

TS and XEN Cell Derivation

Set up matings (natural or super-ovulated) between the mice of interest.

Prepare 4-well plates of EMFIs (5×10^4 cells; 500 μ l of 1×10^5 cells/ml per well in TS medium the day before flushing. This is the same density used to culture TS cells. [TS cell lines have also been derived from blastocysts in the absence of EMFI cells, but with 70%EMFI-CM].

Replace the TS medium with TS + F4H medium (500 μ l per well) on the morning of flushing (day 1).

Flush and collect 3.5dpc blastocysts. Using sterile conditions, place one blastocyst per well in the 4-well plates containing TS + F4H medium and culture at 37°C, 5% CO₂.

The blastocysts should hatch and attach to the wells in 24 to 36 hours (day 2).

On day 3, a small outgrowth is formed from each embryo. Feed each culture with 500 μ l fresh medium.

Day 4 is the day that the outgrowth is usually disaggregated. However, this will depend on its size; it should be smaller than the size of the outgrowth disaggregated for ES cell line derivation. The ideal size for TS cell derivation is illustrated on p. 87, Figure 4b of Robertson reference above, or on p.267 Figure 26 in Hogan et al reference. Larger outgrowths will also work, but with less efficiency.

XEN cell derivation is almost identical to the protocol for TS cell derivation. Although TS colonies will be present, the **vast majority of cells** will be XEN and will frequently take over the culture. An alternate protocol to isolate XEN cells is to leave the outgrowth un-aggregated. Change the media every three days and XEN cells will appear after about 15 days. Both methods have been used successfully.

Once suitable outgrowths have been chosen, they may be disaggregated by different means. The microdrop technique described in Robertson and Hogan references may be used.

However, we perform the disaggregation directly in the wells they were cultured in. Remove the medium and wash the cells with PBS (500 ul). Aspirate the PBS, add 0.1% trypsin/EDTA (100 ul), and incubate for 5 minutes at 37°C, 5% CO₂. Using a P2 pipette or a drawn Pasteur pipette disaggregate the clump by pipetting up and down vigorously until the outgrowth is reduced to a small clump of cells. Immediately stop the trypsinization by adding 70% cond medium + 1.5x F4H (400 ul) and returning to the incubator. Change the medium 8 hours after disaggregation (500ul 70% cond medium+ 1.5x F4H).

On day 6, feed each culture (500ul 70% cond medium+ 1.5x F4H), and continue to re-feed every 2 days.

Between days 6 and 10 (highly variable) TS and XEN cell colonies will begin to appear. TS cells look like flat, epithelial sheets with a distinctive colony boundary, XEN cells more rounded and refractory. Continue to feed the cultures until TS cell colonies get sufficiently large to cover about 50% of the well. For XEN cells, wait until the dish is fairly confluent before passaging. See the attached photo of XEN (top) and TS cells (bottom).

Passage the well to a 6-well plate or 35mm dish of pre-plated EMFIs (1x10⁵ cells/ml- usually between days 15 and 20. Aspirate the medium and wash TS cells with PBS (500ul). Aspirate the PBS, add trypsin/EDTA (100 ul), and incubate for 5 minutes (TS cells) or 3 minutes (XEN cells) at 37°C/5% CO₂. Stop trypsinization by adding TS + 1.5x F4H (400ul) and pipetting up and down to get a near single-cell suspension. Transfer all the cells (TS) or half the cells (XEN) to a 6-well plate or 35mm dish of TS+1.5x F4H medium (2.5ml) on EMFIs. This first passage is crucial; this is the most likely time for the culture to differentiate.

After this, the ratio for passaging the cells can be increased to 1:3. Some differentiation will be observed at the edges of the colonies. This is normal; they are most often giant cells and other unidentified cell-types that may be between a stem cell and giant cell phenotype.

Change the medium 8 hours after passage (3ml TS+1.5x F4H).

Feed the cells every two days (3 ml TS+1.5x F4H). Follow the "Culturing TS cell lines" guidelines provided. After one or two more passages on EMFIs, TS cells may be cultured without them in the presence of 70% cond med+F4H.

Once XEN cells are established and growing well, you may stop adding FGF4 and heparin to the media. See 'XEN maintenance' protocol on Rossant lab website:

www.sickkids.ca/rossant/custom/stemCells.asp

