

## Preparation of ES cells for aggregation

It is important to maintain optimal ES cell culture conditions all the time but particularly for ES cell clones used to generate chimeras. See references below for more details on ES cell culture. ES cells are grown in recommended ES cell medium at sub-confluent stage, i.e. passage is done at densities that are neither high nor low, most of the time splitting (1:3 to 1:6) every other day and used for the experiment when growing exponentially.

Three or four days prior to the aggregation thaw a vial of ES cells on MEF. If necessary, thaw the cells earlier to ensure their timely recovery and the quantity required for experiment but use cells at as low passage as possible. Change the medium the next day.

One day prior to the aggregation experiment, passage subconfluent ES cells on gelatin-coated plates. Sparser than usual passage (e.g. 1:10-1:50) one day before aggregation produces small colonies of 8-15 cells required for aggregations.

*NOTE: It is possible to grow ES cells on MEF up until the day of aggregation. In this case, a very short trypsinization at room temperature should be used to lift the ES cell colonies leaving the majority of feeders behind. Transfer floating ES cell clumps into a new dish with medium, gently pipette if necessary to reach the clumps of the right size.*

- Remove the medium and rinse the cells with PBS. Add trypsin and incubate for 5 minutes at 37° C. Resuspend the cells by gentle pipetting after adding ES cell medium. Ensure to achieve a single cell suspension.
- Spin down the cell suspension (~1000 rpm, 5 minutes), remove the supernatant. Resuspend the pellet in ES cell medium. Leave the tube undisturbed for 3-5 minutes to allow for the majority of the ES cell clumps and feeders to settle. Alternatively, place the cell suspension back into the original plate and put it in the incubator for 15-20 minutes to allow the MEF to reattach.
- Seed the ES cells from the top portion of the cell suspension into a few gelatinized plates, using different dilutions. For example, re-suspend cells from subconfluent 60 mm dish in 5 ml, using a 1ml pipette seed 0.2 ml, 0.4 ml and 0.6 ml of suspension from the top portion on 3 x 60 mm plates. Check the cell density under a microscope and adjust if necessary. Seed the rest of the cells on one or more plates, they will serve as a back up and may be re-frozen.

**On the day of the experiment,** change the medium in the morning or at least one hour before the experiment (optional but highly recommended step). Small colonies of 8-15 cells are lifted by gentle trypsinization immediately before the aggregation experiment.

- Remove the medium, rinse the cells with PBS, then with trypsin (helps to loosen up cells and minimize the amount of trypsin in the next step).
- Add a minimal amount of trypsin to just cover the cells (e.g. 0.3 - 0.5 ml per 60 mm plate), place in the incubator for 1-2 minutes or leave at room temperature.



- Watch under the microscope, gently swirl the plate to detach the colonies and tap at the microscope stage until all colonies are lifted. Do not over-trypsinize, as cells will become sticky and hard to manipulate.
- Quickly add ES cell medium to each plate (~4 ml per 60 mm dish). Do not pipette. However, if the ES cell clumps are larger than required 8-15 cells, a very gentle pipetting can be used to break cells being careful NOT to break them into single cells suspension.
- Cells are now ready for aggregation. Gently transfer the suspension of loosely connected clumps of ES cells into 5 ml tubes (e.g. Falcon 352063 or 352058) without breaking the clumps into single cells. Place the tubes inside sterile 50 ml tubes and label clearly exactly the same way as ESC clone IDs in the submitted service request. Do not place into the incubator, keep at room temperature.

### References

1. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. C. (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **90**, 8424-8428.
2. Wood, S.A., N.D. Allen, J. Rossant, A. Auerbach and A. Nagy (1993). Non-injection methods for the production of embryonic stem cell-embryo chimaeras. *Nature*, **365**, 87-89.
3. Nagy, A. and J. Rossant (1999). Production and analysis of ES-cell aggregation chimeras. In *Gene Targeting: A Practical Approach* (ed. A. L. Joyner, Second Edition), Oxford University Press Inc., New York, pp. 177-206.
4. Maise, M.P., W. Auerbach and Joyner, A.L.(1999) Production of targeted embryonic stem cell clones. In *Gene Targeting: A Practical Approach*, (ed. A.L. Joyner, Second Edition) Oxford University Press Inc, New York, pp. 101-132.
5. *Manipulating the Mouse Embryo: A Laboratory Manual*. 3<sup>rd</sup> Ed. (2003) by Nagy, A, Gertsenstein, M, Vintersten, K, Behringer, R. Cold Spring Harbor Laboratory Press. New York.

