Cell Culture 101 - Tips for Successful Cell Culture

Steven Budd, M.S., M.B.A.
Product Line Business Specialist, ATCC

Kevin Grady, B.S.
Product Line Business Manager, ATCC

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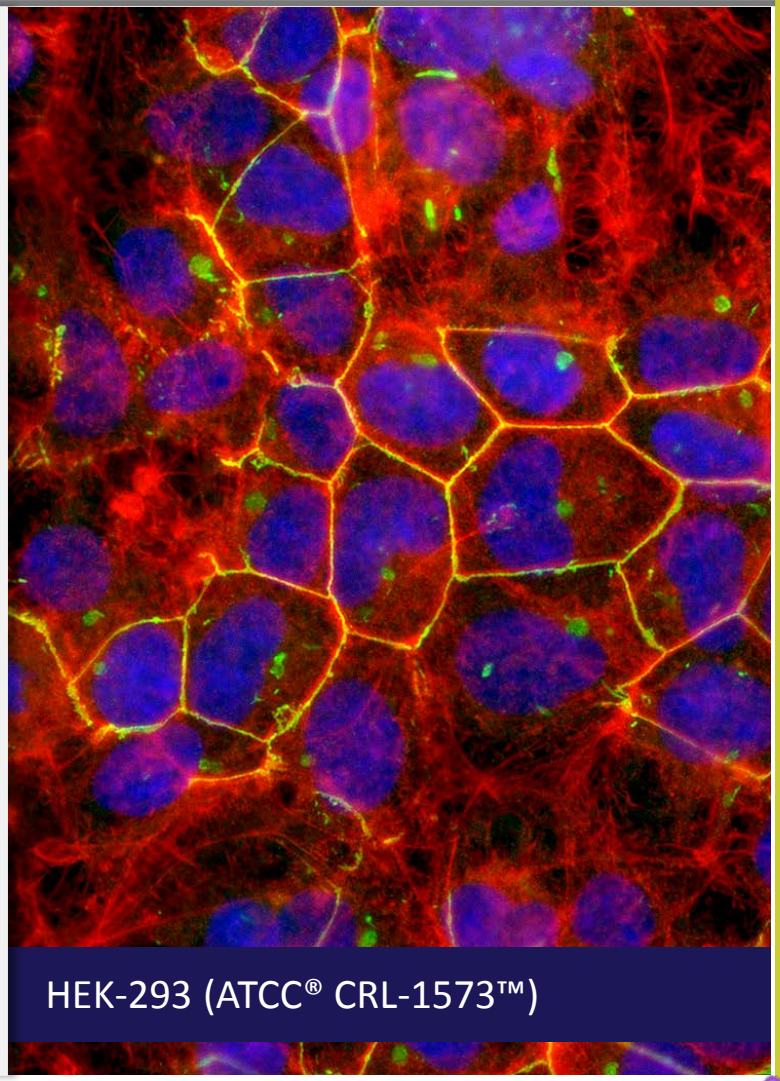
About ATCC

- 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

Established partner to global researchers and scientists

Agenda

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



HEK-293 (ATCC® CRL-1573™)

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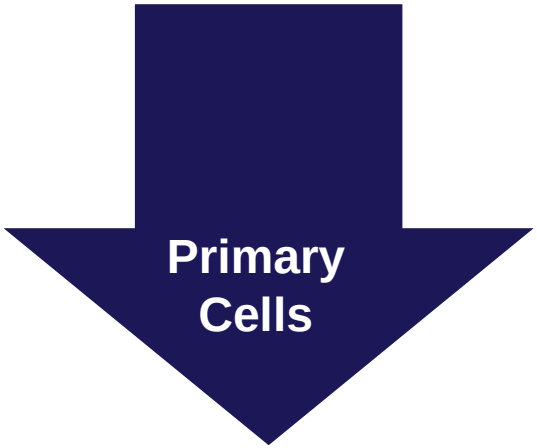
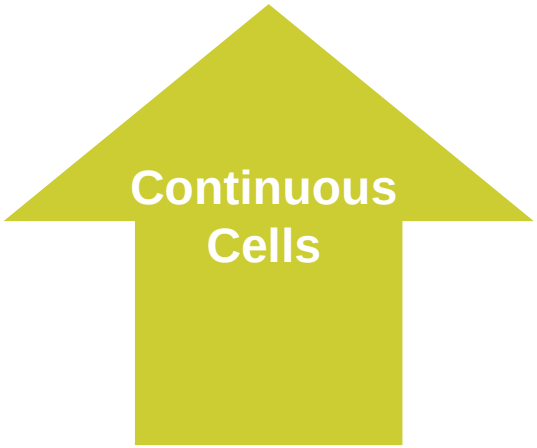
Primary cells versus continuous cells



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

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



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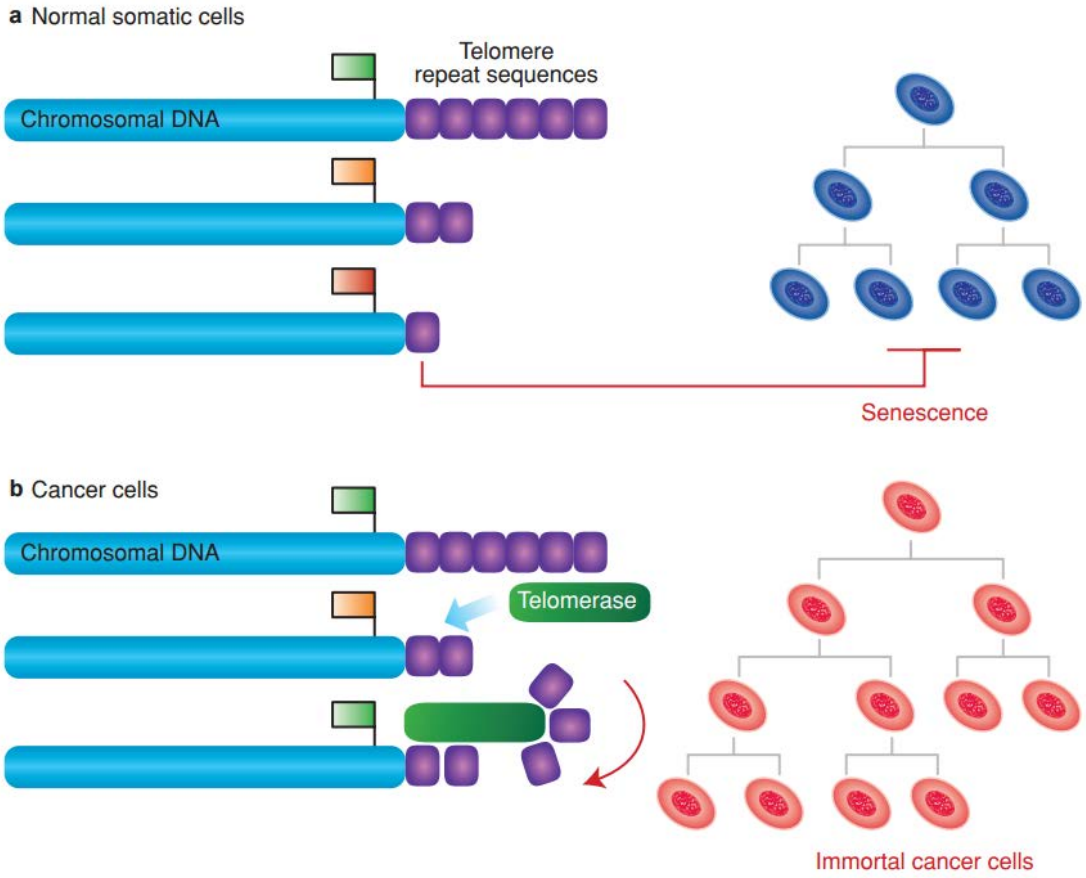
hTERT-immortalized primary cells



Regulation of telomere length in normal and cancer cells by telomerase

Expert Reviews in Molecular Medicine ©2002 Cambridge University Press

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

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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

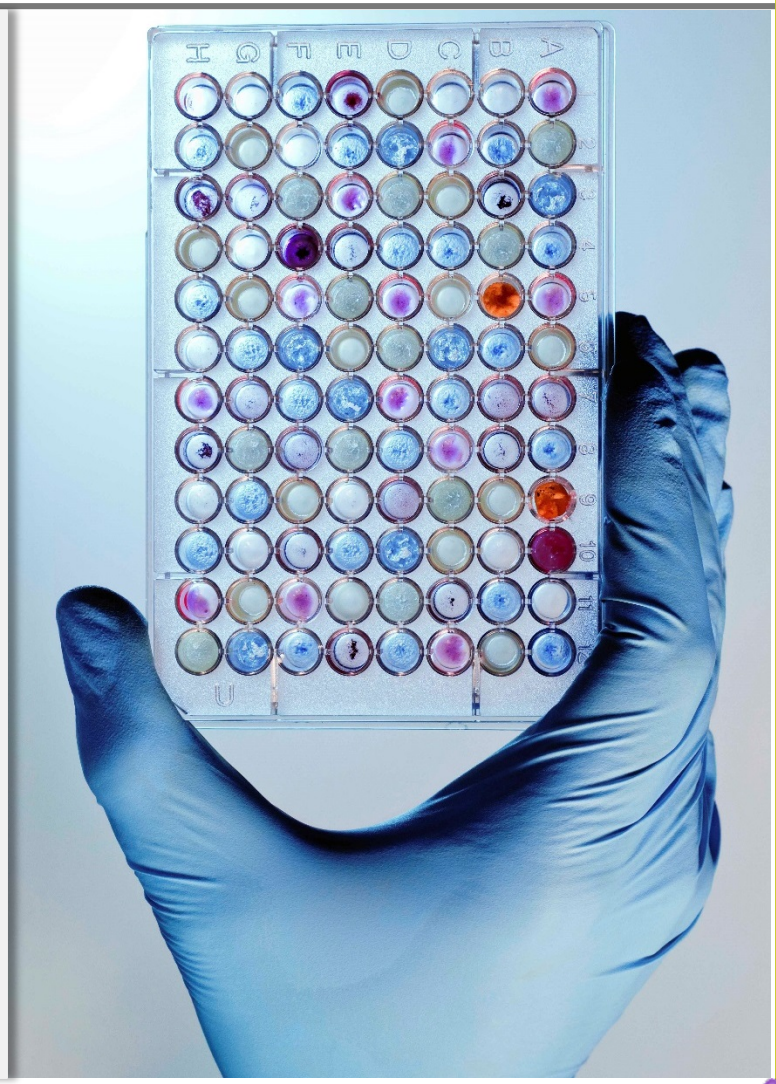
Use primary cells after standardization to further validate the results

- 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)**



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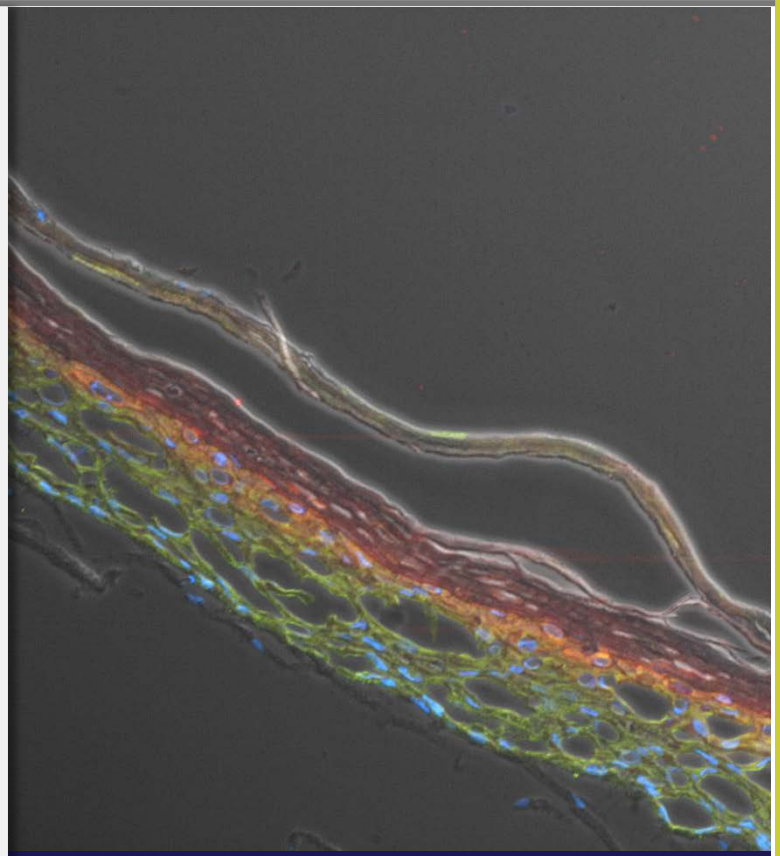
Primary cells as a control

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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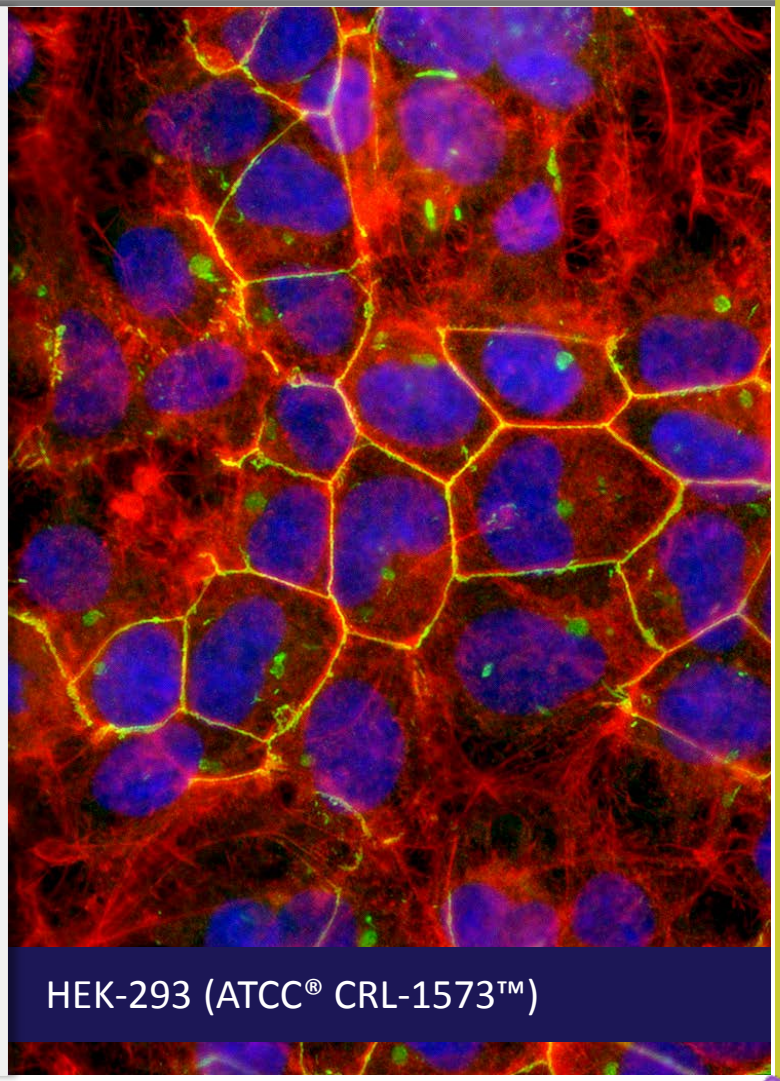

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Thawing cells

- 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**

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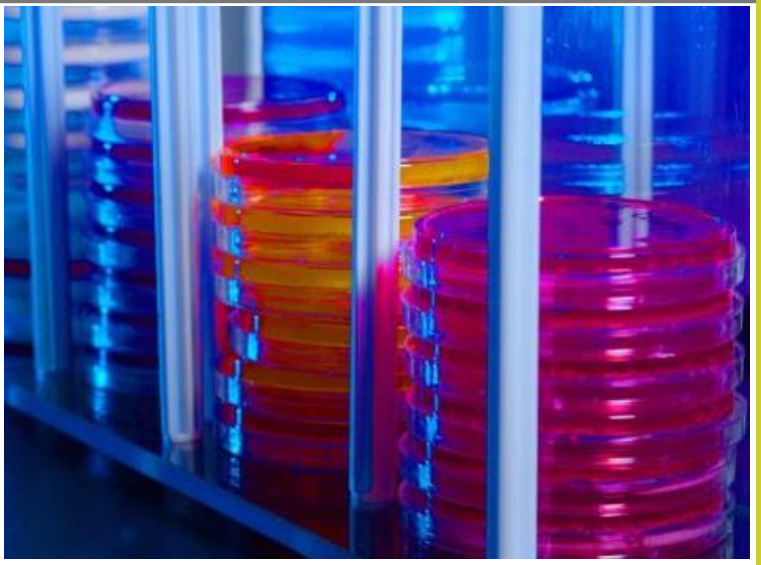
Cell expansion

- After thawing, cells should be plated in an appropriate cell culture vessel with complete media
- 24 hours after seeding, check for confluency

- **Note, primary cells may take up to several days to reach 80% confluency for subculturing**



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Cell expansion

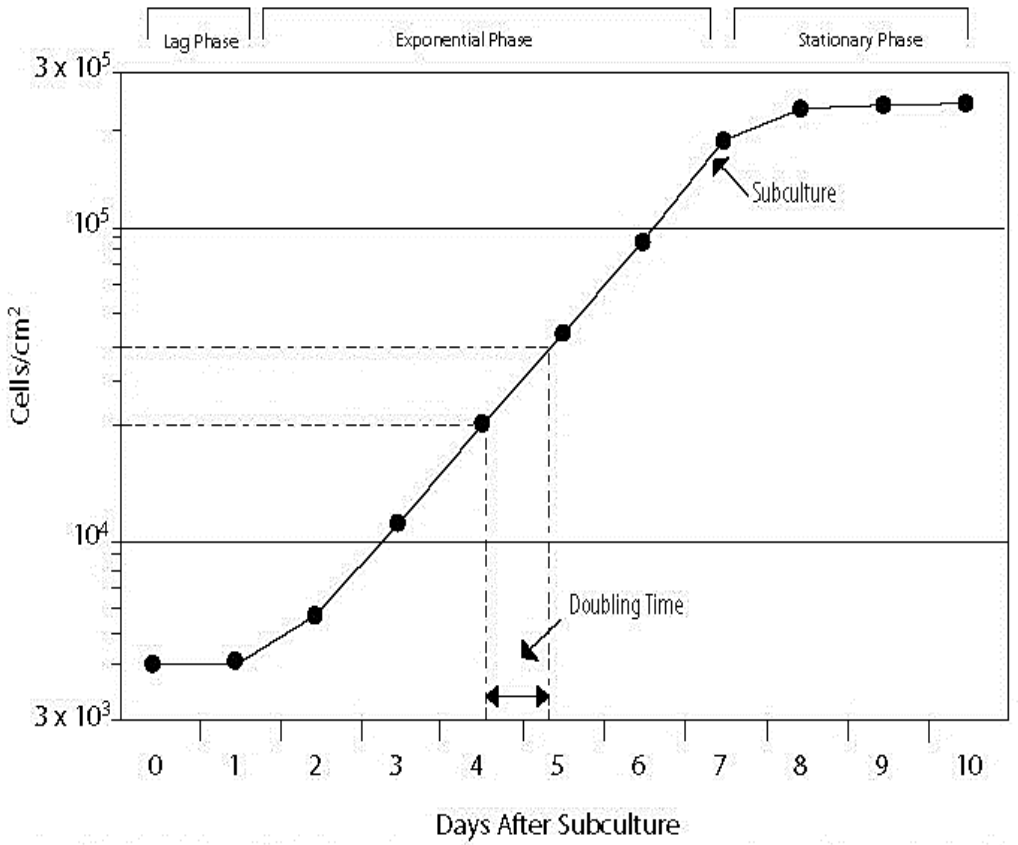


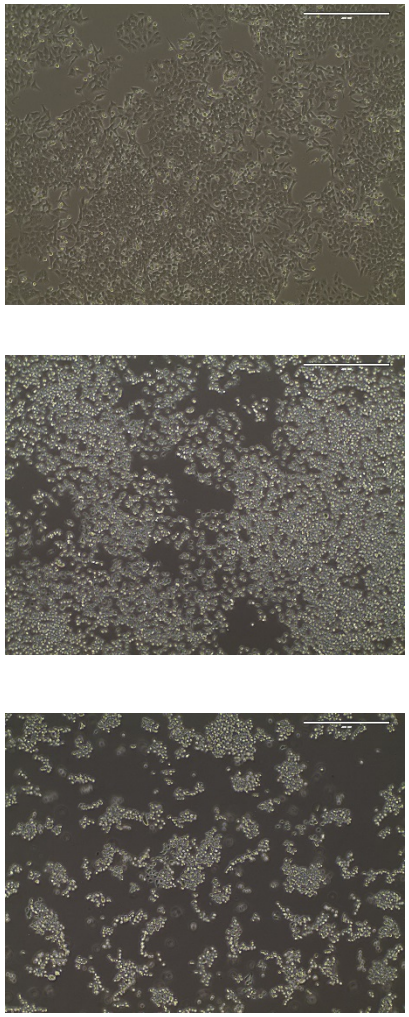
Figure 1. Growth curve for cells grown in culture. Cells should be subcultured while still in the exponential phase.

Trypsinization

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

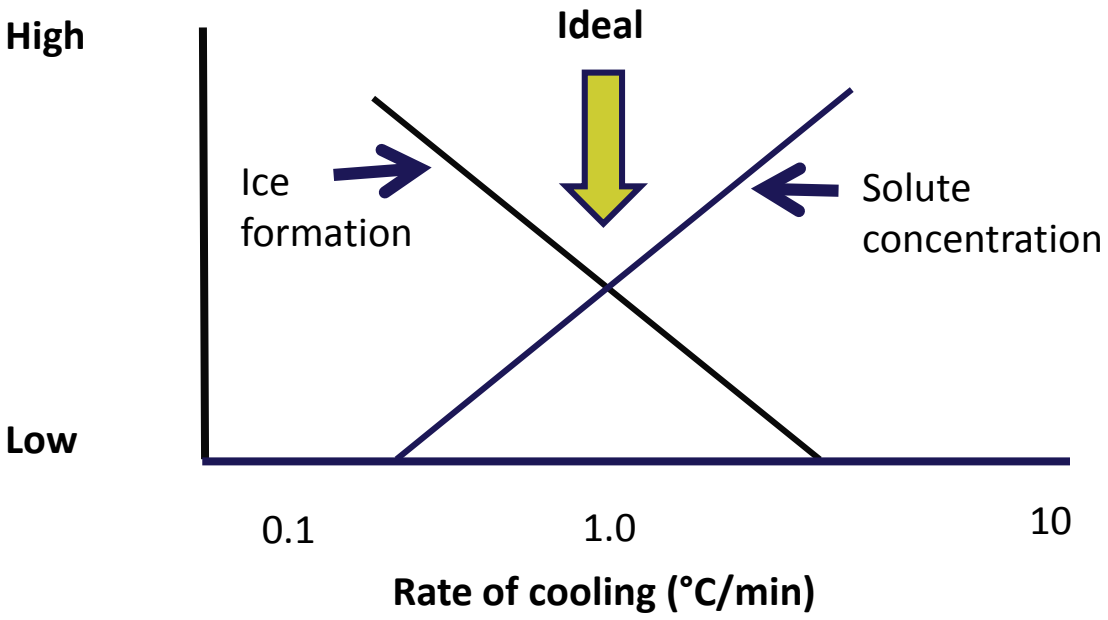
- Using warm trypsin-EDTA for about 3-5 minutes, cells will detach with gentle agitation
- ***Trypsin-EDTA for Primary Cells* (ATCC® PCS-999-003™) *is a low concentration formula (.05% Trypsin and .002% EDTA) – necessary for primary cell survival***
- A Trypsin Soybean Neutralizing Solution (ATCC® 30-2104™) is also needed to prevent cell damage

Monolayer



Fully trypsinized

Cryopreservation



- 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

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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

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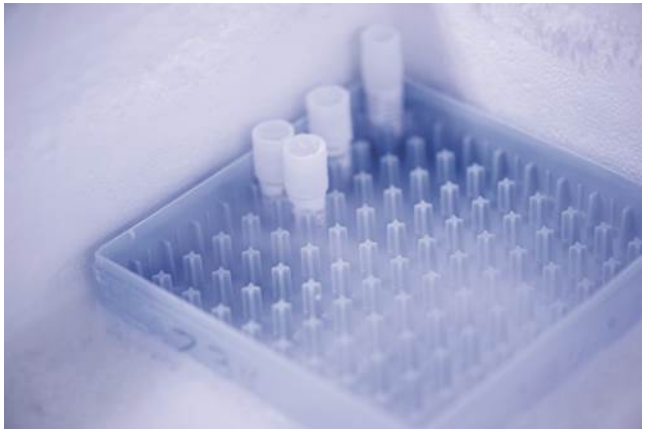
Low temperature storage

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



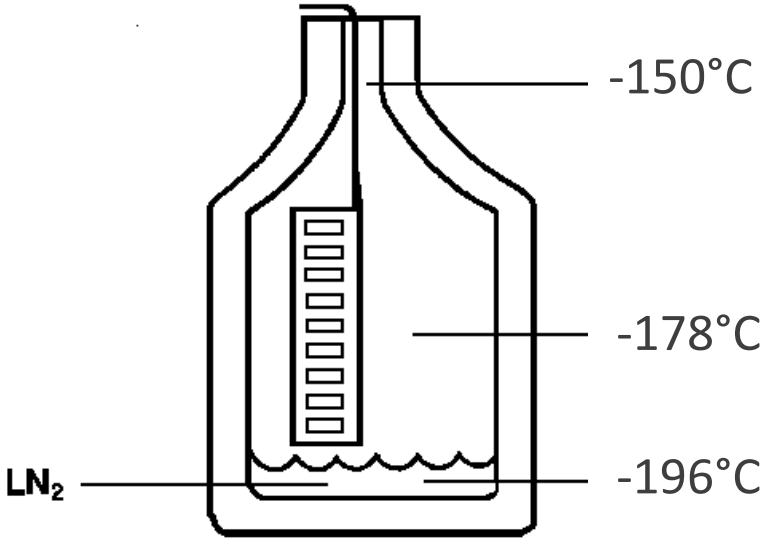
Low temperature storage

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**



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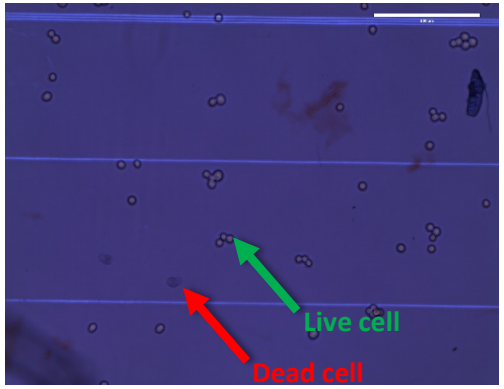
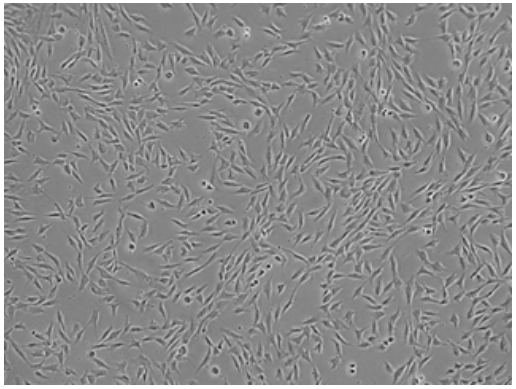
Cell characterization

Characterizing cells

- Cell count before plating
  - Calculating % viability

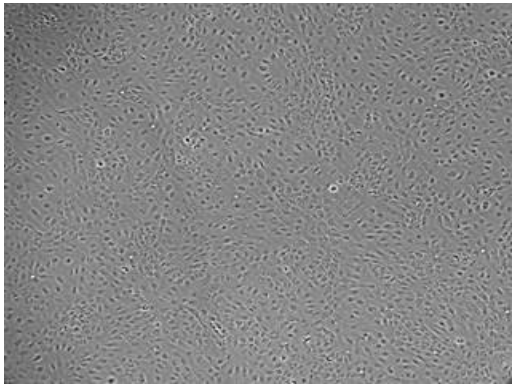




Fibroblasts

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

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



HUVEC

Contamination

Sources

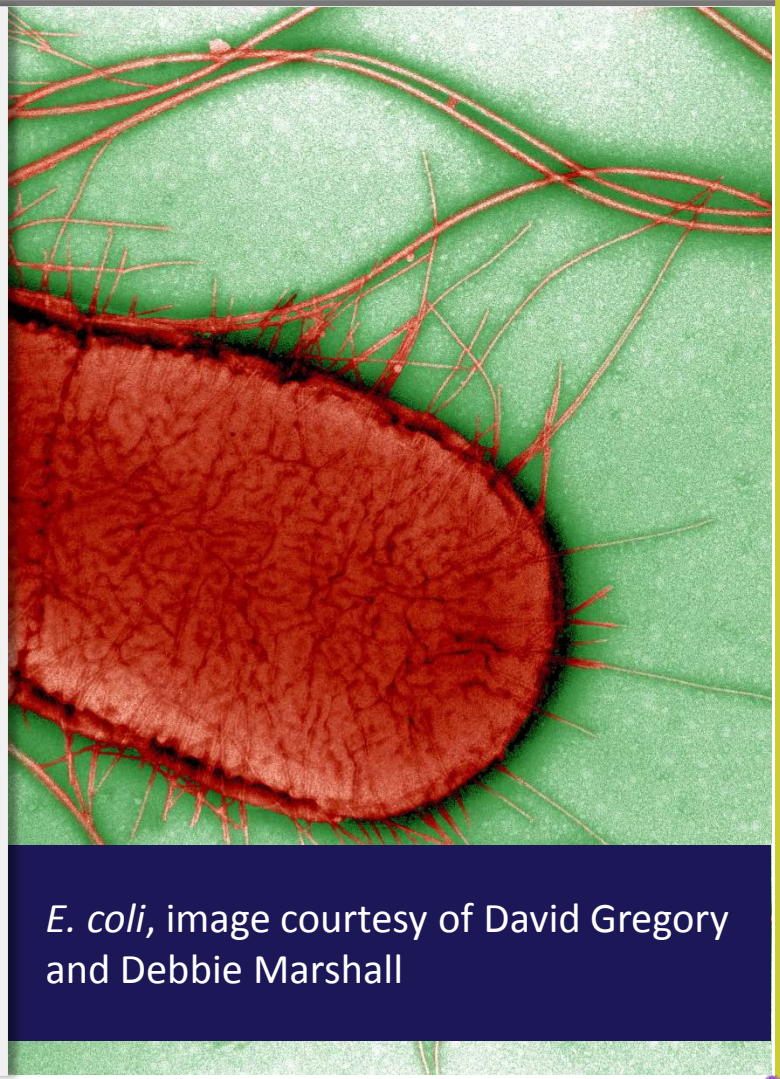
- Contaminated cell lines
- Improper aseptic technique

Types

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

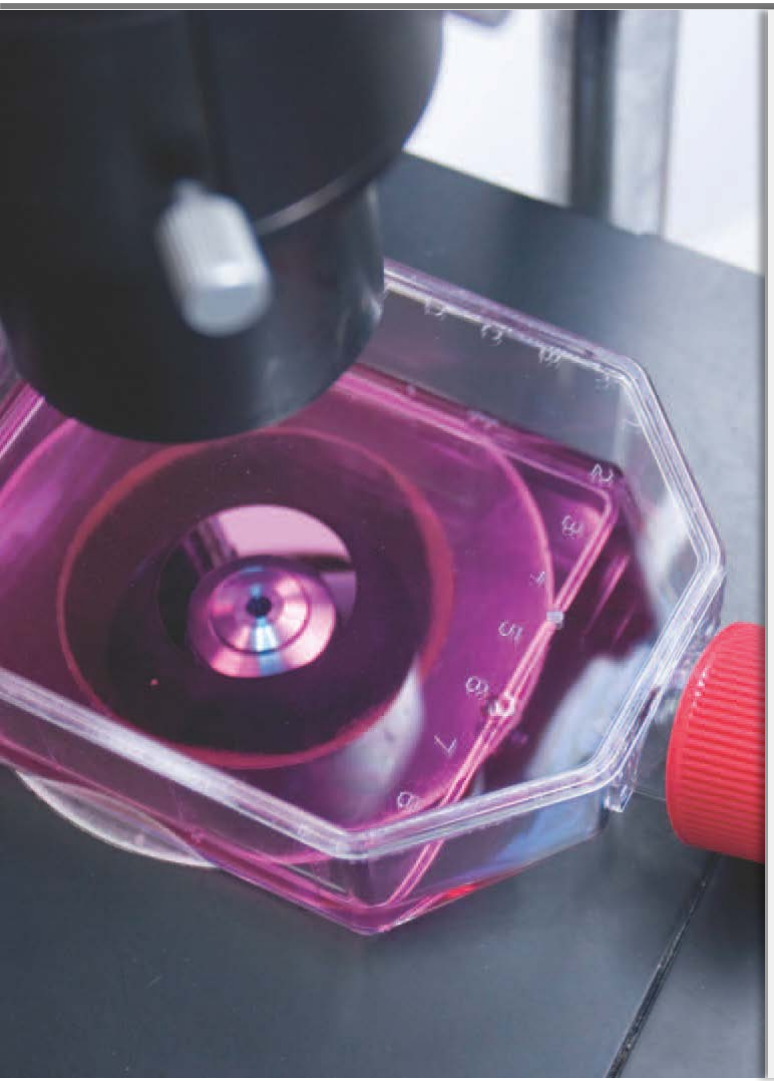
Signs

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



E. coli, image courtesy of David Gregory and Debbie Marshall

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

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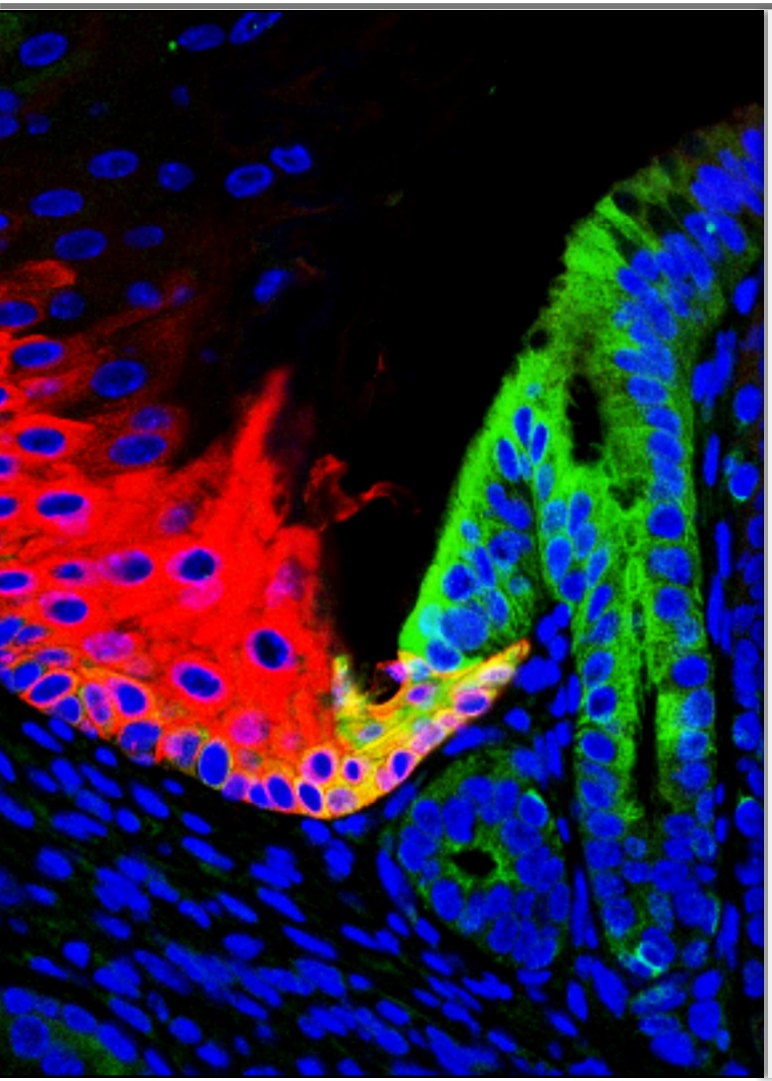
Contamination



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

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Nat Rev Cancer 10(6):441-8, 2010.

Cell characterization

Universal Mycoplasma Detection Kit

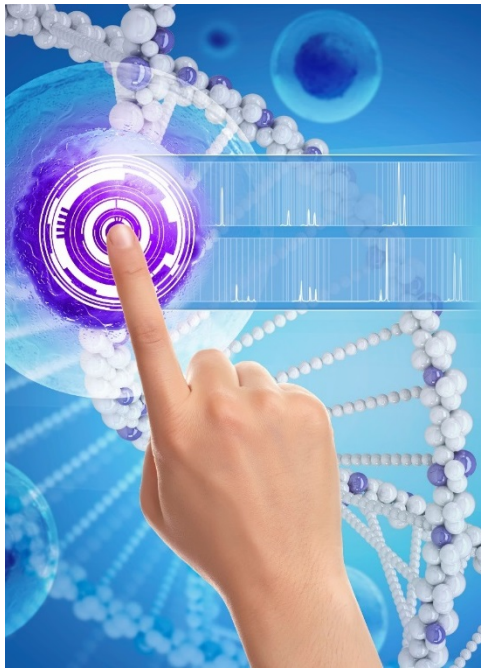
PCR-based kit (ATCC® 30-1012K™)

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



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Contamination

Personnel and equipment

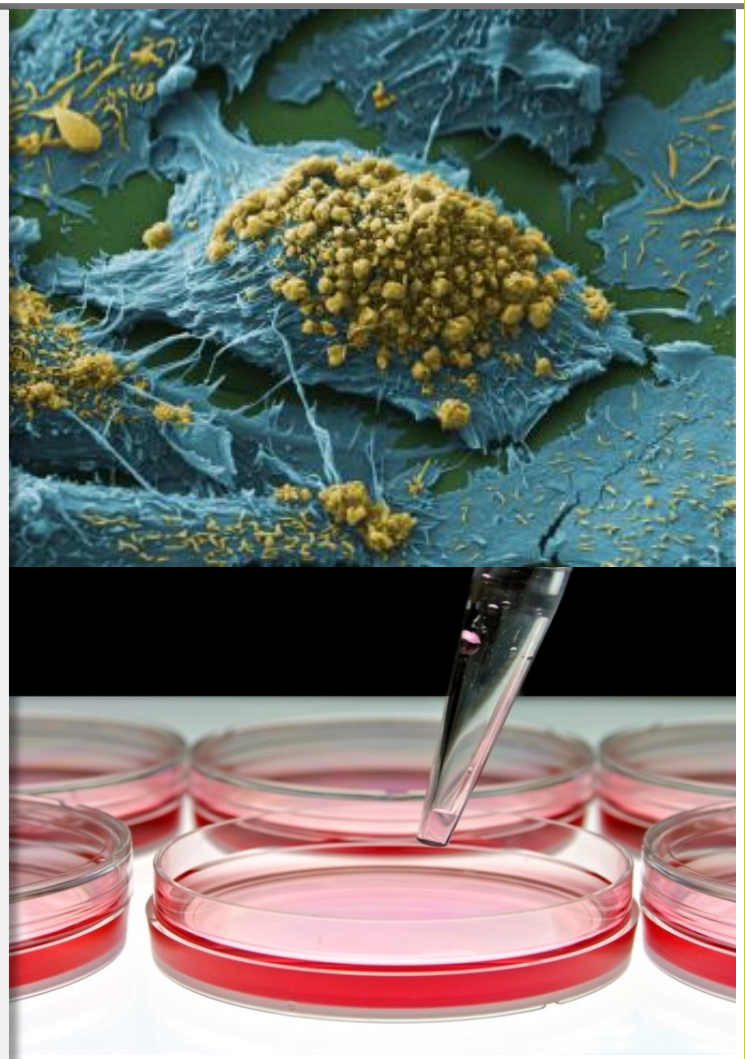
- Poor culturing practices
- Dust and aerosol

Contamination

- Aerosol dispersion of contaminated cell cultures
- Faulty laminar flow

Culture reagents

- Sera
- Media
- Reagents



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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

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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
  - Amphothericin B
  - ***Generally not recommended***

Aspergillus fumigatus, image courtesy of David Gregory and Debbie Marshall

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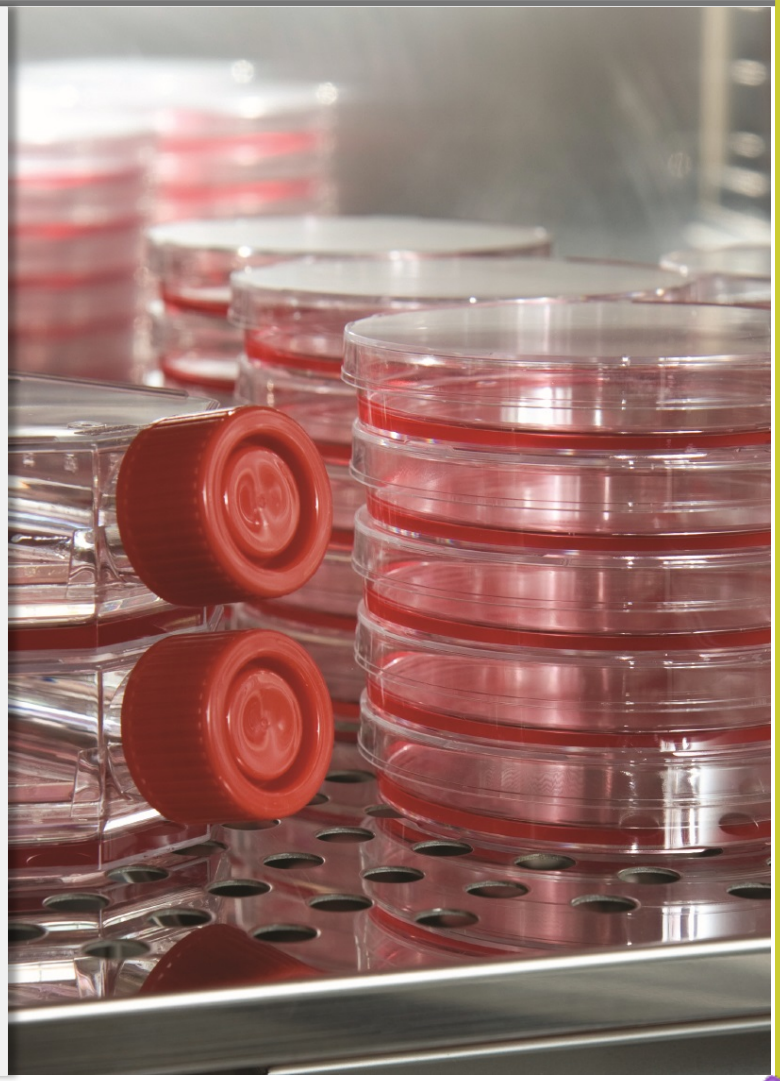
Media choices

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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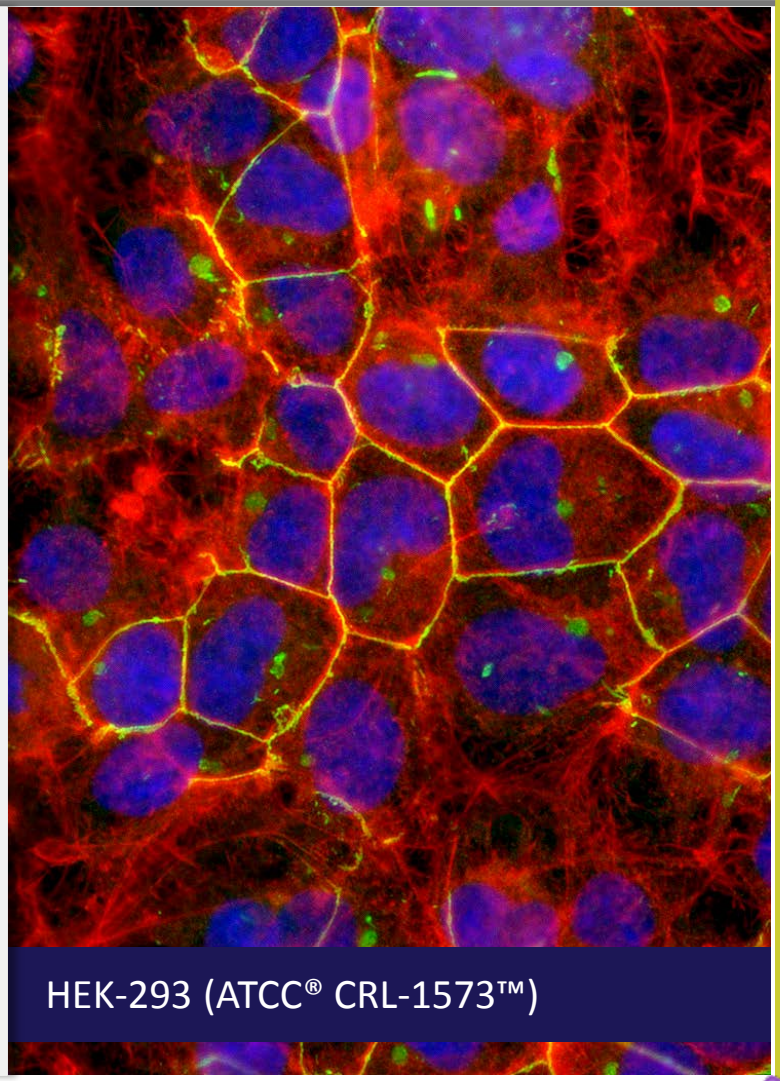

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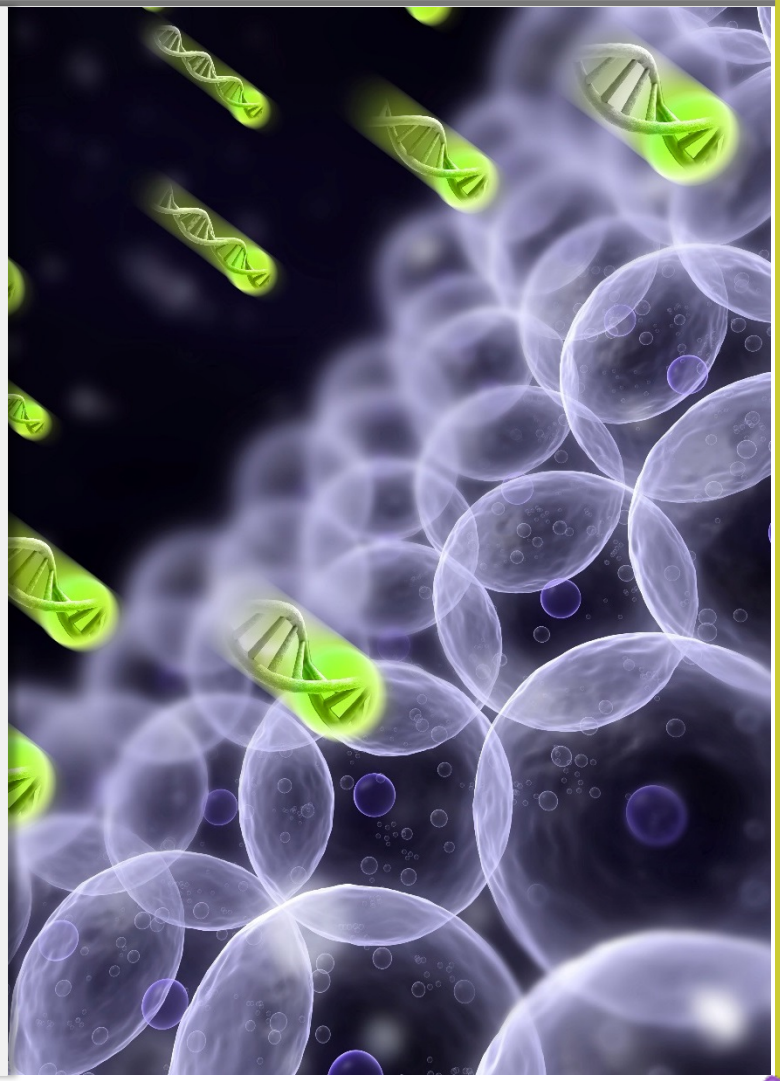
Introduction to transfection

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



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Transfection methods

Lipid	• Easy, most common method • Variable efficiencies • Will not work with all cell types
Viral	• Will transfect 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

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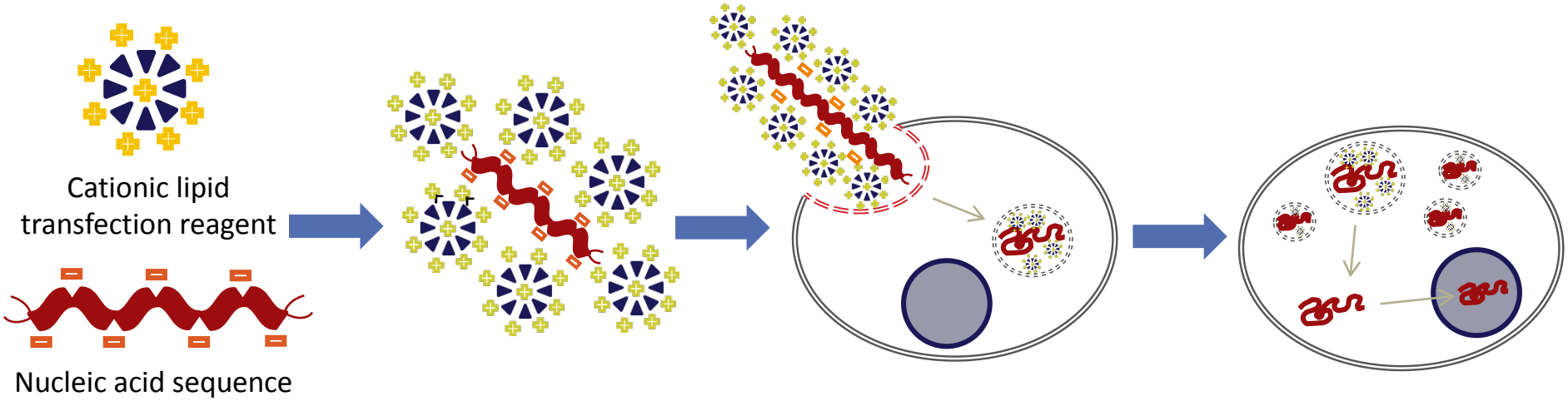
Mechanism of lipid-based transfection



ATCC transfection reagents:

- GeneX*Plus* (ATCC® ACS-4004™)
- TransfeX™ (ATCC® ACS-4005™)

Typical transfection workflow

Day -1

Collect and seed cells into vessel where transfection will be performed

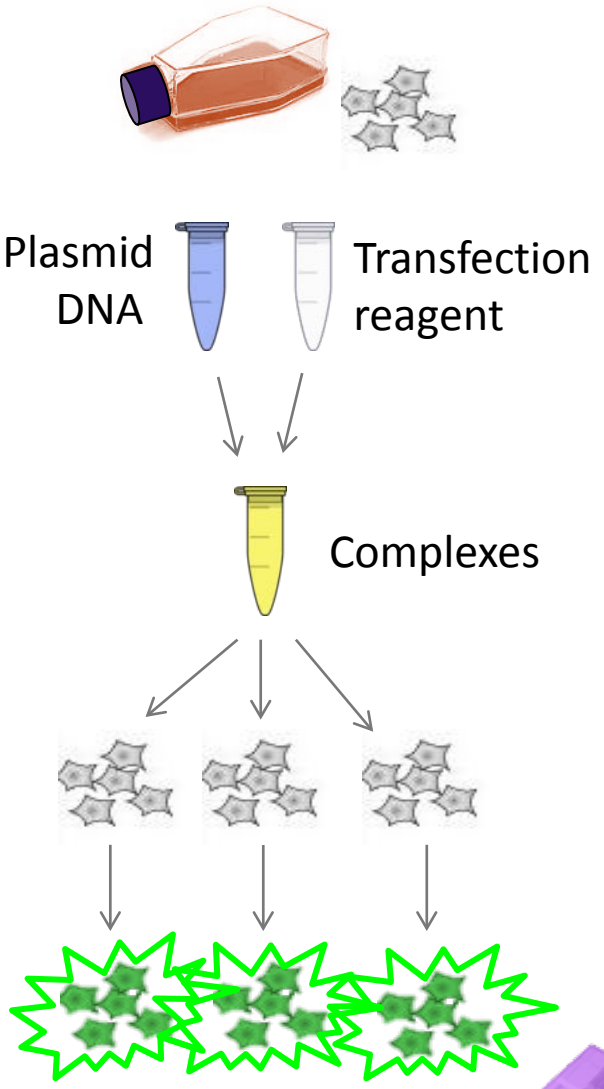
Day 0

Form transfection complexes by combining nucleic acid sequences and transfection reagent

Add transfection complexes to cells

Days 1+

Assess transfection



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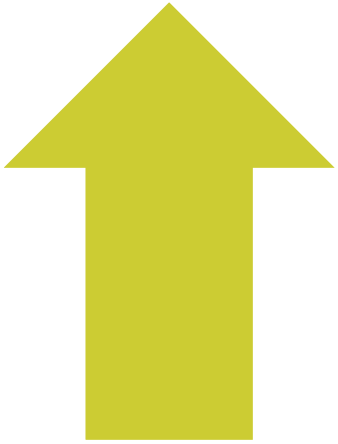
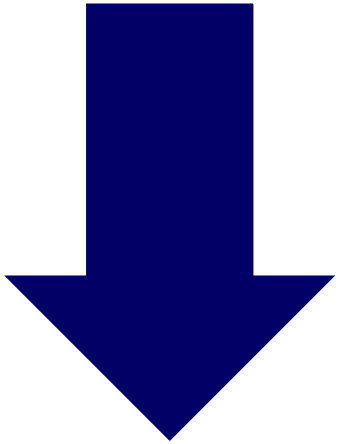
Overexpression vs. knockdown



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

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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

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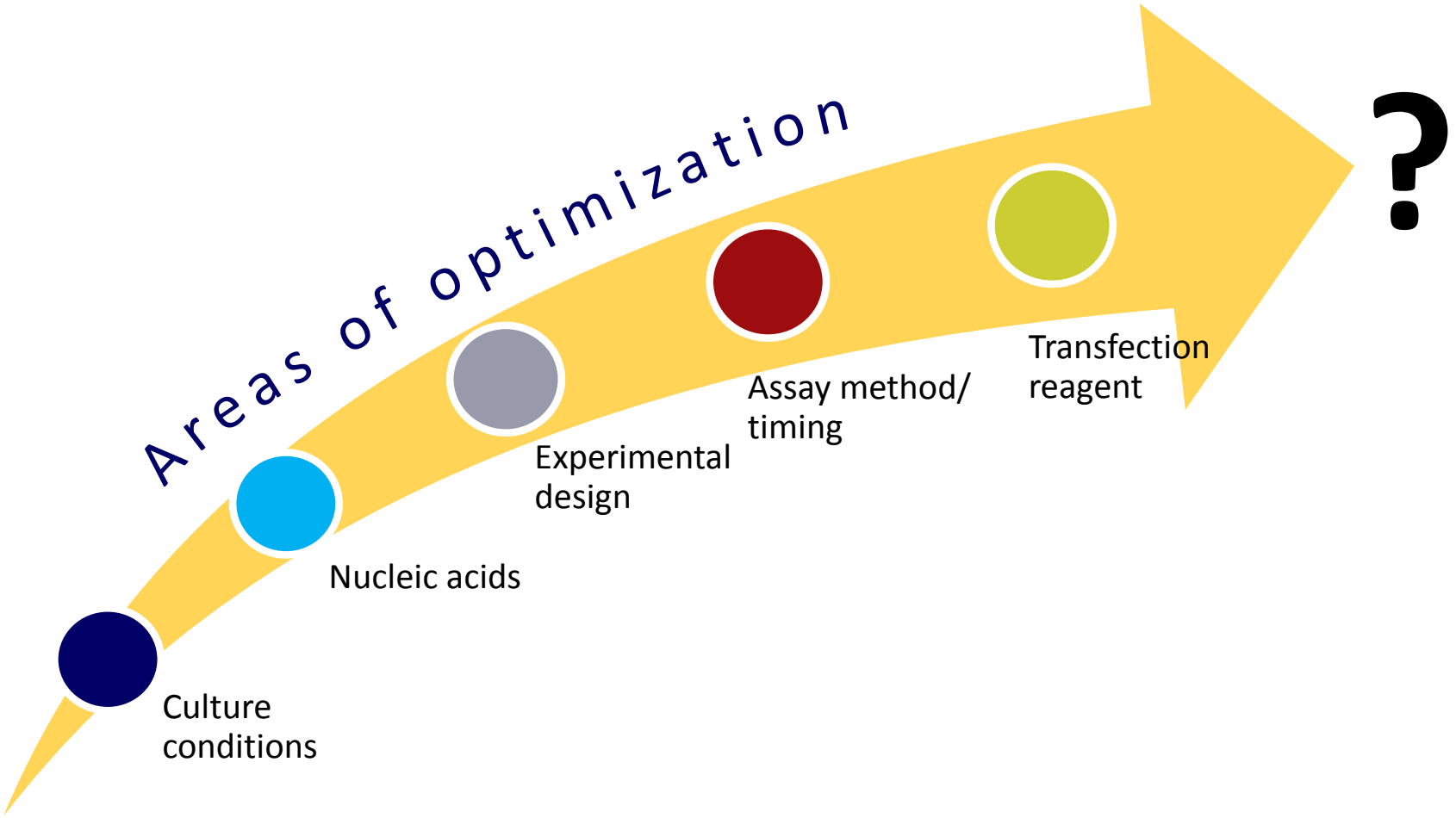
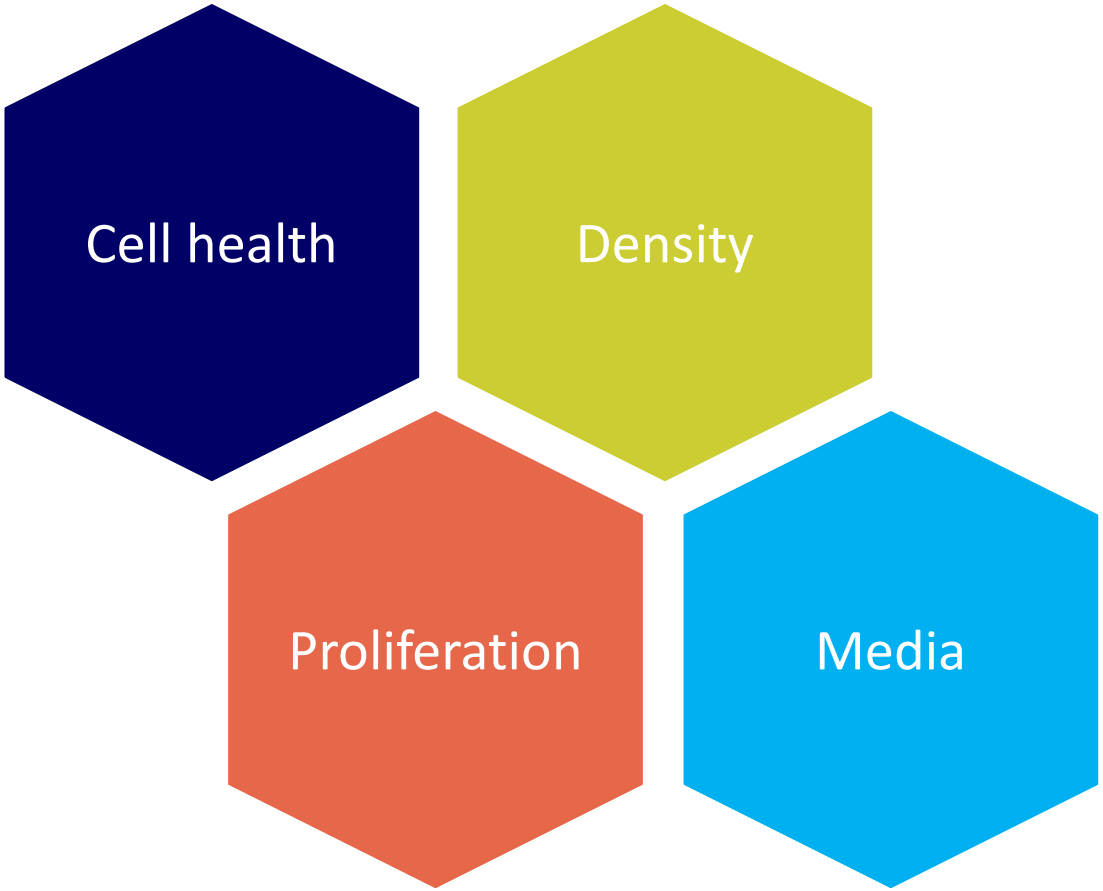
Transfection: Best practices



Cell culture conditions



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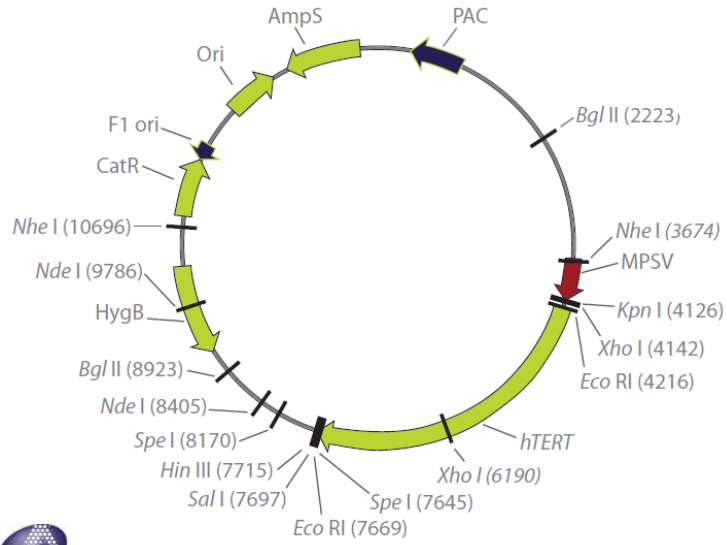
Nucleic acids

All nucleic acids

- High purity
- Endotoxin free
- Validated



Plasmid DNA

- Promoter
- Plasmid size
- Conformation

RNA

- Chemical modifications
- Pooled siRNAs

ATCC hTERT Immortalized Cell Culture Guide, 2014.

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

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Assay methods

mRNA

- Real time RT-PCR

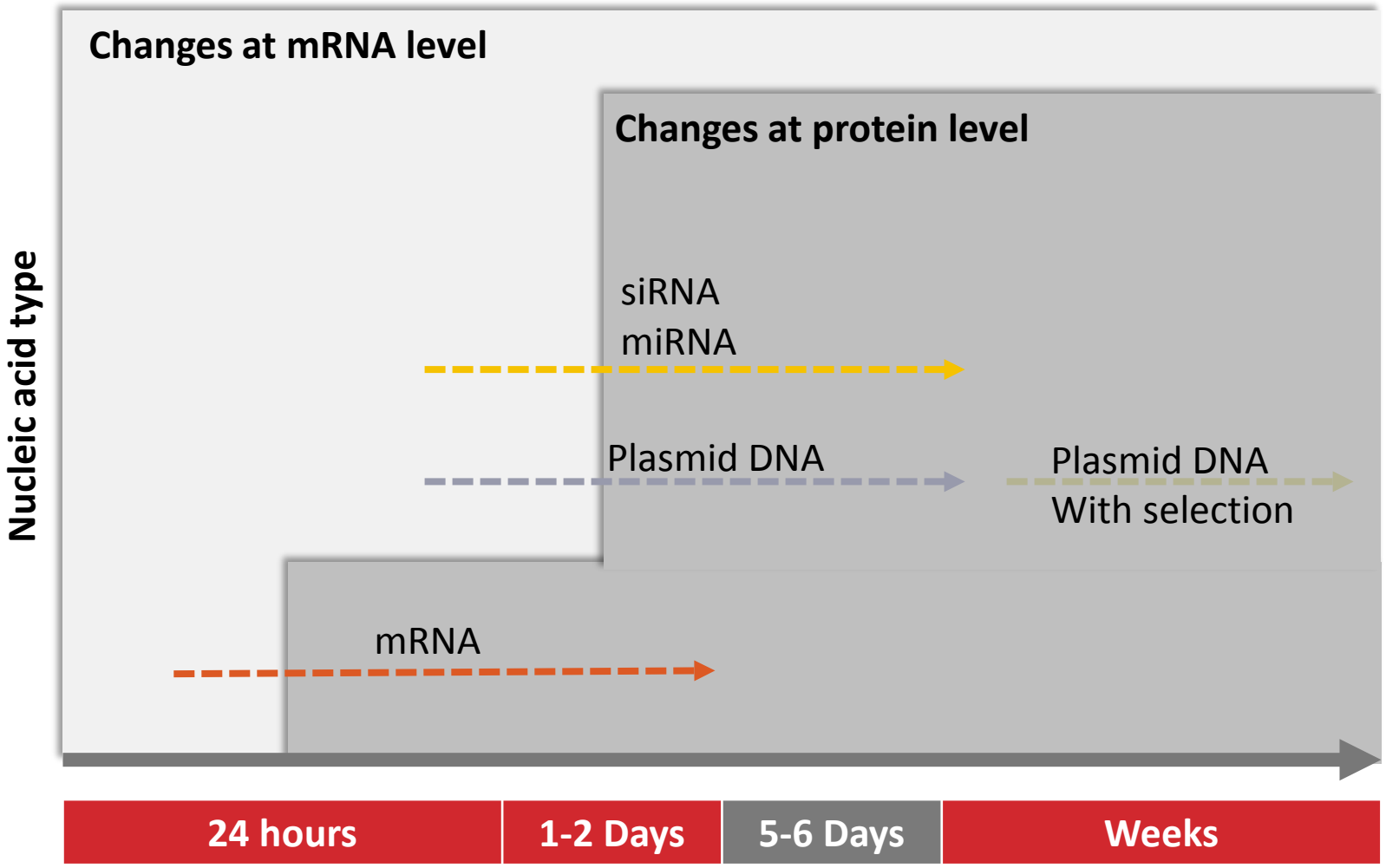
Protein

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

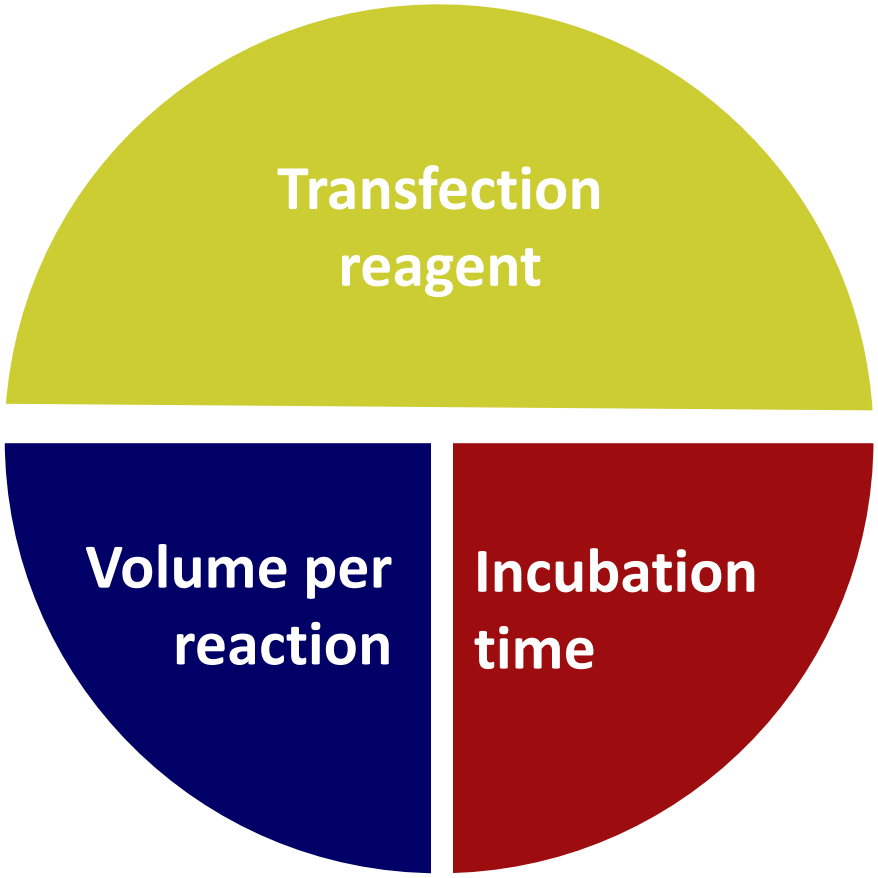
Other

- Morphology
- Functional

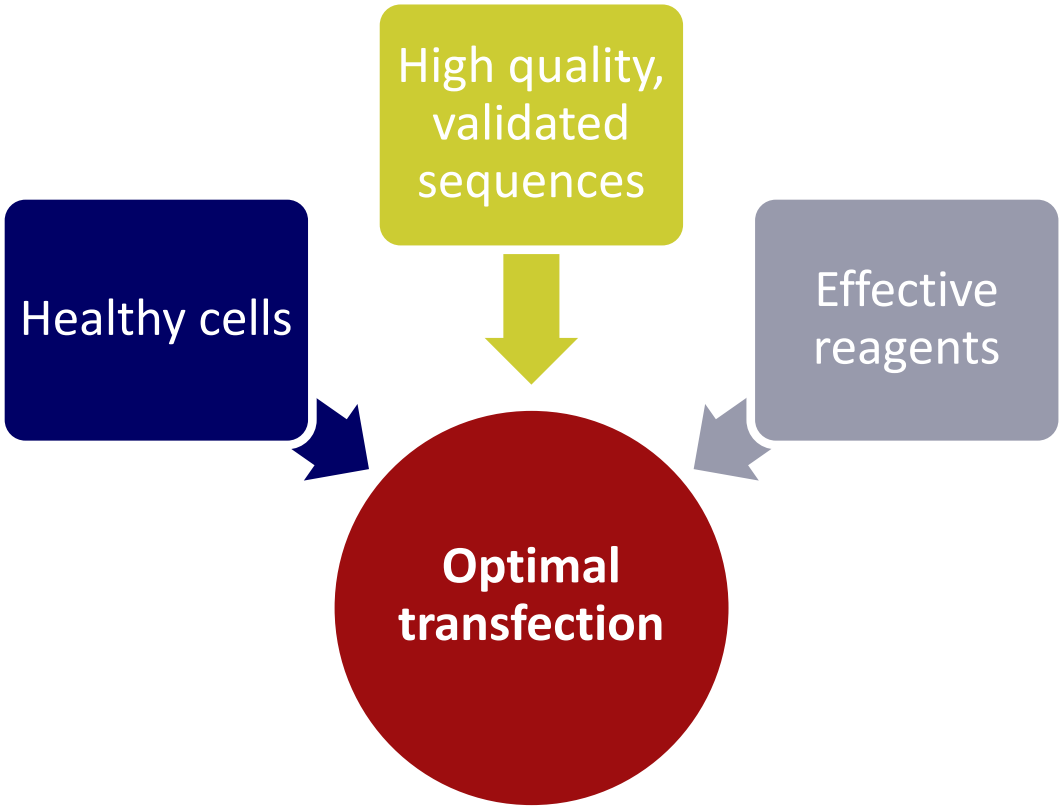
Assay timing



Transfection reagents



Best practices summary



Minimum optimization

1. Seeding density
2. Volume of transfection reagent
3. Amount of DNA/RNA

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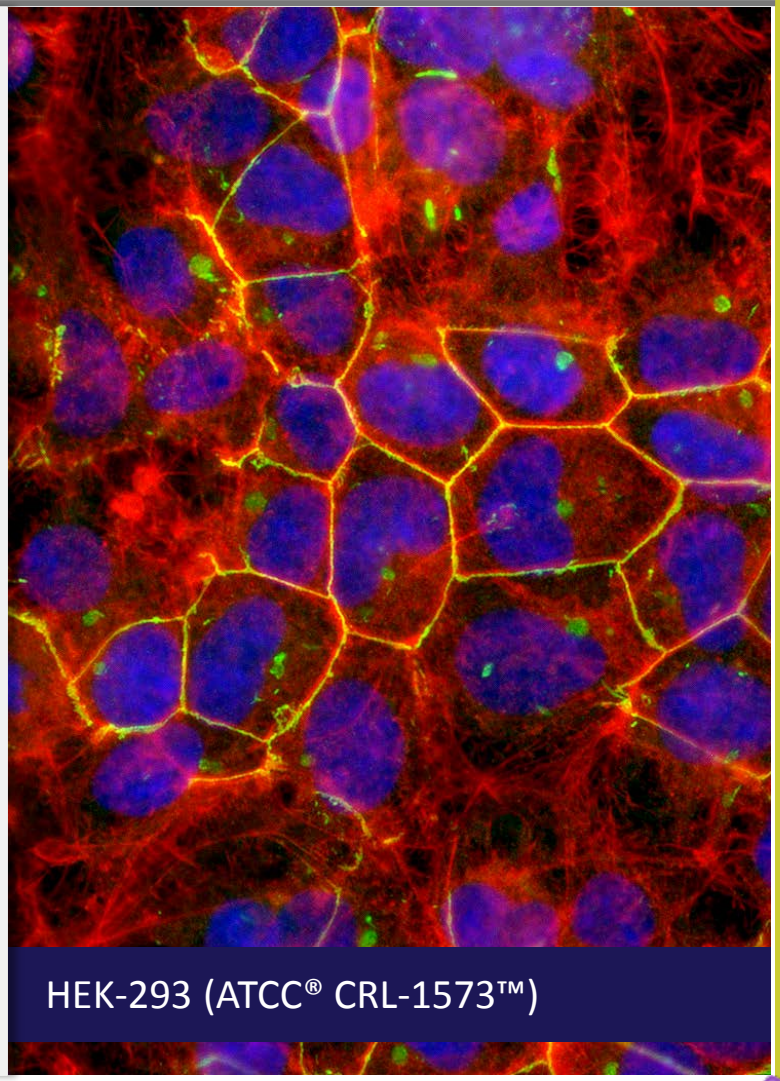


HEK-293 (ATCC® CRL-1573™)

Viability assays

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

- Commonly used for cytoxicity, 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™)

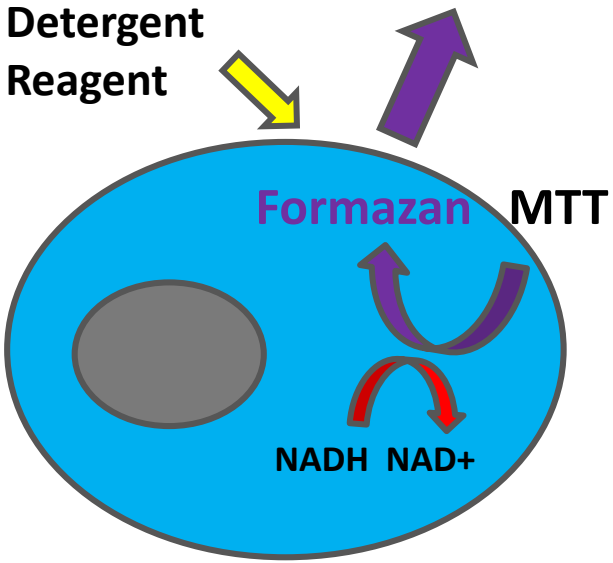
- Tetrazolium XTT (sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium

Viability assays

MTT Reaction

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

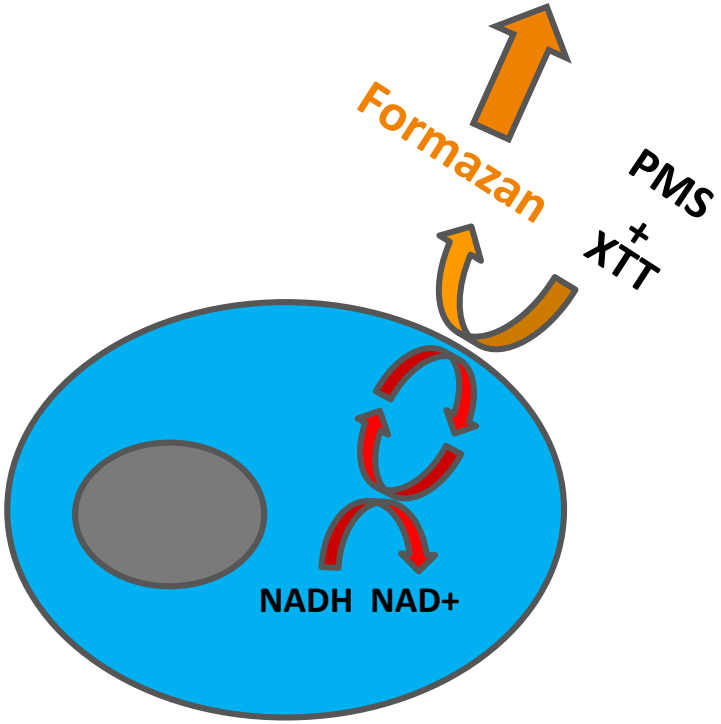
Media turns **PURPLE**



XTT Reaction

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

Media turns **ORANGE**



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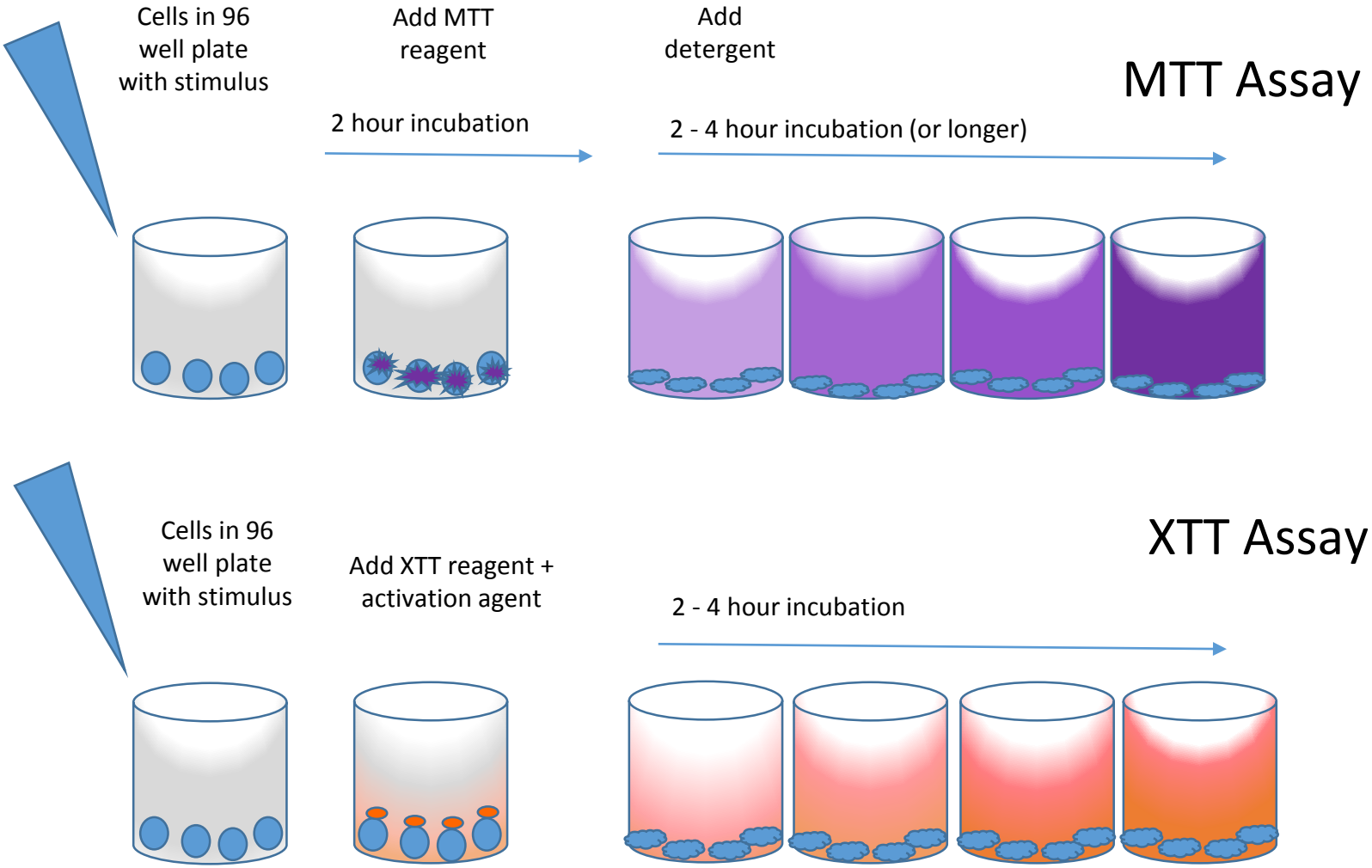
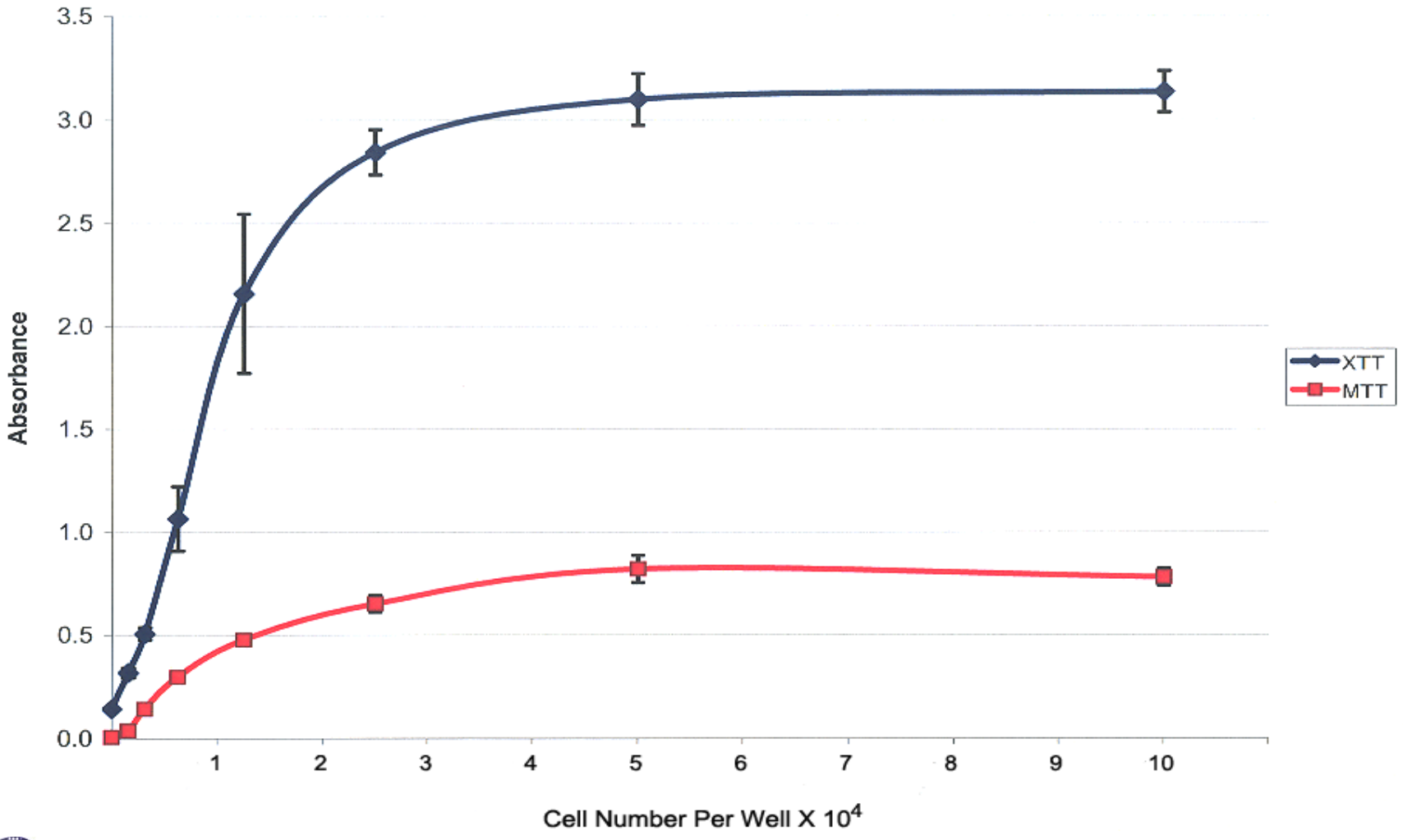
Viability assays

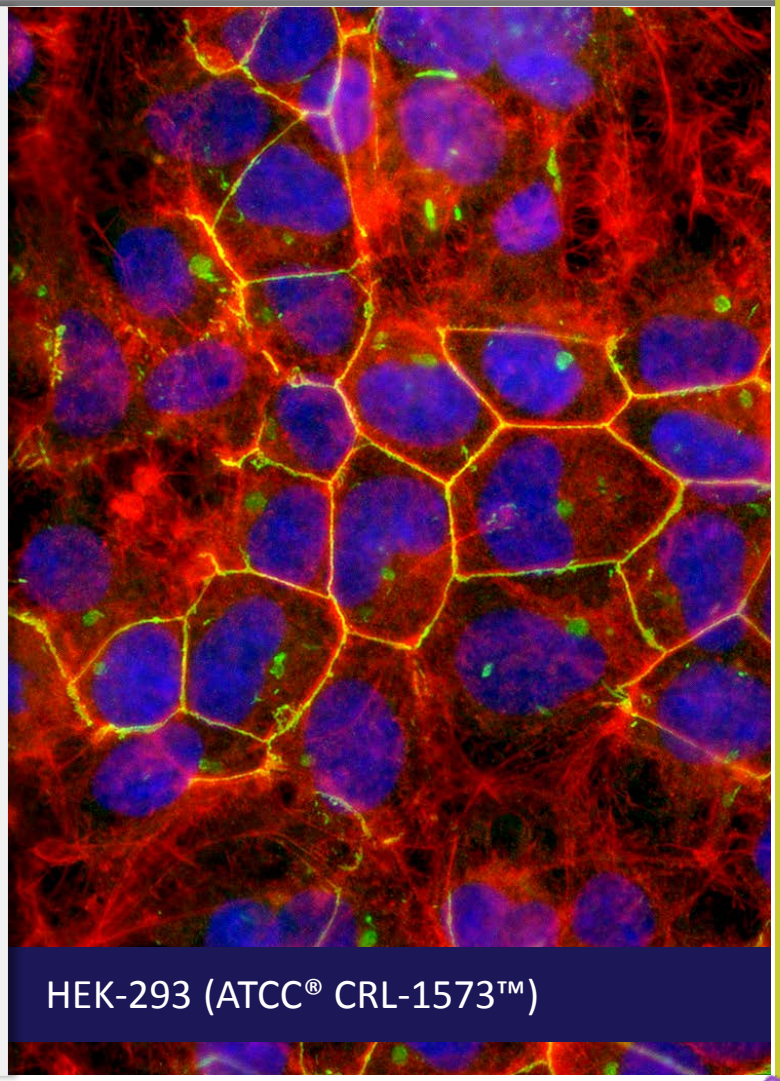


Viability assays



Agenda

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



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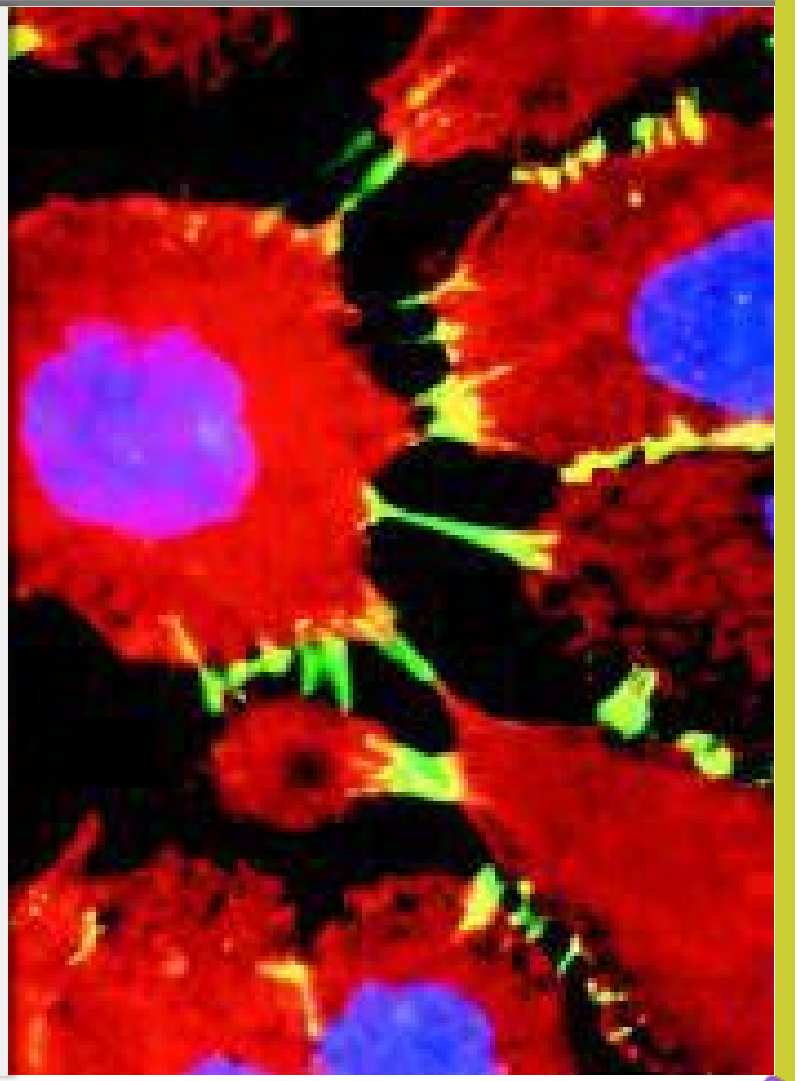
Disclaimers



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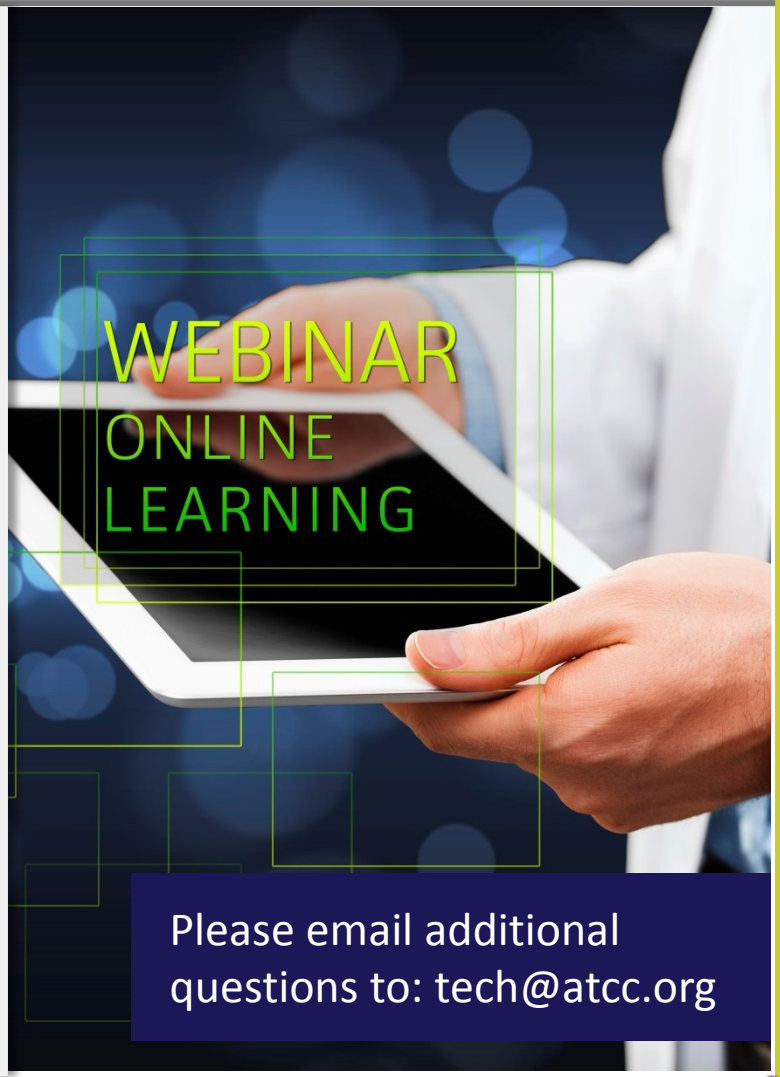

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