Creighton UNIVERSITY

Hoechst 33342 Staining for Side Populations

(Sue Brusnahan & J. Graham Sharp, UNMC; 2008)

Reagents:

Hoechst-IMDM Media (stored at 4°C)

IMDM with 2% FBS, 1mM HEPES and 1% Pen-Strep

Volume	Component
96.5 ml	IMDM (Iscove's Modified Dulbecco's Medium) (Gibco #12440-053)
2.0 ml	FBS (HyClone)
0.5 ml	200mM HEPES Buffer (diluted from 1M HEPES Buffer Stock) (Gibco #15630-080)
1.0 ml	Pen-Strep (Gibco #15140-122; 10,000 U/ml penicillin & 10,000 mg/ml streptomycin)

Hoechst 33342 dye (stored at -20°C)

Purchase from Sigma (B2261-100MG). Comes as 100 mg powder. Add 1 ml sterile H_2O, aliquot 10 μl into snap-cap tubes (100 mg/ml). Dilute with sterile water 1:100 for use (1 mg/ml). Protect from light.

Method:

1) Prepare a single cell suspension of sample.
2) Perform a WBC count on sample.
3) Allow cells to incubate overnight at 4°C in a small volume (1.0 ml) of Hoechst-IMDM media.
   (Use 1ml of Hoechst-IMDM for cell counts ranging from $1x10^6$ to $6x10^7$ cells/ml)
4) Warm Hoechst-IMDM in 37°C water bath in the morning.
5) Obtain an accurate cell count of specimen. Use this number for further processing.
6) Thaw Hoechst dye just prior to use. Keep cool until needed.
7) Using the warm Hoechst-IMDM, adjust the cell concentration to $1x10^6$ cells/ml.
8) Add the appropriate amount of dye for specimen being stained. Place in humidified incubator (37°C with 5% CO_2 in air) and incubate for period indicated. ***See table below.***
9) Return unused Hoechst dye to the freezer (can be used again).
10) After incubation, put cells on ice and analyze by flow cytometry.

Specimen Source	Specimen Status	Specimen Type	Hoechst dye concentration	Incubation time (minutes)
Human	Fresh	BM	6 μl/ml	120
		PB	7 μl/ml	60
	Frozen	BM	6 μl/ml	90
		PB	6 μl/ml	60
Mouse	Fresh	BM or PB	5 μl/ml	90

Notes:

1. Stem cells can be isolated from leukemias, lymphomas, breast cancer, etc. Time and dye concentrations must be optimized for each.
2. We have found that the side population profile differs between media vendors and media batches. For instance, CellGro IMDM will work but requires different incubation times and dye concentrations. Powdered Gibco media vs. premade Gibco IMDM have shown different profiles. Once we had variance between two lot numbers of Gibco premade medias, but only once.
3. Cells need to be incubated overnight in a minimal amount of Hoechst-IMDM. Being crowded, cold and hungry somehow affects (increases) the dye efflux.
4. Different strains of mice require different dye concentrations. Optimize for each strain.
5. HEPES is a critical reagent! You must use 200mM HEPES as the stock.
6. The Hoechst-IMDM has to be pre-warmed to 37^0C just prior to use. It cannot be left in a water bath overnight.
7. Dilution of the cells to 1.0 $x10^6$/ml is another crucial step. If there are $1.2x10^6$ cells/ml in the refrigerated sample, remove 833 µl sample and add 167 µl of the pre-warmed media. Likewise, if there are 67.4 $x10^6$ cells/ml in the refrigerated sample and you want to stain 50.0 $x10^6$ total cells, remove 742 µl and add 49.258 ml pre-warmed media.
8. The Hoechst dye can be frozen and re-thawed multiple times. If thawing, make sure to mix well prior to use. It does eventually lose its brightness; we seldom use a 1.0 ml vial more than 5 times.
9. Stained cells must be kept on ice and protected from light at all times.
10. We often run cell lines or tissues other than bone marrow or peripheral blood through a nylon mesh cell strainer (BD/Falcon #352235) prior to flow analysis.
11. To concentrate cells for high speed sorting, spin stained cells for 10 minutes at 800x g in a refrigerated centrifuge. Carefully pipette off excess supernatant to make $5.0x10^6$ cells/ml, resuspend cells gently.
12. We also transfer our stained, strained, concentrated, and cold samples into 12x75 snap cap tubes that can be directly placed on the flow cytometer. This prevents cell loss and eliminates a potential source of contamination. All tubes, except the sample being sorted, are kept in the dark on ice.
13. Stained cells can be stored for several hours (or more) if kept in the cold and dark.
14. Lastly, we have always performed box titrations of the dye and incubation times to find what works for each cell type. Start with concentrations of 4, 5, and 6 µl/ml dye for 60 and 90 minutes.