amount of the lysates you are going to prepare, and we will send you an appropriate amount.

** Two vials of these reagents are supplied. Please use one vial in 'A. Preparation of Cell Pellets' and the other vial in 'B. Preparation of Cell Lysate in the procedure explained in this document.

Required Reagents and Materials NOT provided

- PBS (*e.g.,* Life Technologies, 14190-144)
- Liquid nitrogen
- Dewar vessel (*e.g.,* KGW-Isotherm, 45-391-01)
- Dry ice
- Forceps

A. Preparation of Cell Pellets

For adherent cells

1. Seed cells in 6-well plates, 60 mm-diameter cell culture dishes, or 100 mm-diameter cell culture dishes and grow to a confluency of no more than 70%.
2. Treat cells according to your experimental design.
3. Dilute the 100x protease inhibitor and 100x phosphatase inhibitor 1 to 100 into PBS (1,300 µl x the number of tubes to be used in step 5. Chill the prepared PBS on ice until it is used in step 5 and 6.
4. Wash the cells once with PBS pre-warmed to 37°C after removing the cell culture medium.
5. Add 650 µL of PBS pre-warmed to 37°C into the dishes or wells. Scrape cells off using cell scrapers, and collect the cell suspension into the provided microtubes for homogenization. Unused stirrer sticks for homogenization should be stored in a clean container.
6. Add an additional 650 µL of chilled PBS prepared in step 3 and collect the remaining scraped cells into the same microtube.
7. Centrifuge the microtubes at 8,000 rpm for 2-3 minutes at 4°C.

8. After discarding the supernatant (the supernatant should be removed as completely as possible), immediately snap-freeze the cell pellets in liquid nitrogen contained in a Dewar vessel. Carefully immerse the cell pellets in liquid nitrogen while gripping them with forceps. Leave the cap exposed to air as illustrated, to avoid the intrusion of liquid nitrogen.
9. Transfer the frozen cell pellets to dry ice.
10. Transfer the tubes to a -80°C freezer. The frozen cell pellets can be stored for a month under these conditions.

For suspension cells

1. Inoculate cells into non TC-treated 6-well plates, 60mm-diameter dishes, T25 flasks or larger culture vessels.
2. Treat cells according to your experimental design.
3. Dilute the 100x protease inhibitor and 100x phosphatase inhibitor 1 to 100 into PBS (1,300 µl x the number of tubes to be used in step 4). Chill the prepared PBS on ice until it is used in step 7.
4. Collect cell suspensions into conical tubes and centrifuge at 1,200-1,500 rpm for 5 minutes.
5. After discarding the supernatant, add 5 ml of PBS pre-warmed to 37°C and resuspend the cells.
6. Centrifuge the cells again at 1,200~1,500 rpm for 5 minutes.
7. After discarding the supernatant, add 1.3 ml of chilled PBS prepared in step 3 and resuspend the cells.
8. Transfer the cell suspensions into the provided microtubes for homogenization. Unused stirrer sticks for homogenization should be stored in a clean container.
9. Centrifuge the cell suspensions in microtubes at 8,000 rpm for 2-3 minutes at 4°C.
10. After discarding the supernatant (the supernatant should be removed as completely as possible), immediately snap freeze the cell pellets in liquid nitrogen contained in a Dewar vessel. Carefully immerse the cell pellets in liquid nitrogen while gripping them with forceps. Leave the cap exposed to air as illustrated above, to avoid the intrusion of liquid nitrogen.
11. Transfer the frozen cell pellets to dry ice.
12. Transfer the tubes to a -80 °C freezer. The frozen cell pellets can be stored for a month under these conditions.

B. Preparation of Cell Lysate

1. Prepare the complete lysis buffer by diluting the 100x protease inhibitor and 100x phosphatase inhibitor 1 to 100 into lysis buffer. Chill the complete lysis buffer on ice.
2. Immediately after thawing pellets, add ice-cold complete lysis buffer into the microtubes for homogenization. Keep the mixture on ice. The following volume of lysis buffer should be added;

   For collected cells,

   1 x 106 to < 3 x 106 cells - lyse in 50 µL buffer

   3 x 106 to < 6 x 106 cells - lyse in 100 µL buffer

   6 x 106 to < 9 x 106 cells - lyse in 150 µL buffer

   Use at least 50 µL lysis buffer to homogenize cell pellets.

3. Fit the stirrer sticks against the walls of the microtubes, and grind the cell pellets into fine homogenate to prepare cell lysate.
4. Centrifuge the cell lysates at 15,000 rpm for 3 minutes at 4°C.
5. Carefully collect the supernatant into the provided microtubes for cell lysate collection. Discard the pellet.

C. Protein quantification of cell lysates

It is necessary to measure the protein concentration with the procedure specified below. The protein concentration must be more than 3 μg/μl for all cell lysates. Carna Biosciences measures protein concentration prior to RPPA analysis, and any lysates of less than 3 μg/μl will need to be prepared by the customer again before the analysis starts.

Use the Bradford protein assay reagent ("Assay Reagent") and BSA included in the kit for protein quantification.

1. Transfer deionized water and the Assay Reagent into microtubes to prepare the diluted Assay Reagent.
2. Transfer the cell lysate or BSA sample into each tube containing the diluted Assay Reagent. Mix the contents of each tube by inverting.

BSA Standard					
Conc. (μg/ml)	0	5	10	15	20
Deionized water	800μl	795μl	790μl	785μl	780μl
Assay Reagent	200μl	200μl	200μl	200μl	200μl
BSA (1.0mg/ml)	0μl	5μl	10μl	15μl	20μl

Lysate	
Deionized water	798μl
Assay Reagent	200μl
Lysate	2μl

3. Incubate at room temperature for 5 minutes.
4. Measure absorbance at 595 nm within 30 minutes to determine the protein concentration of your samples.
5. Fill in the yellow cells in "STEP 2 Protein Quantification" sheet in the application form with the absorbance values of BSA standards.
6. Fill in the orange cells in the same sheet with the absorbance values of diluted lysate samples.
7. Protein concentration values of lysate samples before dilution are automatically calculated, appear in the blue cells, and are transferred to the "STEP 3 Packing List" sheet in the form.

D. Storage and shipment of cell lysates

1. Freeze the cell lysates in microtubes at -80°C by transferring them to a deep-freezer (The frozen cell lysate can be stored for 3 months under these conditions).
2. Ship the frozen lysate on dry ice to the following address. Please send the "STEP 3 Packing List" sheet in the application form by email (info@carnabio.com) in advance or by shipping with the samples.

Shipping address: Carna Bioscience, Inc.
Dr. Y. Kawase
BMA 3F 1-5-5, Minatojima-Minamimachi
Chuo-ku, Kobe, 650-0047
Japan

This protocol was established by following the advise from Dr. Mari Masuda of National Cancer Center, Tokyo, Japan.