

GENERAL CELL CULTURE GUIDELINES AND CONSUMABLES

For growing AN3-12 haploid ES cells, standard cell culture techniques apply. In this manual, we provide information in respect to:

1. MEDIA AND CONSUMABLES
2. GENERAL CELL CULTURE
3. HAPLOIDY/DIPLOIDY - **Please pay special attention to this part if your experiment requires *pure* haploid cells.**
4. HETEROZYGOUSLY TARGETED HAPLOBANK CLONES

1. MEDIA AND CONSUMABLES

ES cell medium (ESCM)

450ml	DMEM	Sigma D1152
75ml	FCS	Invitrogen
5.5ml	Penicillin-Streptomycin	Sigma P0781
5.5ml	NEAA	Sigma M7145
5.5ml	L-Glutamine	Sigma G7513
5.5ml	Sodium pyruvate	Sigma S8636
550µl	β-Mercaptoethanol	Merck 805740 (10 µl βME in 2.85 ml PBS for a 1000x stock)

ESGRO Recombinant mouse LIF Protein (Sigma ESG1107) acc. to instructions by manufacturer.

We recommend performing batch testing of FCS. This allows to select a batch which assures a rapid proliferation and a high rate of colony formation without inducing a massive differentiation of the ES cell lines.

1x Freezing medium (10% DMSO)

5ml	ESCM	
4ml	FCS	
1ml	DMSO	Sigma 41648

2x Freezing medium (20% DMSO)

4ml	FCS
1ml	DMSO

The use of following cell culture dishes is well established:

SUPPLIER	DESCRIPTION	CATALOG NUMBER
Greiner	Cell culture dish, PS, 145/20mm	639160
Nunc	100x17mm Dish, Nunclon™ Delta	150350
	Nunc 6-well Plate	140675
	Nunc 96-Well Flat/U Bottom	167008/163320

Coating of dishes, i.e. with gelatin, is not required.

2. GENERAL CELL CULTURE

AN3-12 should be fed every day with fresh ESCM and split before they become too dense and confluent. As a rule of thumb, the split ratios are 1:3 if the cells should be confluent the next day (i.e. Mon to Tue), 1:10 if they should be confluent 2-3 days later (i.e. Mon to Wed) and at a ratio of 1:30-1:40 to keep them over the weekend without having to split.

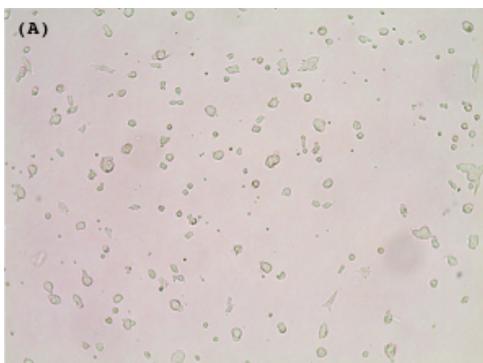
To passage cells, wash cells once with PBS, then add 1x Trypsin (e.g. Invitrogen 10x stock, 15400-054) just covering the cells and incubate for 5min at 37°C. Add ESCM in excess to inhibit trypsin and to obtain single cells by pipetting vigorously up and down for a few times. Pellet cells (310g, 5min) and resuspend the pellet in ESCM. Seed cells in fresh ESCM according to your needs.

Colony formation from single cells takes about 10 days, i.e. colonies are nicely visible by the eye and have an optimal size to be picked (i.e. to generate clones or to generate subclones). For picking colonies manually, wash the cells once with PBS, and pick colonies in 20 µl PBS under a binocular or microscope into a 96U well by sucking up and/or slightly scraping the colonies off the dish. For the dissociation of colonies into single cells, add 5 µl of 5x trypsin to the colony in 20 µl of PBS, incubate for 6min at 37°C, stop with 175 µl of ESCM and split according to your needs, e.g. 1:5 in a 96-well, or directly into a 24-well plate.

For freezing cells, trypsinize, stop with the addition of ESCM, pellet cells and resuspend the cell pellet either in ESCM and subsequently add an equal volume of 2x freezing medium, or alternatively, resuspend the cell pellet directly in 1x freezing medium. Transfer cells into cryogenic vials (e.g. CryoTube Vials, Thermo Scientific), place vials in a freezing container (e.g. Mr. Frosty Freezing Container, Nalgene, Cat.No. 5100-0001) and store at -80°C for 24hrs before transferring into liquid nitrogen for long term storage.

For reviving cells, cells should be thawed as quickly as possible, i.e. in a 37°C water bath. Transfer the cell suspension into a 15ml Falcon tube already containing some ESCM and pellet the cells. Carefully resuspend the cell pellet in fresh ESCM and plate the cells accordingly, i.e. on a 10cm dish if frozen down in a 2ml cryovial.

A typical visual representation of wildtype AN3-12 cells is shown after (A) 20h, and (B) 70h post thawing.



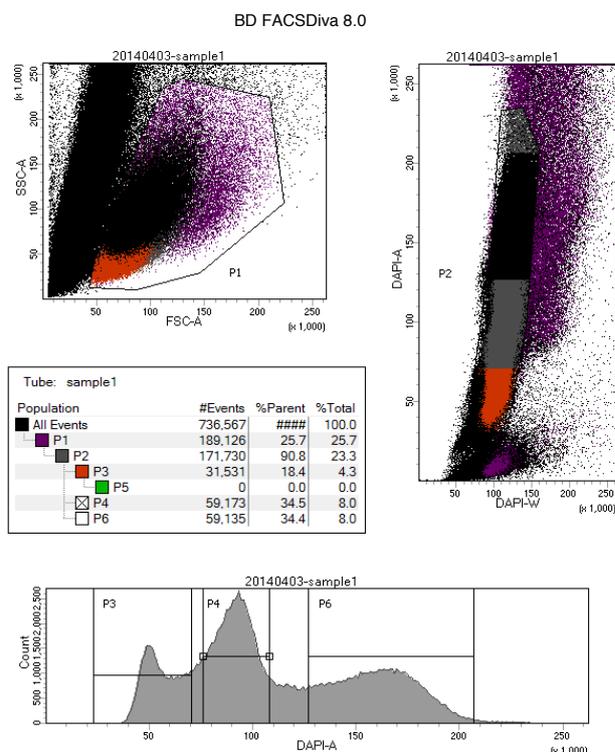
Should you experience any problems reviving wildtype cells and clones, respectively, please get in touch with office@haplobank.at.

3. HAPLOIDY/DIPLOIDY

Please keep in mind that the wildtype AN3-12 cells and HAPLOBANK clones become diploid over time. The rate for which was estimated to be about 2-3% for each day in culture (Elling et al. (2011), Cell Stem Cell, 9(6): 563-74). Consequently, should your intended experiment(s) require "pure" haploid ES cells, you need to FACS sort the cells by gating for the "1n haploid" peak.

To prepare cells growing on a 15cm dish for FACS sorting, wash the cells with PBS, trypsinize with 6ml using 1x trypsin for 5-6min at 37°C, stop the reaction with 12ml of ESCM, carefully and very thoroughly make a single cell suspension (critical step), before adding 30µl of Hoechst DNA stain solution (10mg/ml Hoechst 33342 stock, Sigma B2261) directly to the cell suspension. Immediately swirl the plate to distribute the dye. Subsequently, add another 12ml of ESCM (total volume: 30ml), mix the cells again on the plate, and incubate for 30-40min at 37°C. To avoid settling of the cells during staining, occasionally mix the cells by gently swirling the plate. After staining, collect and pellet the cells, resuspend them in 500µl of ESCM, pass them through a cell-strainer cap of a FACS tube (e.g. Falcon 352235) and keep them on ice until FACS sorting. Collect the haploid peak in ESCM (e.g. 8ml of ESCM in a 15ml collection tube) and pellet the cells prior to plating. It is advisable to exchange the ESCM after around 8h in order to increase cell viability after FACS sorting.

The wildtype AN3-12 cells we provide should give you a FACS profile as illustrated below after being thawed and cultured for 1-2 days.



Again, should you experience any problems FACS sorting wildtype cells or clones, please get in touch with office@haplobank.at.

4. HETEROZYGOUSLY TARGETED HAPLOBANK CLONES

Performing integration site PCR (please also see *Integration Site PCR Protocol* at https://www.haplobank.at/ecommerce/control/haplobank_resource) is part of the QC before a HAPLOBANK clone is handed over to the customer. This PCR allows to confirm the integration of the gene trap in its correct genomic location and to assess if the respective gene was targeted hemizygotously.

We have noticed that a minority of clones are apparently diploid and carry only a heterozygous insertion. We believe that this may be due to a homo-/hemizygous insertion being lethal to the cells. At the time of targeting, only a small fraction of cells, which were diploid and only heterozygotously targeted, survived and thus were enriched through expansion.

As HAPLOBANK comprises > 100 000 clones, targeting approx. 17 000 genes, it is impossible to screen all clones for this scenario. However, once we receive an order, we check whether we have already accumulated data on this aspect due to earlier expansion(s) and QC-checks performed. If the clone of interest is already known to be targeted heterozygotously we will let the customer know and recommend choosing another clone if available.

In case the clone has not been expanded before and upon QC it turns out that it is targeted heterozygotously, following steps may be performed by the customer:

1. FACS sort the cells and collect the 1n peak
2. Plate cells, perform integration site PCR. If there is still a heterozygotous population, please follow point 3.
3. Start drug selection (acc. to: *Elling U. et al: Forward and reverse genetics through derivation of haploid mouse embryonic stem cells. (2011), Cell Stem Cell 9(6):563-74. doi: 10.1016/j.stem.2011.10.012*)

Geneticin (2 mg/ml):	Retro-EGT Lenti-EGT Tol2-EGT
Neomycin (200 µg/ml):	Tol2-polyA

Pick colonies after 10d of selection, expand, perform integration site PCR and select colonies which show a homozygotously targeted pattern for further expansion.

If results show that the colonies remain to be targeted heterozygotously, we recommend choosing another clone if available.

If another clone is not available, the IMBA Stem Cell Core Facility (into which HAPLOBANK is embedded), can offer as a service to perform custom-tailored genetic editing in the AN3-12 cells (i.e. inducible knock-out for targeting essential genes): <https://www.imba.oeaw.ac.at/scientific-facilities/stem-cell-core-facility/>