Cell Culture

Counting Cells in a Hemacytometer

1. Clean the chamber and the cover slip.
2. Put the cover slip above the chamber
3. Add around 10µl cells. D o not overfill
4. Place the chamber in the inverted microscope under a 10X objective. Use phase contrast to distinguish the cells.
5. Count the cells that are in the large gridded square ($1mm^2$). The gridded square is circled in the graphic. Multiply by 10^4 to estimate the number of cells per ml. Prepare the same samples and average the amount of cells that we obtain.

Freezing Cells

1. Prepare freezing medium and store at 2-8^oC until we use it. Different cell lines might need different type or amount of freezing medium.
2. For adherent cells, detach the cells from tissue culture vessel. Resuspend the cells in complete medium that fit that cell type.
3. Determine the total number of cells and percent viability using a hemacytometer、cell counter and Trypan Blue exclusion、or the Countess® Automated Cell Counter。According to the desired viable cell density、calculate the required volume of freezing medium。
4. Centrifuge the cell suspension for 5-10 minutes at 100 – 200 x g. Discard the supernatant.
5. Resuspend the pellet in cold freezing medium at the recommended viable cell density for the specific cell type.
6. Dispense the cell suspension into cryogenic storage vials. Aliquot them frequently and gently mix the cells to maintain a homogeneous cell suspension.
7. Freeze the cells in a controlled rate freezing apparatus. Decreasing the temperature 1^oC every minute. Put the cryovials with the cells in an isopropanol chamber and store at -80^oC overnight.

8. Transfer frozen cells to liquid nitrogen and store in the gas phase above the liquid nitrogen.

Sub Culturing Adherent Cells

Materials

- Culture vessels containing your adherent cells
- Tissue-culture treated flasks, plates or dishes
- Complete growth medium, pre-warmed to 37°C
- Disposable, sterile 15-mL tubes
- 37°C incubator with humidified atmosphere of 5% CO_2
- Balanced salt solution such as Dulbecco's Phosphate Buffered Saline (DPBS), containing no calcium, magnesium, or phenol red
- Dissociation reagent such as trypsin or TrypLE™ Express, without phenol red
- Reagents and equipment to determine viable and total cell counts such as Countess® Automated Cell Counter, Trypan Blue and hemacytometer, or Coulter Counter® (Beckman Coulter)

Procedures

1. Remove and discard the spent cell culture media from the culture vessel.
2. Wash cells using a balanced salt solution without calcium and magnesium (approximately 2 mL per 10 cm_2 culture surface area). Gently add wash solution to the side of the vessel opposite the attached cell layer to avoid disturbing the cell layer, and rock the vessel back and forth several times. **Note:** The wash step removes any traces of serum, calcium, and magnesium that would inhibit the action of the dissociation reagent.

3. Remove and discard the wash solution from the culture vessel.
4. Add the pre-warmed dissociation reagent such as trypsin or TrypLE™ to the side of the flask; use enough reagent to cover the cell layer (approximately 0.5 mL per 10 cm_2). Gently rock the container to get complete coverage of the cell layer.

5. Incubate the culture vessel at room temperature for approximately 2 minutes. Note that the actual incubation time varies with the cell line used.
6. Observe the cells under the microscope for detachment. If cells are less than 90% detached, increase the incubation time a few more minutes, checking for dissociation every 30 seconds. You may also tap the vessel to expedite cell detachment.
7. When ≥ 90% of the cells have detached, tilt the vessel for a minimal length of time to allow the cells to drain. Add the equivalent of 2 volumes (twice the volume used for the dissociation reagent) of pre-warmed complete growth medium. Disperse the medium by pipetting over the cell layer surface several times.
8. Transfer the cells to a 15-mL conical tube and centrifuge then at 200 × g for 5 to 10 minutes. Note that the centrifuge speed and time vary based on the cell type.
9. Resuspend the cell pellet in a minimal volume of pre-warmed complete growth medium and remove a sample for counting.
10. Determine the total number of cells and percent viability using a hemacytometer, cell counter and Trypan Blue exclusion, or the Countess® Automated Cell Counter. If necessary, add growth media to the cells to achieve the desired cell concentration and recount the cells.

Note: We recommend using the Countess® Automated Cell Counter to determine the total number of cells and percent viability. Using the same amount of sample that you currently use with the hemacytometer, the Countess® Automated Cell Counter takes less than a minute per sample for a typical cell count and is compatible with a wide variety of eukaryotic cells. See the protocol on Counting Cells with a Hemacytometer.

11. Dilute cell suspension to the seeding density recommended for the cell line, and pipet the appropriate volume into new cell culture vessels, and return the cells to the incubator.

Note: If using culture flasks, loosen the caps before placing them in the incubator to allow proper gas exchange unless you are using vented flasks with gas-permeable caps.

Thawing Frozen Cells

Materials

- Cryovial containing frozen cells
- Complete growth medium, pre-warmed to 37^{o}C
- Disposable, sterile centrifuge tubes
- Water bath at 37^{o}C
- 70% ethanol
- Tissue-culture treated flasks, plates, or dishes

Procedure

1. Remove the cryovial containing the frozen cells from liquid nitrogen storage and immediately place it into a 37^{o}C water bath.
2. Quickly thaw the cells (< 1 minute) by gently swirling the vial in the 37^{o}C water bath until there is just a small bit of ice left in the vial.
3. Transfer the vial it into a laminar flow hood. Before opening, wipe the outside of the vial with 70% ethanol.
4. Transfer the desired amount of pre-warmed complete growth medium appropriate for your cell line dropwise into the centrifuge tube containing the thawed cells.
5. Centrifuge the cell suspension at approximately 200 x g for 5-10 minutes. The actual centrifugation speed and duration varies depending on the cell type.
6. After the centrifugation, check the clarity of supernatant and visibility of a complete pellet. Aseptically decant the supernatant without disturbing the cell pellet.
7. Gently resuspend the cells in complete growth medium, and transfer them into the appropriate culture vessel and into the recommended culture environment.

Note : The appropriate flask size depends on the number of cells frozen in the cryovial and the culture environment varies based on the cell and media type.

Useful Numbers for Cell Culture

	Surface Area (cmA2)	Seeding Density	Cells at Confluency	Versene (mlof 0.05% EDTA)	Trypsin (mlof 0.0.5% tryspin 0.53 mM EDTA)	Growth Medium (ml)
Dishes						
35mm	9	0.3×10^6	1.2×10^6	1	1	2
60 mm	21	0.8×10^6	3.2×10^6	3	2	3
100 mm	55	2.2×10^6	8.8×10^6	5	3	10
150 mm	152	5.0×10^6	20.0×10^6	10	8	20
Culture Plates						
6-well	9	0.3×10^6	1.2×10^6	2	2	3-5
12-well	4	0.1×10^6	0.4×10^6	1	1	1-2
24-well	2	0.05×10^6	0.2×10^6	0.5	0.5	0.5 - 1.0
Flasks						
T-25	25	0.7×10^6	2.8×10^6	3	3	3-5
T-75	75	2.1×10^6	8.4×10^6	5	4	8-15
T-160	162	4.6×10^6	18.4×10^6	10	10	15-30