Troubleshooting in Cell Culture
Factors that Influence Cell Culture

CO₂-Incubator

Cell culturist and cell culture technique

Laminar flow hood

Plastic ware

Media

Cells

Reagents

Flasks/Dishes
Bacterial Contamination

- Turbid and cloudy media
- Media turning yellow (pH shift)
- Microscopically: moving black dots or rods
- Size approx. 1 µm in diameter
- Source: cell culture scientist/water bath

Culture can no longer be maintained → DISCARD!
Fungal Contamination

- Relatively slowly growing
- pH shift in media
- Source: airborne
- Microscopic detection possible
  - Hyphae (5-10 µm Ø): main mode of vegetative growth
  - Branching hyphae
  - Spore formation
- Macroscopic: mycelium

Don‘t open  ➔ DISCARD!
Yeast contamination

- Cloudiness of the media
- Oval organisms, budding yeast cells and chains
- Approx. 3-5 µm in diameter
- Source: airborne

→ Treatment with antimycotics possible (e.g. Amphotericin B or Nystatin)
Mycoplasma Contamination

- First discovered in 1898 and classified as viruses
- Smallest self-replicating prokaryotes known
  - 0.3-0.8 µm in diameter → pass cellulose and polyvinyl filters with 0.2 µm pore width (due to the ability to deform!)
  - Lack a peptidoglycan cell wall → resistant to penicillin
  - Presence generally not obvious in cultures
    (neither macro-, nor [light-]microscopically)
- Occurring extracellularly, only rarely intracellularly
Consequences of Mycoplasma Contamination

- Negative effects on growth rates
  - Inhibition of cell proliferation up to 50% by nutrient withdrawal and secretion of harmful metabolic products (McGarrity et al., 1984)
    - Fast glucose reduction and formation of acids: pH shift
    - Arginine depletion: inhibits protein biosynthesis and cell growth
- Cause chromosomal aberrations and multiple translocations (McGarrity et al., 1978)
- Significant changes in microarray and gene expression profiles
- Can constitute up to 50% of total protein and 15-30% of isolated DNA

They influence almost all functions of the host cell metabolism
Perform tests regularly for the presence of mycoplasma
Mycoplasma Detection

- **DNA fluorescence stains**
  *e.g.* Bisbenzimide, DAPI

- **PCR technique**

  Specific primers have been designed from DNA sequences coding for the highly conserved 16S ribosomal RNAs. These sequences are very similar in the various *Mycoplasma spec.* and will not detect bacterial or animal DNA.
Tests necessary for:

- New cell and virus cultures
- Monthly for continuous cell lines; weekly in cases of laboratory contamination
- Prior to every liquid nitrogen storage
- Upon modification of cell characteristics
- In case of problems with reproducibility of results
- Editors of scientific journals are aware of the significance of mycoplasma contamination!
Handling of Contaminated Cultures/Virus Stocks

- Isolate the culture immediately (separate incubator) and test corresponding cryopreserved cell samples
- Inform all sorts of recipients
- Perform standard disinfection of the laboratory (laminar flow, incubator, water bath, centrifuge, ...)
- Initiate immediate treatment of irreplaceable, valuable cells
- Autoclave/discard cells that can be replaced
Mycoplasma Elimination

- **Antibiotics:**
  - Fluoroquinolone derivatives
    - Ciprofloxacin (*e.g.* BIOMYC 3 from PromoKine)
    - Mycoplasma Removal Agent (MRA)
  - Tetracyclines
    - Tiamulin and Minocyclin (*e.g.* BIOMYC-1 and 2 from PromoKine)

- **Special reagents:**
  - Reagents that effectively kill mycoplasma
    - *e.g.* Mycoplasma-EX from PromoKine; non-antibiotic/antibiotic combination
Prophylactic use of antibiotics may cause lab workers to neglect sterile handling of cell cultures leading to poor aseptic conditions.

Antibiotics are only stable for a certain time at 37°C (thermolabile; Pen/Strep: 3 days, Genta/Ampho: 5 days).

Only use antibiotics if absolutely needed:
- Isolation of primary cells
- Very valuable and irreplaceable cell lines
- Inducible promoter system

Whenever possible: Operate free of standard antibiotics.
Side effects:

- Toxic and anti-proliferative effects
- Negative effects on the differentiation of several cell types (e.g. blood progenitor cells)
- Reduced proliferation rates of primary cells (up to 40 %)
  [Review article: *The prophylactic use of antibiotics in cell culture*, Ingrid Kuhlmann, 1996]
- Influences on gene/protein expression
- May lead to antibiotic resistances
Good Cell Culture Practice (GCCP)

Before work is set out:

- Disinfect the work bench in laminar flow cabinet
- Disinfect hands / gloves
- Put all material and equipment (media bottles, pipette tip boxes, pipette aids) into the cabinet prior to the start of your work and disinfect everything prior to use with 70% ethanol
- Check whether your cells are free of any contamination
Good Cell Culture Practice (GCCP)

Whilst working:

- Do not work above opened flasks or culture plates
- Movements within the cabinet must not be rapid
  - Will impede the airflow to circulate properly
- Do not contaminate gloves by touching anything outside the cabinet (especially face and hair)
  - If gloves become contaminated re-disinfect before proceeding
- Do not block the airflow by crowding the hood with equipment that you will not need/use
- Wipe away any liquids that have potentially been spilled out
- Speaking, sneezing and coughing must be directed away from the cabinet to not to disrupt the airflow/introduce contamination
Good Cell Culture Practice (GCCP)

After the work is completed:

- Disinfect all equipment and material before removing from the cabinet
- Disinfect work bench in laminar flow cabinet
- Periodically clean cabinet surfaces with a disinfectant according to the manufacturers’ instructions
- Periodic maintenance of laminar flow hood (+ “expiry“ of UV lamp) by manufacturer’s service is important
Thawing – General Considerations

- **Aim**: Maintain as many healthy and well proliferating cells as possible

- **However**: The thawing procedure is stressful for cells
  - Freezing media contain DMSO as cryo-protectant
  - Liquid DMSO (during the process of thawing) is toxic for cells:
  Final concentration of DMSO should not exceed 1%
  (can be tolerated for 16-24 h)

- **Therefore**:
  - Thaw cells as fast as possible at 37°C
  - Make sure to have everything prepared before taking cells out of the liquid nitrogen tank
  - Dilute liquid DMSO as fast as possible
Thawing of Cells – Protocol

- Prepare a cell culture flask with GM
- Prewarm in a CO₂ incubator for approx. 30 min
- Take cryo vial out of the liquid nitrogen (transport on dry ice or in liquid nitrogen), open/close cap
- Thaw cells for 2 min at 37°C in the water bath
- Disinfect the vial and transfer content immediately into prewarmed medium
- Incubate in a CO₂ incubator (37°C, 5% CO₂) for approx. 16-24 h (Do NOT spin them down)
Parameters:

- Temperature
- CO₂ concentration
- Relative humidity

→ Maintains „physiological“ conditions for cells

However:

- Optimal conditions for yeast, fungi and bacteria (→ keep clean!)
- Frequent opening of the door leads to variations in temperature, CO₂ (→ pH changes) and humidity (→ evaporation of H₂O from the medium)
Frequency of media change and subcultivation has to be adapted to the growth rate of the respective cell type.

In general, media change for primary cell culture has to be performed every 2 - 4 days:
- **Monday – Wednesday – Friday** (fast growing cultures)
- **Monday – Thursday – Monday** (slower growing cell cultures)

Too frequent as well as too few media changes have negative effects:
- Growth arrest
- Cell death
Serum (FCS, FBS) is the most undefined component in classical media formulations!

→ Extreme lot-to-lot variations (growth factors, hormones, proteins, enzymes, salts, glucose, vitamins, trace elements, endotoxin, pH)

Recommendations:

- Perform pre-testing of different lots and buy or reserve a lot that is working well in your experiments (1-3 years)
- If possible, use serum-reduced or serum-free media (standardization of cell culture conditions!)
- If not required, don't use "heat-inactivated" serum*

*Heating serum (30 min, 56°C) is intended to inactivate the complement but also reduces the concentration of growth factors and other proteins.
Subculture of Adherent Cells – Protocol

- Prewarm detachment reagents
- Aspirate growth media
- Wash cell monolayer with PBS w/o Ca^{++} and Mg^{++} or Hank’s BSS or HepesBSS at room temperature
- Aspirate washing solution
- Add Trypsin/EDTA (0.04%/0.03%)
- Watch detachment under microscope (cells are getting round in shape)
  → Concentration and incubation time are dependent on cell type and confluence of cells
Subculture of Adherent Cells – Protocol

- Stop trypsination by trypsin neutralisation solution or media with 10% FCS
- Resuspend cells by pipetting up and down, transfer suspension to a centrifuge tube
- Spin down at 220 x g for 3-5 min
- Remove clear supernatant and resuspend cell pellet in growth media
- Determine cell number and seed cells at recommended seeding density into a new cell culture flask (or apply split ratio)
- Incubate in a CO₂ incubator at 37°C
PromoCell do not determine the number of passages but instead calculates the **Population Doublings** (PD) that can be performed with the cells.

The term **Passage** only describes the process of detachment and replating and does not tell how **old** a cell culture population is as subcultivation can be performed:
- with different split ratios/dilutions
- at different confluence stages
Freezing of Primary Cells

To get as healthy as possible cells after resuscitating them

- Freeze them down at early passages / PDs
- Freeze them gradually (1°C per minute), e.g. using „Mr. Frosty“
- 0.5 to about 4 million cells per vial (1 ml)
- Long-term storage in liquid nitrogen (or gas phase)
- If possible, use serum-free freezing medium
- Only cells that are free of any kind of contamination
- Don’t forget to label the vial properly
In Addition and in Summary

Factors that influence the reproducibility of your experiments:

- Microbial contaminations
- Thawing of cells/re-freezing
- Cell density
- Changes in media composition (e.g. addition of serum)
- Ageing of the cell culture
- Problems with parameters of the incubator
Checklist:

- New protocol/equipment/scientist?
- FCS added? New lot? Tested? Concentration?
- Plates/vessels: New plastic ware? Coating?
- Contamination?
- Incubator?
- Thawing: Modification of protocol?
- Cells: Age? Morphology?
Why Documentation?

Documentation of:
- performed manipulations
- used reagents and cells

will facilitate to discover possible reasons when experiencing problems in cell culture
Thank you for your attention!