Mammalian Cell Culture Maintenance Protocol

Complete Guide to Cell Culture, Passaging, and Cryopreservation

Overview

This protocol covers standard procedures for maintaining mammalian cells in culture, including routine maintenance, media changes, passaging/subculturing, and cryopreservation techniques for both adherent and suspension cells.

SAFETY

Always wear appropriate PPE: lab coat, gloves, and eye protection. Work with human or primate cell lines in BSL-2 facility. Dispose of biological waste in appropriate biohazard containers. Handle liquid nitrogen with cryogenic gloves and face protection. DMSO can facilitate absorption of toxic compounds - use with care.

Routine Maintenance

- Daily monitoring
- Media changes every 2-3 days
- Maintain log phase growth

Passaging

- Passage at 80-90% confluence
- Trypsin or gentle dissociation
- Split ratios: 1:3 to 1:10

Cryopreservation

- Freeze slowly (-1°C/min)
- Thaw rapidly (37°C bath)
- Store in liquid nitrogen

Incubation Conditions

Parameter	Standard Conditions	Purpose
Temperature	37°C	Physiological temperature
CO_2	5%	Maintains pH 7.2-7.4
Humidity	95%	Prevents media evaporation

Required Materials

Equipment

- Biological safety cabinet (BSC) / Laminar flow hood
- CO_2 incubator (37°C, 5% CO_2)
- Inverted microscope
- Water bath (37°C)
- Centrifuge
- Hemocytometer or automated cell counter
- Pipettes (serological and micropipettes)
- Controlled-rate freezing container (CoolCell, Mr. Frosty)
- -80°C freezer
- Liquid nitrogen storage tank

Reagents & Consumables

- Cell culture medium (appropriate for cell line)
- Fetal bovine serum (FBS)
- Trypsin-EDTA (0.25% or 0.05%)
- Gentle cell dissociation reagent (Accutase, TrypLE, Versene)
- PBS without Ca^{2+}/Mg^{2+}
- DMSO (dimethyl sulfoxide)
- Culture vessels (flasks, dishes, plates)
- Cryovials (1-2 mL, sterile)
- 70% ethanol for surface disinfection
- 0.4% Trypan blue (for viability)

Culture Vessel Volumes

Vessel	Medium Volume
T25 flask	5-7 mL
T75 flask	12-15 mL
T150 flask	25-35 mL
T175 flask	25-30 mL
T525 flask	75-100 mL
T875 flask	125-175 mL
6-well plate (per well)	2-3 mL
10 cm dish	10-12 mL

Freezing Medium Options

- **Option 1:** 90% FBS + 10% DMSO
- **Option 2:** 50% medium + 40% FBS + 10% DMSO
- **Option 3 (Serum-free):** 90% complete medium + 10% DMSO
- **Option 4:** Commercial freezing medium (CryoStor, etc.)

Routine Cell Culture Maintenance

Daily Monitoring

Mark Done

Check medium color (phenol red indicator):

- **Red/pink** = alkaline pH (too high, poor cell growth)
- **Orange/salmon** = optimal pH 7.2-7.4 ✓
- **Yellow** = acidic pH (cells overgrown or contaminated)

Examine cell morphology under microscope:

- **Adherent cells:** Consistent attachment, characteristic shapes

- **Suspension cells:** Evenly dispersed, round, refractile
- **Healthy cells:** Bright and refractile
- **Dead cells:** Dark and granular

Check for contamination:

- Bacterial: Medium becomes cloudy/turbid
- Fungal: Visible mycelia or spores under microscope
- Mycoplasma: No visible signs (requires specific testing)

Media Change (Adherent Cells)

Mark Done

Frequency: Every 2-3 days or when media color changes

1. Pre-warm fresh medium to 37°C in water bath
2. Work in biosafety cabinet using aseptic technique
3. Aspirate spent medium from culture vessel
4. (Optional) Gently wash cells with PBS to remove metabolic waste
5. Add fresh pre-warmed medium (see volumes in Materials section)
6. Gently rock flask to distribute medium evenly
7. Return flask to incubator
8. Label flask with date of media change

IMPORTANT:

- Never reuse pipettes between flasks (prevents cross-contamination)
- Avoid excessive agitation when handling flasks
- Do not overfill vessels (prevents spillage and contamination)

When to Passage | Trypsinization | Gentle Dissociation | Suspension Cells | Cell Counting

When to Passage Cells

Adherent Cells

- Passage when cells reach **80-90% confluence**
- Before cells become contact-inhibited
- While cells are in logarithmic (log) phase of growth
- Viability should be **>90%** at time of subculturing
- DO NOT allow cells to become overconfluent

Suspension Cells

- Passage when cells reach optimal density for the cell line
- Typically **0.5-2 × 10^6 cells/mL**
- Before cells enter stationary phase

Passaging Frequency

Cell Type	Frequency
Most mammalian cell lines	Every 2-4 days
Fast-growing lines	Every 1-2 days
Slow-growing lines	Every 5-7 days

⚠️ Do NOT passage adherent cells more than once every 48 hours

Passaging Adherent Cells (Trypsinization)

> **Materials: Trypsin-EDTA, PBS (no Ca^{2+}/Mg^{2+}), complete medium with serum**

1. **Examine cultures**
   - View cells under inverted microscope
   - Confirm 80-90% confluence
   - Check for contamination, ensure viability >90%
2. **Prepare reagents**
   - Pre-warm trypsin-EDTA to room temperature or 37°C
   - Pre-warm complete medium to 37°C
   - Label new culture vessel(s)
3. **Remove medium**: Aspirate spent medium, tilt flask to remove all traces

4. **Wash cells**: Add PBS (2 mL per 10 cm^2), gently rock flask, aspirate completely

PBS volumes: T25 (3-5 mL) | T75 (5-10 mL) | T175 (10-15 mL)

5. **Add trypsin**: Add minimal volume (1 mL per 25 cm^2), rotate to cover monolayer, decant excess

Trypsin volumes: T25 (1 mL) | T75 (2-3 mL) | T175 (5 mL)

6. **Incubate**: Return to 37°C incubator for 2-10 minutes (depends on cell line), tap gently after 5 min

7. **Check detachment**: Examine under microscope - cells should appear rounded and floating
   - If cells remain attached, return to incubator for 1-2 more minutes
   - Avoid over-trypsinization (damages cells)

8. **Neutralize trypsin**: Add complete medium with serum (≥ trypsin volume), pipette gently to create single-cell suspension

9. **Seed new culture**: Dilute cells into new flask with fresh medium

Standard Split Ratios:

- **1:2** - slow-growing cells, want high density
- **1:3 to 1:5** - routine maintenance (most common)
- **1:6 to 1:10** - fast-growing cells, want low density

10. **Incubate**: Gently rock flask, place in 37°C incubator, allow cells to attach 4-24 hours

11. **Record keeping**: Label with cell line, passage number (e.g., P15), date, initials

Gentle Cell Dissociation (Sensitive Cells)

When to use gentle dissociation:

- Cells sensitive to trypsin
- Weakly adherent cells
- Stem cells, primary cells, neurons
- Serum-free cultures

Common Gentle Dissociation Reagents:

- **Accutase** - proteolytic and collagenolytic enzyme blend
- **TrypLE** - recombinant trypsin alternative
- **Versene (EDTA)** - chelates Ca^{2+}/Mg^{2+}, very gentle

- **Cell Dissociation Buffer** - enzyme-free, very gentle
- **Dispase** - gentle protease for clumps and tissue

1. Examine cultures under microscope, confirm appropriate confluence
2. Pre-warm dissociation reagent to room temperature or 37°C
3. Aspirate spent medium from flask
4. (Optional) Wash cells with PBS - some reagents work better without washing
5. Add dissociation reagent (same volumes as trypsin)
6. Incubate at room temperature or 37°C for **5-15 minutes** (check manufacturer's instructions)
7. Gently tap flask periodically to aid detachment
8. Check detachment under microscope
9. Add complete medium directly to flask (most gentle reagents don't require neutralization)
10. Pipette gently to create single-cell suspension
11. Transfer to new vessel and incubate

> **Advantages:**
> - Less harmful to cell surface proteins and receptors
> - Often do not require neutralization with serum
> - Can be used with serum-free cultures
> - Better viability for sensitive cell types

Passaging Suspension Cells

> **Suspension cells do not require trypsinization since they are already in suspension.**

1. Remove flask from incubator
2. Mix cell suspension by gentle pipetting
3. Take small sample for cell counting
4. Count cells and determine density
5. Calculate volume needed for desired seeding density
6. Transfer calculated volume to new flask
7. Add fresh medium to achieve desired final volume and cell concentration

8. Typical seeding density: **2-5 × 10^5 cells/mL**
9. Return to incubator

Example Calculation:

Current density: 2 × 10^6 cells/mL in 10 mL = 2 × 10^7 total cells
Target density: 3 × 10^5 cells/mL in 30 mL total volume
Cells needed: 3 × 10^5 × 30 = 9 × 10^6 cells
Volume to transfer: 9 × 10^6 ÷ 2 × 10^6 = **4.5 mL**
Add **25.5 mL fresh medium** to reach 30 mL total

Cell Counting Methods

Using Hemocytometer

1. Mix cell suspension thoroughly
2. Take 10 µL sample
3. Load hemocytometer chamber
4. Count cells in 4 corner squares (each 1 mm^2)
5. Calculate: Cells/mL = (Average count) × 10^4 × (dilution factor)

Automated Counter

1. Follow manufacturer's instructions
2. Typically requires 10-20 µL sample
3. Provides cell concentration and viability automatically

Trypan Blue Exclusion (Viability)

1. Mix 10 µL cell suspension + 10 µL 0.4% trypan blue (1:1 dilution)
2. Load hemocytometer
3. Count viable (clear) and non-viable (blue) cells separately
4. Viability (%) = (Viable cells / Total cells) × 100
5. Should be **>90%** for passaging

Freezing Cells | Thawing Cells

Freezing Cells (Cryopreservation)

When to freeze cells:

- Passage cells 1-2 days before freezing
- Cells should be in logarithmic growth phase
- Confluence: >80% for adherent cells
- Viability: >90%
- Use low passage number cells (typically P5-P15)

Freezing Medium Options

Option 1 (FBS-based):
90% FBS + 10% DMSO

Option 2 (FBS-based):
50% medium + 40% FBS + 10% DMSO

Option 3 (Serum-free):
90% complete medium + 10% DMSO

Option 4 (Commercial):
Vendor-supplied freezing medium (CryoStor, Synth-a-Freeze, etc.)

Preparation Notes

- Prepare freezing medium fresh on day of use
- Keep cold (on ice or 4°C) until ready to use
- Add DMSO last and mix thoroughly
- For serum-free cultures, use Option 3 or 4
- Target concentration: 1-5 × 10^6 cells/mL

Freezing Protocol

1. **Prepare cells**
   - Passage cells as normal (trypsinization or gentle dissociation)
   - Collect cell suspension in sterile centrifuge tube

2. **Pellet cells**: Centrifuge at 200-300 × g for 5 minutes at room temperature, carefully aspirate supernatant

3. **Resuspend in freezing medium**
   - Resuspend pellet in ice-cold freezing medium
   - Target concentration: 1-5 × 10^6 cells/mL
   - Example: 10 × 10^6 cells at 2 × 10^6/mL = 5 mL freezing medium

4. **Aliquot into cryovials**
   - Label vials: cell line, passage, concentration, date, initials
   - Aliquot 1 mL per cryovial
   - Work quickly to minimize time in DMSO at room temperature

5. **Controlled-rate freezing**
   - Place cryovials in freezing container (CoolCell, Mr. Frosty)
   - Add isopropanol to container if required
   - Place in -80°C freezer
   - Container ensures **-1°C/minute** cooling rate
   - Leave at -80°C for 24 hours minimum

6. **Long-term storage**
   - After 24 hours, transfer to liquid nitrogen storage
   - Store in vapor phase (-150°C to -190°C)
   - Record location in cell bank inventory

> CRITICAL NOTES:
> • Freeze cells slowly (1°C/min) but thaw quickly (rapid warming)
> • DMSO is toxic at room temperature - work quickly during aliquoting
> • Never refreeze thawed cells
> • Keep detailed records of frozen stocks

Thawing Cryopreserved Cells

> KEY PRINCIPLE: Thaw rapidly but freeze slowly

1. **Prepare reagents**
   - Pre-warm complete medium to 37°C
   - Prepare culture vessel with pre-warmed medium (T25: 5 mL, T75: 12 mL)
   - Set water bath to 37°C

2. **Retrieve cryovial**
   - Remove from liquid nitrogen storage
   - Handle with cryogenic gloves

- Transport on dry ice if moving between locations

3. **Rapid thaw**
   - Immediately place cryovial in 37°C water bath
   - Gently swirl vial in water bath
   - Thaw until only small ice crystal remains (60-90 seconds)
   - Do NOT allow to thaw completely in water bath
   - Wipe vial with 70% ethanol before opening

4. **Transfer to hood**: Move to biosafety cabinet, work quickly to minimize DMSO exposure time

5. **Dilute cells**

Method A (Direct seeding - less trauma):
• Transfer entire contents to prepared vessel
• Rock gently to mix
• Cells attach within 4-24 hours
• Change medium after 24 hours to remove DMSO

Method B (Centrifugation - removes DMSO immediately):
• Transfer to 15 mL tube with 10 mL medium
• Centrifuge at 200-300 × g for 5 min
• Aspirate supernatant (contains DMSO)
• Resuspend in fresh medium, transfer to vessel
• Recommended for suspension cells

6. **Incubate**: Place in 37°C, 5% CO_2 incubator, do not disturb for at least 24 hours

7. **Assess recovery**
   - After 24 hours, examine under microscope
   - Adherent cells should be attaching
   - Change medium to remove dead cells and remaining DMSO
   - Continue normal culture maintenance

8. **First passage after thawing**
   - Allow cells to reach 80-90% confluence before first passage (3-7 days)
   - First passage may have lower viability (70-80% acceptable)
   - Subsequent passages should return to normal >90% viability

> **IMPORTANT:**
> • Remove DMSO within 24 hours (media change or centrifugation)
> • Do not passage cells immediately after thawing
> • Some cell death is normal; viability improves with passages

Troubleshooting Common Problems

Passaging Issues

Problem	Possible Cause	Solution
Cells not attaching after passage	Trypsinization too harsh Medium lacks serum	Reduce trypsin time Pre-coat flask with ECM proteins Check medium contains serum
Clumping after trypsinization	Incomplete dissociation DNA from dead cells	Pipette more thoroughly Increase trypsinization time DNase treatment
Slow growth rate	Wrong incubator settings High passage number	Check CO_2 (should be 5%) Verify medium is appropriate Thaw new low-passage stock
Cells differentiating or morphology changing	Overconfluent Too high passage	Passage before confluence Increase split ratio Thaw new vial from frozen stock

Cryopreservation Issues

Problem	Possible Cause	Solution
Low viability after thawing	Slow thawing Freezing rate incorrect	Thaw more rapidly (full immersion 37°C) Use centrifugation to remove DMSO Check controlled-rate freezing
Cells not recovering after thaw	DMSO exposure too long Cells frozen at wrong phase	Work quickly during thawing Remove DMSO within 24 hours Freeze only log-phase cells

Contamination

Type	Signs	Prevention
Bacterial	Cloudy medium, rapid pH drop	Strict aseptic technique Discard contaminated cultures
Fungal	Visible mycelia or spores	Clean incubator regularly Discard contaminated cultures
Mycoplasma	No visible signs	Test every 2-3 months Use validated cell sources Quarantine new cells

Best Practices & Quality Control

DO ✓

- Maintain cells in logarithmic phase of growth
- Pre-warm all media and solutions to 37°C
- Label all flasks clearly with cell line, passage number, and date
- Keep detailed records in laboratory notebook
- Use appropriate vessel coatings if required (collagen, fibronectin, poly-D-lysine)
- Monitor cell morphology daily
- Freeze stocks at low passage numbers
- Test for mycoplasma every 2-3 months
- Use STR profiling to verify cell line identity

DON'T ✗

- Overgrow cells (leads to differentiation or senescence)
- Reuse pipettes between flasks
- Allow cells to enter stationary phase
- Passage cells more frequently than every 48 hours
- Use cells beyond recommended passage number
- Forget to record passage numbers
- Add trypsin to cells in the presence of serum
- Leave cells in trypsin longer than necessary
- Refreeze thawed cells

Aseptic Technique

- Always work in biological safety cabinet
- Spray gloves, bottles, flasks with 70% ethanol before placing in hood
- Never place items above open culture vessels
- Keep work area uncluttered
- Clean BSC before and after use with 70% ethanol
- UV sterilize BSC for 15-30 minutes before use (optional)

Quality Control

- **Morphology:** Check daily under microscope
- **Growth rate:** Monitor population doubling time
- **Viability:** Should be >90% for healthy cultures
- **Mycoplasma testing:** Every 2-3 months
- **STR profiling:** Verify cell line identity periodically
- **Passage records:** Maintain detailed logs
- **Frozen stocks:** Create at regular intervals

Record Keeping

Cell Culture Log should include:

- Date
- Cell line name and passage number
- Procedure performed (passage, media change, freezing, thawing)
- Split ratio (for passaging)
- Confluence or cell density
- Viability percentage
- Morphology observations
- Any anomalies or concerns
- Initials of person performing work

General Guidelines

- L-Glutamine is essential but labile - use within 1-2 weeks or use GlutaMAX
- Most mammalian cells double every 18-24 hours under optimal conditions

- Some cell lines require specialized media, supplements, or coated surfaces
- Always refer to ATCC or provider datasheet for cell line-specific requirements
- Maintain master cell bank (low passage) and working cell bank (higher passage)