

## Freezing Embryonic Stem (ES) Cell Clones in 96-Well Plates

1. Refeed cells 2-4 hours before freezing. Your plate should be mostly confluent – not every well will be, of course, but overall, the plate should be confluent.
2. Aspirate off all of the media and wash 2 times with PBS.
3. Using the multi-channel pipetter, add 50  $\mu$ l of trypsin to each of the wells. Change pipette tips between wells!
4. Incubate 15' at 37° C.
5. Add 50  $\mu$ l of 2X Freezing Media (60% DMEM, 20% FCS, 20% DMSO) to each well and break up the colonies by pipetting up-and-down 5-12 times.
6. Using the multi-channel pipetter, add 100  $\mu$ l of filter-sterilized (0.22  $\mu$ m) Light Paraffin Oil to each well. This prevents degassing and evaporation during storage.
7. Replace the lid on the plate and secure by completely sealing with tape around all the edges. Place the plate in a polystyrene cuvette box, cover with a polystyrene lid that fits, secure with tape, and freeze at -80° C. Optimally, the temperature should drop about 1°C/minute. The next day, transfer the plate to your space in the -80°C freezer.
8. To retrieve ES cell clones that have been frozen by this method, take the 96-well plate from the -80° C freezer and place directly into the 37° C incubator. Allow all of the wells to thaw completely (this may take 15-25 minutes for the wells near the center of the plate), then remove the clones from the wells and transfer to appropriately labelled wells in 24-well feeder plates pre-fed with 1 ml of M-15 per well. For maximum recovery of sample, add another 200 $\mu$ l of M-15 to the well to rinse it out. It is important to vigorously pipette the thawed cells to dislodge them from the bottom of the plate (where they settle during the freezing process. There is no need to remove the DMSO and the paraffin oil until the cells have replated (24 hours after thawing). The plate can be re-taped and re-frozen.