CULTURING OF SELECTED COLONIES ON 96-WELL PLATES

1. ES cell colony culture in 96-well plates

- One day before picking of colonies: prepare 96-well plate with MEFs:
  . gelatinize 96 well plate (flat bottom).
  . resuspend 30 cm^2 MEFs in 10 ml CM + β + LIF; add 100 µl per well.
- Prepare 96-well plate (U-bottom) with 15 µl PBS per well using multichannel pipet.
- Pick series of 24-36 colonies and transfer to 96-well plate.
- Add 15 µl 2xTVP to each well and incubate 5 min at 37 °C.
- Add 70 µl medium and resuspend.
- Trypsinized colonies are then transferred (in total 100 µl) to a 96-well plate with MEFs (flat bottom) and 100µl medium.
- Culture cells overnight and refresh medium (200µl).
- Culture cells to (semi)confluency (2-3 days).
- Wash cells with PBS and trypsinize with 25 µl 10x TVP (5 min 37 °C).
- Add 175 µl complete medium and resuspend.
- Transfer two 100 µl portions of cell suspension into two 96-well plates with MEFs and 100 µl medium.
- One plate is cultured to semi-confluency (2-3-days) and processed for freezing.
- The other plate is cultured as far as possible for DNA isolation (± 5 µg DNA per well).

2. Freezing procedure

- Prepare complete medium (without LIF) with 11.43 % DMSO at 4 °C.
- Wash cells with PBS and trypsinize with 25 µl 10xTVP.
- Add 175 µl complete medium plus 11.43 % DMSO and resuspend carefully.
- Add to individual wells of Greiner 975561.
- Store in liquid nitrogen.

<u>ALT.</u>

- Leave cells resuspended in DMSO in 96-well plate.
- Wrapp plate in Kleenex tissues and place in small plastic box at -20 °C for several hours and then at -80 °C (cells can be stored for at least 6 weeks).

3. DNA isolation

- Wash cells 1x with PBS.
- Optional: cells can be stored 'dry' in 96-well at -20 °C.
- Add 50 µl lysis buffer with 100 µg/ml Proteinase K.
- Carefully seal 96-well plate and incubate minimal 2 hours at 55 °C.
- Add to lysates 100 µl ethanol 100 % and mix at RT for 60 min.
- Centrifuge plates at 4000 rpm for 30 min at RT.
- Remove supernatant bij inverting the plate.

- Add 150 μl 70% ethanol, centrifuge at 4000 rpm for 15 min at RT.
- Remove supernatant with Gilson pipet, allow to dry.
- Add 100 μl TE plus RNAase 10 μg/ml and incubate for 2 h at 55 °C.
- Use 25-35 μl DNA for Southern analysis.

LYSIS BUFFER

Tris pH 8.0	100 mM
EDTA	5 mM
SDS	0.2%
NaCl	200 mM
Proteinase K (Merck 24568.0100)	100 μg/ml (freshly added)

4. Thawing procedure

- Cut the Greiner tube with the right clone and thaw at 37 °C immediately.
- ALT.: thaw right clone in 96-well plate by adding as much as possible complete medium at 37 °C to the well and pipet up and down.
- Transfer cells to sterile eppendorf tube or 15 ml Falcon tube.
- Add medium up to 1-2 ml and centrifuge cell suspension (5 min 1200 rpm).
- Resuspend cell pellet in complete medium + β + LIF and transfer cells to 96-well or 4-well (with MEFs).

NOTE: cells are plated on the same surface as they came from.