Cell culture:

1. Media and supplements
2. Cell culture conditions
3. Serum
4. Thawing and freezing cells
5. Standard harvesting procedure
6. Mycoplasma
7. ES Culture

1. Media and supplements:

- Choose the proper cell culture medium for your cells. ATCC provides this information for each cell line.
- Wherever possible maintain separate bottles of media for each cell line.
- Add required supplements to the media (e.g. glutamax, sodium-pyruvate..)
- Prepare the amount of medium that you need for approx. one week
- Store media at 4°C, pre-warm media (the volume that you need) before working with cells
- Avoid unnecessary warm-up of trypsin

2. Cell culture conditions:

- Choose the proper cell culture conditions (temperature and CO_2)

3. Serum:

- Since serum is subject to batch-batch variation one has to compare different batches (seed defined number of cells in 6 well plate and use three different concentrations of FBS (10%, 20%, 30%). Culture two to three days and monitor cell morphology and cell proliferation.
- In particular serum is screened fort he presence of bovine viral diarrhea virus (BVDV) and mycoplasma

Optional: Heat inactivation of serum (incubation at 56°C for 30 min) can help to reduce the risk of contamination

4. Thawing and freezing cells

Thawing: Frozen cultures should be processed immediately upon arrival or taking out of the N2. Cell lines should be thawed rapidly. Place the tube in a water bath at 37°C and incubate until its contents have thawed completely. Add 1 ml of pre-warmed medium to the tube and transfer the contents to a 15ml tube in a total volume of 10 ml pre-warmed medium Spin 100xg (~700rpm) for 5 minutes. Aspirate carefully in order to avoid losing the pellet. Resuspend cell pellet in medium and transfer the cells in a cell culture dish.

Freezing: Start with the standard harvesting procedure (see below). Count the viable cell number and centrifuge the appropriate volume of cells ($2x10^6$ cell/ml freezing medium) to obtain a soft pellet (100xg for 5min). Remove the supernatant and resuspend cells gently in x ml of normal medium. Add x ml of 2 x freezing medium (dropwise). Mix gently and transfer instantly to Nalgene freezing container

Note: Protection against freezing damage is not provided by cryoprotection agents (like DMSO or glycerol) but by rather careful control of the freezing rate. Therefore, use freezing containers and store for 24h at -80°C, than transfer to the N2 tank. (Replace the isopropanol after five times use in the freezing containers)

Optional: Frozen cells can be stored in a freezer between -70 and -80°C for short periods (1 to 5 days). Frozen cultures are stable in the long term only when stored at -130°C or below. Viability of cultures stored between -80 and -130°C will vary with the nature of the cryoprotective agent and the strain and can only be determined empirically.

Freezing medium (2x): prepare always fresh

10 ml complete medium
4 ml DMSO (ultrapure)
6 ml FBS

Alternatively: High serum concentrations may help cells survive freezing. Therefore one can use 10%DMSO in FBS as freezing medium.

5. Standard harvesting procedure: Avoid cell cultures becoming fully confluent. Subculture at 80-90% confluency.

- Aspirate off the medium.
- Remove cell debri and FBS by washing the cells with PBS.
- Add trypsin/EDTA and incubate at 37°C (optimal temperature for the enzyme trypsin) and check with microscope if the cells are detached and individualized.
- Harvest cell suspension by adding 10 ml of pre-warmed medium (containing serum to inactivate the trypsin by an excess of protein) and transfer in 10 ml tube.
- Centrifuge at 100xg (~700rpm) for 5 min.
- Sub-cultivate cell suspension in a ratio that is recommended for the cell type (depending on the proliferation).

Note: Long incubation with trypsin can damage your cells.
If you culture your cells without serum you have to inactivate trypsin with inhibitors.
Optional: Pre-treatment with EDTA (before adding trypsin) can help to destroy cell-cell contacts. There are also other enzymes (e.g. dispase) available that are less aggressive.
Note: Some cells do need a matrix to attach. Therefore, coating with collagen or gelatine might be important.

6. Mycoplasma: We use Ciproflaxin to treat cells that are contaminated. For the treatment see protocol on M (Mykoplasmenbehandlung).
Routinely we check for contamination by PCR and DAPI staining.

ES Culture:

1. Serum Containing medium
   - At the moment we use 15% ES cell culture tested serum without heat inactivation. Old protocol used 5% NCS + 5% FCS. We are attempting to go back to the old culture conditions since ES cells at the moment are showing a high tendency for differentiation.
   - Prepare sufficient medium for 1 week culture. Mix individual components well before preparing medium.
   - Before use, bring the medium to room temperature for about 30 minutes, warm to 37°C for 10 minutes, and use directly. Before use, make sure that the media is mixed properly. The same requires to be done with trypsin as well.

2. Cell culture conditions / specification:

- It is very important that ES cells should be free of Mycoplasm. Use dedicated hoods and test routinely for mycoplasm contamination as mentioned above.
- It is highly recommended to culture ES cells free of antibiotics. When cells are freshly thawed, Pen/Strep (1:200) can be used. In routine culture, remove antibiotics.
- ES cells are cultivated at 37°C in a humidified incubator with 5% $CO_{2.}$
- Only use Falcon plasticware for the culture of ES cells
- Coat the dishes with 0.1% gelatine and leave on bench for a minimum of 10 minutes before seeding cells onto the dish.
- Freezing and thawing of ES cells is carried out as outlined above.
- Once cells are seeded onto the dish, swirl the dish clockwise a few times and anti-clockwise a few times in order to facilitate even distribution of cells.
- ES cells grow as tight colonies, where the form a kind of a dark cap at the top of the colony and they have shiny edges.
- ES cells should be split when they are about 80-90% confluent. Trypsinization is a very crucial step for ES cell culture.
- Add 1ml of pre-warmed trypsin / 25cm^2 flask to wash cells through the sides of the dish and wash for about 5 seconds.
- Aspirate the trypsin and add 1ml of fresh trypsin and allow to flow through the sheet of confluent cells gently.

- Place the flask at 37ºC for about 3 – 5 minutes. Check to see if the ES cells dislodge as small clumps on gentle agitation. This is the right time for trypsin-inactivation.
- Add 5 ml of serum containing medium using preferably a 10 ml pipette and pipette about 5-6 times from a distance of 2 cm and about 5 – 6 times from a distance of about 5 – 6 cm. Check under the microscope for presence of single cell suspension. It is important that ES cells are not seeded as clumps.
- Spin cells at 100xg for 2 minutes. Aspirate medium, and re-suspend the pellet in about 5 ml medium and split cells at appropriate density. Normally 1:3 and 1:6 are appropriate.