Experiment Protocol 401

Preparation of cell extract (Large scale method)

1. Materials

1.1 Cell

- Cultured cells(Floating cells, Adhesive cells) >10^9 cells

1.2 Reagents

- PBS(-)
- 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)
- Sodium hydroxide (NaOH)
- Potassium chloride (KCl)
- Magnesium chloride ($MgCl_2$)
- Sodium chloride (NaCl)
- Calcium chloride ($CaCl_2$)
- Ethylenediamine tetraacetic acid (EDTA)
- Glycerol (Glycerin)
- Nonidet P-40 (NP-40)
- Dithiothreitol (DTT)
- Phenyl fluoride methane sulfonyl (PMSF)

1.3 Apparatus

- High-speed cold centrifuge(HITACHI CF15RXII, BECKMAN COULTER Avanti HP301, etc.)
- Homogenizer(Kontes all glass Dounce homogenizer (B type pestle), etc.)
- Magnetic stirrer

2. Method

2.1 Preparation for reagent solutions

1) Buffer A: 10 mM HEPES-NaOH (pH7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT
2) Buffer B: 0.3 M HEPES-NaOH (pH7.9), 1.4 M KCl, 30 mM $MgCl_2$
3) Buffer C: 20 mM HEPES-NaOH (pH7.9), 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25%(v/v) glycerol, 0.5 mM DTT, 0.5 mM PMSF
4) Buffer D: 20 mM HEPES-NaOH (pH7.9), 100 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM EDTA, 10%(v/v) glycerol, 1 mM DTT, 0.2 mM PMSF

2.2 Procedures

2.2.1 Harvest cells from cell culture media

For cells in suspension:

1) Collect cells in an appropriate centrifuge tube, and centrifuge at 500 ×g for 10 min at 4 °C. and remove the supernatant.
2) Suspend cells in PBS(-) and transfer to an appropriate centrifuge tube.
3) Centrifuge at 2,000 rpm for 5 min at 4 °C and remove the supernatant.

For adherent cells:

1) Wash cells with PBS(-), once or two times.
2) Add PBS(-), collect cells by scraper, and transfer to an appropriate centrifuge tube.
3) Centrifuge at 1,000 rpm for 5 min at 15 °C and remove the supernatant.

2.2.2 Crushing of whole cells

4) Wash with five packed cell volumes(5PCV) of PBS(-).
5) Centrifuge at 2,000 rpm for 5 min at 4 °C, and remove the supernatant.
6) Suspend in 5PCV of Buffer A, and stand on ice for 10 min.
7) Centrifuge at 2,000 rpm for 5 min at 4 °C, and remove the supernatant.
8) Suspend in 2PCV of Buffer A, and lyse by 10-20 strokes of a Kontes all glass Dounce homogenizer (B type pestle).
9) Check the homogenate microscopically for cell lysis.
10) Centrifuge at 600 ×g for 10 min at 4 °C, and transfer the supernatant (cytoplasmic extract) and the pellet (nuclei) to each flesh tube, and go to those methods.

2.2.3 Separation of cytoplasmic fraction and cell membrane fraction

11) Add 0.11PCV of Buffer B to the supernatant.
12) Centrifuge at 100,000 ×g for 60 min at 4 °C, and transfer the supernatant to a flesh tube.

(Precipitation contains cell membrane fraction)

13) Dialyze against with 50 volumes of Buffer D at the collected supernatant at 4 °C.
14) Centrifuge at 100,000 ×g for 60 min at 4 °C, and transfer the supernatant to a flesh tube. (Determine protein concentration by Bradford protein assay.)
15) Freeze with liquid nitrogen and store at -80 °C. [Cytoplasmic fraction]

2.2.4 Solubilization of cell membrane fraction

16) Add the surfactant to the precipitate of Step 12) so that the final concentration is as follows, and suspend by pipetting. Furthermore leave overnight at 4 °C.
    Types of surfactants:
    a) 1 %-3 % Octylglucoside
    b) 1 % CHAPS
    c) 1 % NP-40
    d) 1 % Tween20
    e) 1 % Triton X-100
17) Octylglucoside is removed by dialysis. Other surfactants are diluted with Buffer D to a surfactant concentration of 0.1 %.
    (Determine protein concentration by Bradford protein assay.)
18) Freeze with liquid nitrogen and store at -80 °C. [Membrane fraction]

2.2.5 Preparation of nuclear extract

19) Centrifuge the pellet from Step 10) at 20,000 ×g for 20 min at 4 °C and remove the supernatant.
20) Suspend in 1PCV of Buffer C, and lyse by 10 strokes of a Kontes all glass Dounce homogenizer (B type pestle).
21) Stir gently with a magnetic stirring bar in beaker for 30 min at 4 °C.
22) Centrifuge at 20,000 ×g for 30 min at 4 °C, and transfer the supernatant to a flesh tube.
23) Dialyze against 50 volumes of Buffer D for 5h at 4 °C.
24) Centrifuge at 20,000 ×g for 30 min at 4 °C, and transfer the supernatant to a flesh tube. (Determine protein concentration by Bradford protein assay.)
25) Freeze with liquid nitrogen and store at -80 °C. [Nuclear extract]

3. Supplements

- It is desirable that the amount of cells(PCV) before fractionation in Step 4) is about 5-10 ml.
- When using a homogenizer, pull it up slowly without foaming.
- If the detergent concentration is 1 % or higher, it may interfere with the binding of the ligand to the target protein during affinity purification. Reduce the surfactant concentration to 0.1 % before use.

References: J.D.Dignam, R.M.Lebovitz, and R.G.Roeder, Nucleic Acids Res. 11, 1475 (1983)