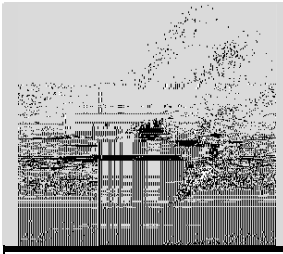

The GOODELL laboratory

Author	Diego Vieyra	February 23, 2009
Title	**Identification and isolation of SP cells from ESC cultures**	
Introduction	This protocol describes experimental conditions to identify and isolate side population (SP) cells from embryonic stem cell (ESC) cultures. The protocol is based on the Hoechst 33342 (Ho) staining method developed by Goodell et al. for bone marrow-derived hematopoietic SP stem cells (1), and has been customized for ESC cultures (2). The SP fraction of ESCs can be identified by its ability to efflux Ho in a dose and Verapamil sensitive manner (2); however, it differs phenotypically and functionally from that of adult bone marrow, as it contains cells that express antigens and molecular markers of undifferentiated ESCs, reconstitute ESC cultures and display *in vitro* and *in vivo* pluripotency (2).	
Materials	1. Healthy mESC culture 2. ESC medium: 85% DMEM-high glucose (GIBCO), 15% ES-screened FBS (Hyclone), 0.1 mM non-essential amino acids (GIBCO), 2mM glutamine (GIBCO), 1 mM sodium pyruvate (GIBCO), 0.1mM beta-mercaptoethanol (Sigma) and 1000 U/mL leukemia inhibitory factor (LIF) (Chemicon). 3. Trypsin inactivating medium (TBM): 90% DMEM-high glucose (GIBCO), 10% ES-screened FBS (Hyclone) 4. 0.05% Trypsin/EDTA (Invitrogen) 5. Hoechst 33342 (Sigma) 6. Verapamil (Sigma) 7. Propidium Iodide (Sigma)	
Protocol		*Notes*
1.	Dissociate ESCs with 0.05% Trypsin/EDTA. Stop the enzymatic reaction by adding identical volume of TBM. Gently resuspend to produce a single cell solution.	Avoid long exposure of ESCs to trypsin (usually, no more than 3-5 minutes).

2.	Centrifuge at 100Xg for 8 minutes, discard the supernatant and gently resuspend the pellet at 10^6 cells per mL in prewarmed (37°C) ESC medium containing 4 µg/ml of Ho.	
3.	Incubate at 37°C for 90 minutes.	Ensure that temperature remains constant.
4	Stop the efflux reaction by transferring the cell samples to ice for five minutes	
5	Centrifuge at 100Xg for 8 minutes at 4°C.	Ensure that temperature remains constant.
6	Gently resuspend cell pellet in ice-cold ESC medium containing 2µg/mL of Propidium Iodide. Maintain samples on ice in the dark until FACS analysis/sorting. FACS settings: Excitation at 350 nm. Emissions at 405/30 nm -Hoechst blue- and 670/40 nm –Hoechst red-.	Ensure that temperature remains constant.

References.

1. Goodell MA, K Brose, G Paradis, AS Conner and RC Mulligan. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. ***J Exp Med*** 183:1797-1806.

2. Vieyra DS, Rosen A and MA Goodell. (2009). Identification and characterization of SP cells in ESC cultures. ***Stem Cells Dev*** (2008 Dec 29. [Epub ahead of print]. PMID: 19113897).