SOP: **Cell purification using Percoll step gradients**
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The method reduces abnormal cell contamination in cell populations for the purpose of improving assays of chromatin accessibility, chromatin immunoprecipitation, specific RNA levels, or other epigenetic assays. Examples of abnormal cells include nonviable cells of various types (apoptotic, necrotic, etc.), pre-apoptotic cells, and cell-cell-fusions/aneuploid cells of unusual size or density. Percoll gradient density steps and length of centrifugation time are designed so that the majority of healthy cells in a population migrate from a less dense start position to a more dense interphase position; abnormal cells either migrate above this healthy cell interphase because of their larger size and/or abnormally low density, or they migrate past the healthy interphase because they are smaller and/or higher density (see references below). The method can be applied to a variety of cell types, including suspension cells and protease-released adherent cells. Gradient behavior is somewhat cell type dependent and should be piloted before performing large-scale preparations.

Materials List

1. Accutase Enzyme Cell Detachment Medium (for attached cells; Innovative Cell Technologies, Inc., Cat# AT 104)
2. Phosphate Buffered Saline (1X PBS without calcium or magnesium) (Corning/Cellgro, Cat# 21-040-CM)
3. Percoll (GE Healthcare, Cat# 17-0891-01)
4. HEPES Buffer, 1M Solution (Corning/Cellgro, Cat# 25-060-Cl)
5. Phosphate Buffered Saline (10X PBS) (Corning/Cellgro, Cat# 46-013-CM)
6. 0.4% Trypan Blue Stain (Invitrogen/Life Technologies, Cat# T10282)
7. Biosafety Cabinet (Nuaire, Model# NU-425-600)
8. Refrigerated Benchtop Centrifuge (Eppendorf, Model# 5810R)
9. Swinging Bucket Rotor (Eppendorf, Cat# A-4-62)
10. Inverted Phase Contrast Microscope (Nikon, Model# TS-100)
11. Phase Hemocytometer (VWR, Cat# 15170-263)
12. Clear Corning Polypropylene Conical Centrifuge Tubes (15mL and 50mL)
13. Graduated Serological Pipets (2, 5, 10, 25, and 50mL)
14. ThermoScientific Matrix S1 Pipet Filler
15. Pipetman P20 (Rainin, Cat# L-20) and P20 pipet tips
16. Pipetman P200 (Rainin, Cat# L-200) and P200 pipet tips
17. Pipetman P1000 (Rainin, Cat# L-1000) and L1000 pipet tips

Procedure

Percoll Step Gradient Preparation

The Percoll step gradient is generally prepared using four steps and kept on ice; for most human cell types studied, the following steps have been successful for enriching in healthy cell populations (from the top layer down): 20% (loading density), 30%, 40%, and 50%. The cells are made as a suspension in 20% Percoll and are loaded onto the gradient as the top layer immediately before centrifugation. The following describes how to make 25mL of each step for this Percoll gradient example, which is sufficient for two preparative gradients using 12mL per

step in 50mL conical centrifuge tubes. The 30% layer can be skipped in some cases without appreciable loss of healthy cell purity; trials using 5% increments may be useful for determining preparative step densities.

Prepare a stock of "100%" Percoll:
36.0mL Percoll
4.0mL10X PBS
0.4mL 1M HEPES Buffer

Make the steps by diluting the 100% Percoll with 1X PBS as shown in the following table:

Step#	Percoll Final Concentration	1X PBS	100% Percoll	Trypan Blue
1	20%	20mL	5mL	
2	30%	17.5mL	7.5mL	30uL
3	40%	15mL	10mL	
4	50%	12.5mL	12.5mL	30uL

All solutions are freshly made and kept on ice prior to use.
Trypan Blue is added to alternate density steps to provide visual contrast for the different phases.

Gradient Examples

For small or trial purifications of 30 million or less cells, make the gradient in a clear 15mL conical centrifuge tube using ~3mL density steps.

Using a P1000 pipet, carefully layer the first 3 steps starting with highest concentration. Carefully place gradients on ice until ready to load the cell suspension (20% layer). Resuspend the 1X PBS-washed cell pellet in 20% Percoll immediately prior to loading and centrifugation (can resuspend and load cells in 2-3mL 20%).

For larger-scale purifications, make the gradient in a clear 50mL conical centrifuge tube using ~12mL density steps.

Using a 10mL plastic serological pipet and the lowest speed on the ThermoScientific Matrix S1 Pipet Filler, carefully layer the first three density steps starting with highest Percoll concentration. Carefully place gradients on ice until ready to load the cell suspension (20% layer). Resuspend the 1X PBS-washed cell pellet in 20% Percoll immediately prior to loading and centrifugation (can resuspend and load cells in 5-10mL 20%).

Density Gradient Centrifugation

1. Cultured cells should be grown under optimal conditions to limit the number of abnormal cells in the population.
2. Harvest cells using standard methods and wash once with 1X PBS.
3. Resuspend cells in cold 20% Percoll solution at ~10-15 million cells/mL.
4. Carefully layer cells onto the top layer (e.g., the 30% Percoll layer).
5. Carefully place gradient into the pre-cooled centrifuge with a swinging bucket rotor and centrifuge for 30 minutes at 2000 x g, 0 acceleration, and 0 brake (4°C).

6. Carefully remove gradient and visually inspect for the presence of cells within and between phases. The majority of healthy cells will likely be visible as a white layer at the 30-40 interface (*or at the 40-50 interface in some cell types)*.
7. The healthy cell layer is carefully removed with a 5mL plastic serological pipet and placed into a 50mL conical centrifuge tube on ice. Top layers can be removed first to make collection easier, if necessary; cells in unwanted layers can also be collected for comparisons of viability, cell size, etc.
8. Collected layers are diluted with 4-5 volumes of cold 1X PBS and thoroughly mixed by inversion before centrifugation at 500 x g for 5 minutes at 4°C.
9. After removal of the supernatant, the cells are now ready for further processing for epigenetic analysis (e.g., nuclei preparation for the DNaseI chromatin sensitivity assay, or cryopreservation for liquid nitrogen storage and subsequent assay).

References:

Kreamer BL, Staecker JL, Sawada N, Sattler GL, Hsia MT, Pitot HC. Use of a low-speed, iso-density Percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. In Vitro Cell Dev Biol. 1986 Apr; 22(4):201-211.

Roome AJ, Reading CL. Frequency of B-lymphocyte transformation by Epstein-Barr virus decreases with entry into the cell cycle. Immunology. 1987 (60):195-201.

Resnicoff M, Medrano EE, Podhajcer OL, Bravo AI, Bover L, Mordoh J. Subpopulations of MCF7 cells separated by Percoll gradient centrifugation: a model to analyze the heterogeneity of human breast cancer. Proc Natl Acad Sci USA. 1987 Oct; 84(20):7295-7299.

Patterson SD, Grossman JS, D'Andrea P, Latter GI. Reduced numatrin/B23/nucleophosmin labeling in apoptotic Jurkat T-lymphoblasts. J Biol Chem. 1995 Apr 21; 270(16):9429-9436.

Scriba A, Luciano L, Steiniger B. High-yield purification of rat monocytes by combined density gradient and immunomagnetic separation. J Immunol Methods. 1996 Feb 5; 189(2):203-216.

Chakravarti A, Rusu D, Flamand N, Borgeat P, Poubelle PE. Reprogramming of a subpopulation of human blood neutrophils by prolonged exposure to cytokines. Lab Invest. 2009 Oct; 89(10):1084-1099. doi: 10.1038/labinvest.2009.74.

Rosca AM, Burlacu A. Isolation of a mouse bone marrow population enriched in stem and progenitor cells by centrifugation on a Percoll gradient. Biotechnol Appl Biochem. 2010 Apr 12; 55(4):199-208. doi: 10.1042/BA20090356.