Cell Culture 101 - Tips for Successful Cell Culture

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About ATCC

- Founded in 1925, ATCC is a non-profit organization with headquarters in Manassas, VA
- World’s premiere biological materials resource and standards development organization
- ATCC collaborates with and supports the scientific community with industry-standard biological products and innovative solutions
- Strong team of 400+ employees; over one third with advanced degrees
Agenda

- Cell culture workflow - what cells to use
- Cell handling/media handling
- Transfection and analysis issues
- Viability assays
- Summary
Primary cells versus continuous cells

- Prepared directly from tissue
- Physiologically relevant
- Low risk for phenotypic or genotypic drift

Primary Cells

- Easy to propagate *in vitro*
- Easy to generate large quantities of cells
- Inexpensive to maintain

Continuous Cells
hTERT-immortalized primary cells

- Bypass replicative senescence by telomerase
- Maintain primary cell function with the lifecycle of a continuous cell line

Regulation of telomere length in normal and cancer cells by telomerase

Expert Reviews in Molecular Medicine ©2002 Cambridge University Press
Potential workflow situations: Standardization and validation

Use cell lines for standardization and confirmation of each experiment
- Large number of cells needed
- Samples with limited variability
- Generally easy to manipulate

Use primary cells after standardization to further validate the results
- Donor variability
- Biological relevance
Potential workflow situations: High-throughput screening

Screening work flow

- Initial screening in cell lines
  - Large number of cells needed
  - Samples with limited variability
- Next level in hTERT-immortalized primary cell lines
  - Large number of cells needed
  - Samples with limited variability
  - More physiologically relevant results
- Final screen in primary cells *(Results with the most biological relevance)*
Primary cells as a control

Continuous cell lines are cells isolated from primary tissue (often a tumor) that have mutated to survive a “crisis”

Continuous cell lines have deviated from original source

In every continuous cell line experiment, primary cells should be used as one of the controls

Primary neonatal keratinocytes (ATCC® PCS-201-010™) differentiated into physiological epidermis
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HEK-293 (ATCC® CRL-1573™)
Thawing cells

- Thaw in 37°C water bath for approximately 2 minutes with gentle agitation
- Spray vial with 70% ethanol
- Transfer to 10 mL centrifuge tube with 9 mL of appropriate growth media (10% FBS)
- *Centrifuge, resuspend in 2 mL of growth media
- Transfer to cell culture vessel

When bringing out of liquid nitrogen, thaw as quickly as possible

*For certain primary cells, centrifugation may be detrimental, refer to specific protocol
Cell expansion

- After thawing, cells should be plated in an appropriate cell culture vessel with complete media
- 24 hours after seeding, check for confluence
- Note, primary cells may take up to several days to reach 80% confluency for subculturing
Cell expansion

Figure 1. Growth curve for cells grown in culture. Cells should be subcultured while still in the exponential phase.
Trypsinization

At 80% confluency (primary cells), cells can be passed using Trypsin-EDTA

- Using warm trypsin-EDTA for about 3-5 minutes, cells will detach with gentle agitation

- **Trypsin-EDTA for Primary Cells (ATCC® PCS-999-003™)** is a low concentration formula (.05% Trypsin and .002% EDTA) – necessary for primary cell survival

- A Trypsin Soybean Neutralizing Solution (ATCC® 30-2104™) is also needed to prevent cell damage
High levels of ice formation and increased solute concentration have a negative impact on cell viability.

- Optimal cooling rate for cell viability is 1 to 3°C/min.
Freezing down cells

-70°C

Controlled-rate freeze chamber

-1°C/min cooling rate

A few hours to 24 hours

-140°C

Liquid nitrogen tank
Low temperature storage

For the best security, always store your cells in liquid nitrogen freezers
Low temperature storage

Mammalian cells
Long-term storage should be below -140°C

Vials should be stored in a liquid nitrogen unit above the volume of liquid at the bottom of the tank

This temperature should be between -140°C and -180°C
Cell characterization

Characterizing cells

- Cell count before plating
  - Calculating % viability

- Morphology
  - Make sure the morphology is consistent with cell type

- Doubling time
  - Contamination from other cell types can affect growth rate

Fibroblasts

HUVEC
Contamination

Sources
- Contaminated cell lines
- Improper aseptic technique

Types
- Microbial – bacteria, mycoplasma, fungi, viruses
- Cellular – cross contamination

Signs
- Turbid media
- Rapid decline in pH – color change
- Morphological changes
- Filamentous structures
Mycoplasma contamination

Not easily detected
- Does not cause media turbidity
- Does not alter the pH of the media
- Few metabolic byproducts
- Cannot be detected by microscopy

Results in a number of deleterious effects
- Chromosomal aberrations
- Disruption of nucleic acid synthesis
- Changes in membrane antigenicity
- Inhibition of cell proliferation and metabolism
- Decreased transfection rates
- Changes in gene expression profiles
- Affects virus production
- Cell death
Contamination

Cross Contamination

Leads to the replacement of the original cell line with the contaminant

Causes

- Multiple cell lines under the hood at the same time
- Failure to change out pipettes
- Receiving cell lines from other labs

20% of scientific publications include misidentified cultures

50% of preclinical research is not reproducible

Cell characterization

Universal Mycoplasma Detection Kit

PCR-based kit (ATCC® 30-1012K™)

Detects any of the 60 most common mycoplasmas

ATCC STR Profiling

Ensures your cells are what you think they are

- STR profile of your cell line
- Comparison of your cells against ATCC STR Profile database at www.atcc.org/str
- Electropherograms supporting the allele calls at each locus
- Comprehensive interpretation of results
Contamination

Personnel and equipment
- Poor culturing practices
- Dust and aerosol

Contamination
- Aerosol dispersion of contaminated cell cultures
- Faulty laminar flow

Culture reagents
- Sera
- Media
- Reagents
Contamination prevention and aseptic technique

**Good aseptic technique**

- Make it difficult for microorganisms to invade culture vessels
  - Sealed cultured vessels
  - Vented cap flasks
- Disposable aspirators
  - Cell culture hoods with good laminar flow
  - Do not use as a storage area!
- Spray media bottles/reagents with alcohol
Contamination prevention and aseptic technique

- **Use small volumes of reagents at a time**
  - Aliquot stock solutions and reagents

- **Always wear clean lab coats and protective clothing**

- **Use seed stocks**
  - Create master stocks

- **Avoid using antibiotics in media!**
  - Can contribute to chronic contamination
  - Rarely prevents contamination
  - Toxic to cells
Media choices

Animal cell lines – media + 10% FBS

- Eagle’s Minimum Essential Medium (EMEM; ATCC® 30-2003™)
- Dulbecco’s Modified Eagle’s Medium (DMEM; ATCC® 30-2002™)
- Iscove’s Modified Dulbecco’s Medium (IMDM; ATCC® 30-2005™)
- Kaighn’s Modification of Ham’s F-12 Medium (ATCC® 30-2004™)
- DMEM/ F12 Medium (ATCC® 30-2006™)
- McCoy’s 5A (ATCC® 30-2007™)
- RPMI-1640 (ATCC® 30-2001™)
- Leibovitz’s L-15 (ATCC® 30-2008™)

Primary Cells – Primary Cell Basal Media and Growth Kits

- Primary cells require their own specially formulated media, specific to each cell type
Media choices

Media ingredients/additives

- Nonessential amino acids
  - Can be added to reduce the metabolic burden on cells

- L-glutamine
  - Present in ATCC Classical Cell Culture Media
  - Relatively stable in bottles kept at 4-8°C
  - *Glutamine degradation increases ammonia toxicity*
  - *Generally not recommended to “spike” media with L-glutamine*

- Antibiotics and antimycotics
  - Penicillin-streptomycin, gentamicin sulfate
  - Amphotericin B
  - *Generally not recommended*
Media choices

Special notes:

- Maintain cells in the same media
- Vendor to vendor media variability
  - Possible osmotic shock

- When transferring to new media:
  - Use 1:1 mix (50% old, 50% new media)
  - 1:2 mix
  - 1:3 mix
  - 1:7 mix

- Heat inactivation of FBS? Not recommended
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Introduction to transfection

Method for introducing exogenous nucleic acid sequences into mammalian cells

Widely used technique that has made expressing DNA or RNA in most types of cells relatively easy

A variety of approaches have been developed for use across a range of applications

No single approach will work for all conditions/cell types/application
## Transfection methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
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| **Lipid**  | - Easy, most common method  
             | - Variable efficiencies  
             | - Will not work with all cell types                                         |
| **Viral**  | - Will transfec non-dividing cells  
             | - Technically challenging, expensive  
             | - Safety issues, immune response, mutagenesis                               |
| **Electroporation** | - Requires specialized equipment  
             | - Cells must be in suspension  
             | - Toxicity can be an issue                                                  |
| **Physical** | - Technically challenging, expensive  
             | - Requires specialized equipment  
             | - Works with non-nucleic acids; single cell transfection                     |
| **Other**  | - Not common, may be technically challenging  
             | - Non-lipid based chemicals  
             | - Nanoparticles/laser/ultrasound/magnetic                                   |
Mechanism of lipid-based transfection

Nucleic acid and cationic lipids form a complex

Nucleic acid-lipid complex enters cell

Sequences are released, enter nucleus

ATCC transfection reagents:
- GeneXPlus (ATCC® ACS-4004™)
- TransfeX™ (ATCC® ACS-4005™)
**Typical transfection workflow**

**Day -1**
Collect and seed cells into vessel where transfection will be performed

**Day 0**
Form transfection complexes by combining nucleic acid sequences and transfection reagent

Add transfection complexes to cells

**Days 1+**
Assess transfection

Diagram:
- Plasmid DNA
- Transfection reagent
- Complexes
- Cells (green)
Overexpression vs. knockdown

Introduce foreign plasmid DNA/mRNA to induce expression of a desired transcript/protein

Utilize RNAi pathway to degrade or inhibit translation of mRNA transcripts and subsequently reduce the amount of protein
Transient versus stable transfection

**Transient**
- Foreign gene not integrated into genome
- Expression persists for limited time
- Foreign gene lost due to cell division, degradation, or other factors

**Stable**
- Initially a transient transfection
- Use co-expressed selection markers
- Long term, only cells that have integrated the foreign gene persist
Transfection: Best practices

Areas of optimization:
- Culture conditions
- Nucleic acids
- Experimental design
- Assay method/timing
- Transfection reagent

?
Cell culture conditions

- Cell health
- Density
- Proliferation
- Media
Nucleic acids

All nucleic acids
- High purity
- Endotoxin free
- Validated

Plasmid DNA
- Promoter
- Plasmid size
- Conformation

RNA
- Chemical modifications
- Pooled siRNAs
## Experimental design and execution

| Transfection protocol | • Use master mixes  
|• Distribute complexes evenly  
|• Store DNA/RNA properly |
|------------------------|--------------------------------------------------|
| Proper controls        | • Positive and negative controls  
|• Transfected and un-transfected controls |
| Monitor toxicity/off-target effects | • Morphological changes  
|• Presence of vacuoles  
|• Changes in proliferation |
| Validate results       | • Multiple assays  
|• For siRNA: test multiple sequences  
|• For miRNA: increase & suppress |
Assay methods

- mRNA
  - Real time RT-PCR

- Protein
  - Indirect (e.g., enzymatic assays)
  - Reporter assays
  - Western blots
  - Immunocytochemistry
  - ELISA

- Other
  - Morphology
  - Functional
Assay timing

<table>
<thead>
<tr>
<th>Nucleic acid type</th>
<th>Changes at mRNA level</th>
<th>Changes at protein level</th>
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<tbody>
<tr>
<td>siRNA</td>
<td></td>
<td></td>
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<tr>
<td>miRNA</td>
<td></td>
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<tr>
<td>Plasmid DNA</td>
<td></td>
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<tr>
<td>mRNA</td>
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<table>
<thead>
<tr>
<th>Time post-transfection</th>
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<tbody>
<tr>
<td>24 hours</td>
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<tr>
<td>1-2 Days</td>
</tr>
<tr>
<td>5-6 Days</td>
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<tr>
<td>Weeks</td>
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Transfection reagents

- Transfection reagent
- Volume per reaction
- Incubation time
Optimal transfection

Healthy cells

High quality, validated sequences

Effective reagents

Minimum optimization
1. Seeding density
2. Volume of transfection reagent
3. Amount of DNA/RNA
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Viability assays

Quantitative evaluation of cell proliferation rate and response to external factors that affect cell viability

- Commonly used for cytotoxicity, high-throughput screening (e.g., drug development)
- Uses tetrazolium salts in a colorimetric method for evaluating cell populations

**MTT Cell Proliferation Assay (ATCC® 30-1010K™)**
- Tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)

**XTT Cell Proliferation Assay (ATCC® 30-1011K™)**
- Tetrazolium XTT (sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium
Viability assays

MTT Reaction

MTT salt is **reduced** within cellular matrix to Formazan, lysed with detergent to solubilize crystals.

Media turns **PURPLE**

![Diagram of MTT Reaction](image)

XTT Reaction

XTT salt is **reduced** at cell membrane with PMS agent.

Media turns **ORANGE**

![Diagram of XTT Reaction](image)

NADH  NAD⁺ → Formazan (MTT)

NADH  NAD⁺ → Formazan (XTT)
Viability assays

**MTT Assay**
- Cells in 96 well plate with stimulus
- Add MTT reagent
- 2 hour incubation
- Add detergent
- 2 - 4 hour incubation (or longer)

**XTT Assay**
- Cells in 96 well plate with stimulus
- Add XTT reagent + activation agent
- 2 - 4 hour incubation
Viability assays
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