

OptiPrep™ Application Sheet S06

Homogenization of mammalian cells

1. Introduction

Unlike an intact tissue such as rat liver, there are no definitive protocols for the homogenization of tissue culture cells that can be applied in all cases. The protocol depends crucially on whether the cells are grown as a monolayer or as a suspension culture. The former are much more easily disrupted than the latter. See ref 1 for a discussion of the methodology for homogenizing cultured cells.

The aim of the homogenization procedure must be to produce at least 90% cell breakage, reproducibly, under the mildest conditions. Methods that employ hypoosmotic media and protracted use of homogenizers should be avoided, if at all possible. In all cases the homogenization procedure must be carried out at 4°C. For monolayer cells a very satisfactory method that uses an isoosmotic homogenization medium is based on the method of Marsh et al [2].

2. Use of an isoosmotic medium

2a. Solutions required

- A. Phosphate-buffered saline (PBS)
- B. 0.25M sucrose, 10 mM triethanolamine-10 mM acetic acid, pH 7.8 (adjust to the correct pH with either triethanolamine or acetic acid, not HCl or NaOH)
- C. Solution B containing 1 mM EDTA

Keep the following stock solutions at 4°C:
100 mM triethanolamine: 1.49 g per 100 ml water.
100 mM acetic acid: 0.60 g per 100 ml water
100 mM EDTA ($\text{Na}_2 \bullet 2\text{H}_2\text{O}$): 3.72 g per 100 ml water

Solution B: Dissolve 17 g sucrose in 100 ml water; add 20 ml of triethanolamine stock; adjust to pH 7.8 with the 100 mM acetic acid and make up to 200 ml.

Solution C: As solution B, but add 2 ml of EDTA stock before adjusting pH.

2b. Protocol

1. Use a near confluent monolayer.
2. Remove the medium and rinse the monolayer at least three times with Solution A (at room temperature). Then wash the monolayer at least twice with Solution B (also at room temperature).
3. Add ice-cold Solution C to the dish (about 2 ml for a 9 cm dish) and scrape the cells into the medium with a rubber policeman. Do not try to produce a single cell suspension.
4. Transfer the crudely resuspended monolayer to a beaker on ice, washing the dish with a further 1 ml of Solution C to recover any remaining cells if necessary. Repeat the procedure for each dish.
5. If you end up with too large a volume, centrifuge the cells and resuspend the pellet in a smaller volume of Solution C. Again do not try to produce a single cell suspension.
6. Homogenize the cells using 10-25 strokes of the pestle of a tight-fitting Dounce homogenizer. Observe the suspension after 10 strokes under the phase contrast microscope. Continue homogenization until about 90% of cells have been broken.
7. The buffer is critical for the success of this method, no substitute is satisfactory.

2c. Problems

One of the major problems with cultured cells is the severity of the shearing forces required to effect efficient cell disruption. The greater the number of strokes of the pestle, the greater the possibility of causing nuclear rupture. Release of DNA, even from a few nuclei will cause severe aggregation of material: this will lead to the loss of large amounts of material into the nuclear pellet. It may therefore be advisable to add DNAase I to the homogenate to minimize this problem.

Proteins from the cytoskeleton may also form a gel-like structure and cause aggregation of subcellular components. Inclusion of 10-15 mM KCl in the homogenization medium may alleviate this.

3. Alternative hypoosmotic homogenization media

Cells, which fail to homogenize in isoosmotic media, may require hypoosmotic swelling to render them susceptible to lysis by Dounce homogenization. Generally most suspension culture cells require osmotic stress.

Osmotic stress involves exposing the cells to a hypoosmotic medium, normally at 4°C for a few minutes prior to disruption by one of the liquid-shear techniques. There are many such media: 1 mM bicarbonate or any organic buffer at approx 10 mM concentration. Divalent cations Mg^{2+} or Ca^{2+} at 1-2 mM may be added to protect the nuclei against lysis but this may also have an unwanted stabilizing effect on the plasma membrane. Sometimes sufficient osmotic stress to produce lysis can be achieved by using a reduced sucrose concentration of 0.1 M. One of the most successful strategies, adapted from ref 3 is described below.

3a. Solutions required

- A. 15mM KCl, 1.5 mM magnesium acetate, (MgOAc)
1 mM dithiothreitol (DTT), 10 mM Hepes-KOH,
pH 7.5.
- B. 375mM KCl, 22.5 mM MgOAc, 1mM DTT, 220
mM Hepes-KOH, pH 7.5
- C. Hepes-buffered saline (HBS).

Keep the following stock solutions at 4°C:
1 M Hepes (free acid): 23.8 g per 100 ml water.
100 mM Mg (OAc)₂•4H₂O: 2.15 g per 100 ml water
100 mM DTT: 1.54 g per 100 ml water
1 M KCl: 7.45 g per 100 ml water

Solution A: Into 50 ml of water mix 1.5 ml each of KCl and Mg(OAc)₂ stocks, 1 ml each of DTT and Hepes stocks; adjust to pH 7.5 with 1 M KOH and make up to 100 ml.

Solution B: Mix 37.5 ml, 22.5 ml, 1 ml and 22 ml respectively of the KCl, Mg(OAc)₂, DTT and Hepes stocks; adjust to pH 7.5 with 1 M KOH and make up to 100 ml.

Solution C: Dissolve 0.85 g NaCl in 50 ml water; add 1ml of Hepes stock; adjust to pH 7.4 with NaOH and make up to 100 ml

3b. Protocol

1. Wash the cells twice in Solution C to remove all traces of the culture medium.
 2. Suspend the cells in 10 ml of Solution A and allow them to swell on ice for 10 min.
 3. Centrifuge the cells and remove sufficient supernatant to leave a volume equivalent to 3.5x that of the cell pellet.
 4. Homogenize in a tight-fitting Dounce homogenizer and then add 1/5th of the volume of Solution B.
- ◆ The ionic composition of the medium tends to avoid any "gel" formation by cytoskeletal proteins and by homogenizing in a small volume, the organelles, which are released, are protected from hypoosmotic shock by the cytosolic proteins.

4. Other means of shear

The other principal liquid shear device, the Potter-Elvehjem homogenizer is generally less efficient than the Dounce type for cultured cells. However a third and very simple alternative for imposing a liquid shear force - repeated aspiration and ejection of a cell suspension through the narrow orifice of a syringe needle is a frequently used technique. Syringe needle gauges of 23-25G are common. Sometimes passage through a high gauge number needle is prefaced by using either a lower gauge number (larger i.d.) needle or by Dounce homogenization.

There are several commercially available devices, which can make the liquid shearing process more reproducible. In the **Cell Cracker** (ball-bearing homogenizer) the cell suspension is repeatedly passed (using two syringes) through the narrow annulus between a ball and a metal block. This is now regarded as one of the most reliable and gentle methods of homogenizing cultured cells (see ref 4). One source of the ball-bearing homogenizer is Isobiotec of Heidelberg, Germany. In the **Stansted Cell Disruptor** the cell suspension is forced, under high pressure from a piston or compressed nitrogen

through a narrow orifice. The big advantage of this device is that the shear force is applied once to the entire cell suspension rather than repeatedly as in manually-operated versions.

Nitrogen cavitation involves the exposure of a stirred cell suspension to nitrogen gas at about 800 psi (5516 kPa) at 4°C for about 15 min within a stainless-steel pressure vessel. The suspension is then forced through a needle valve by the gas pressure, at which point cell rupture occurs by a combination of the sudden expansion of gas dissolved within the cytosol and the formation of bubbles of nitrogen gas in the medium. The method is successful with all types of cell. Gas equilibration parameters (time and pressure) and solution composition need to be tested to optimize the results.

5. References

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