# Cell culture fundamentals: Your questions answered

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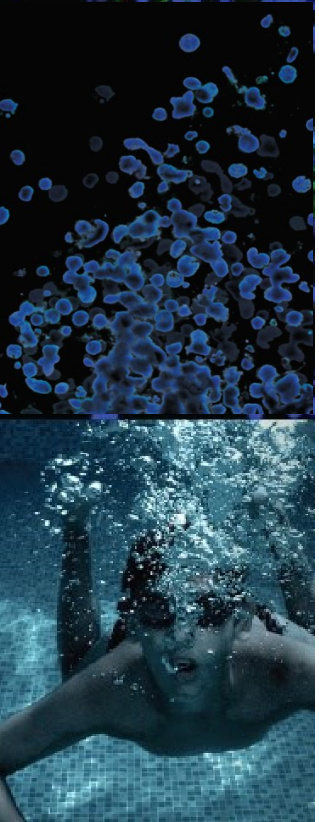
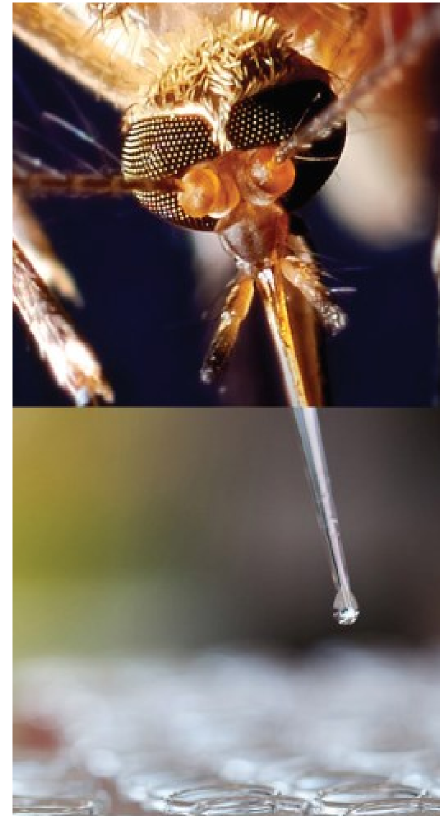
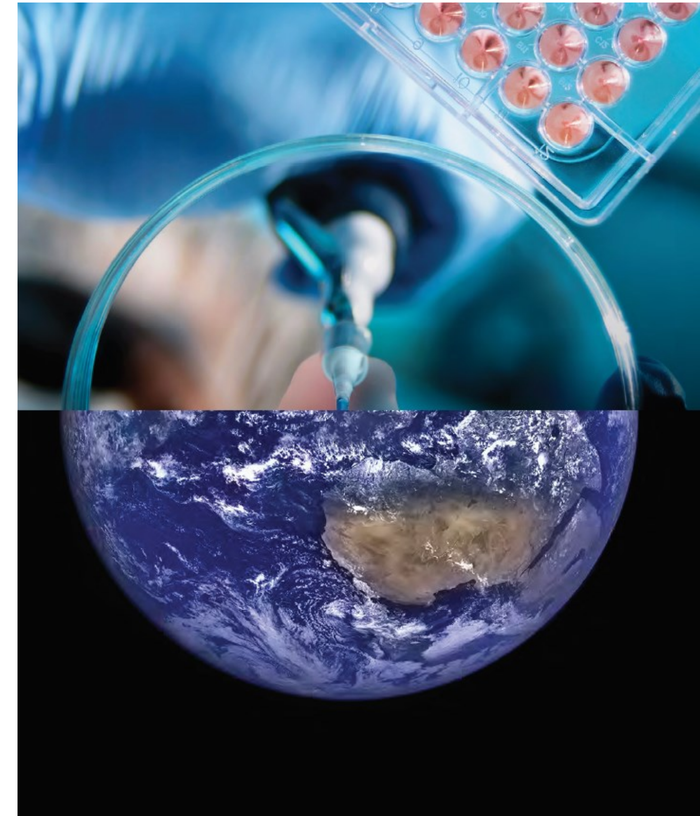


Credible Leads to Incredible™





# ATCC – Credible leads to Incredible

- ATCC has provided credible biomaterials for over 90 years
- We continue to cultivate collaboration
  - Among scientists across disciplines
  - Essential for accelerating innovative research
  - Leading to incredible, high-impact results
- Our Cultivating Collaboration pledge: We bring scientists together to discuss
  - Breakthroughs in the state of science
  - Multidisciplinary approaches to key areas of research
  - Breaking the silos that impede research
- Our partnership with you, the scientific community, allows us all to reach the incredible

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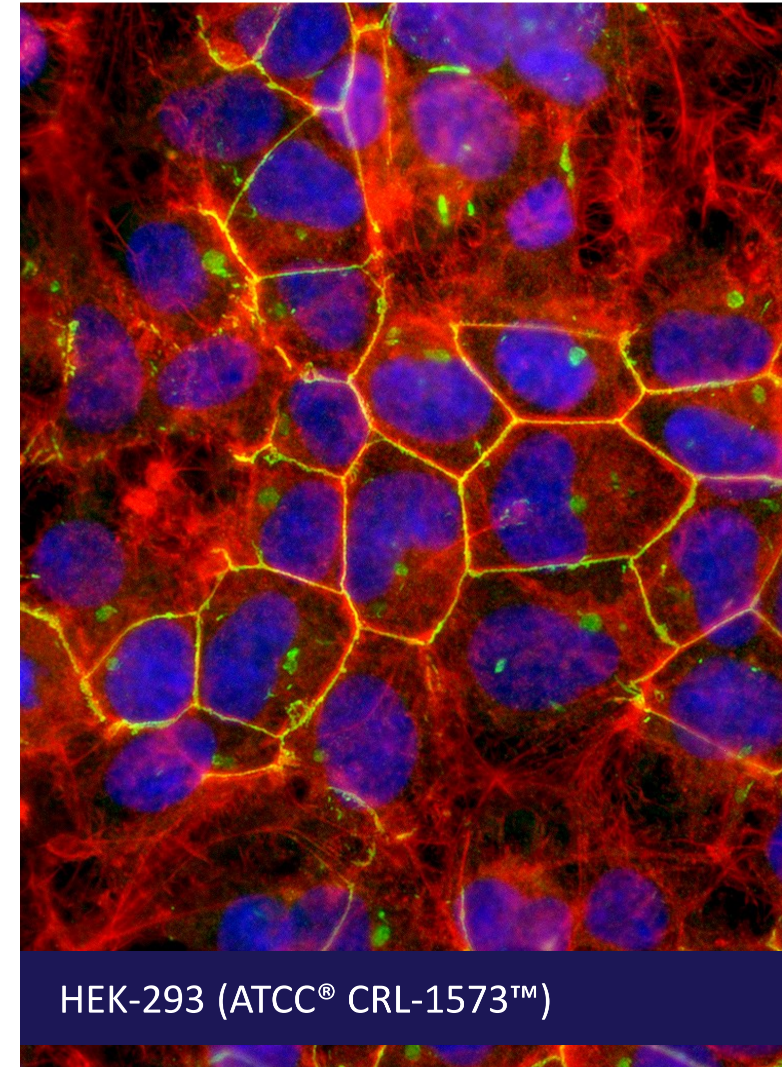
# Agenda

- Cell culture workflow - what cells to use
- Cell handling/media handling
- Viability assays
- Summary


HEK-293 (ATCC® CRL-1573™)



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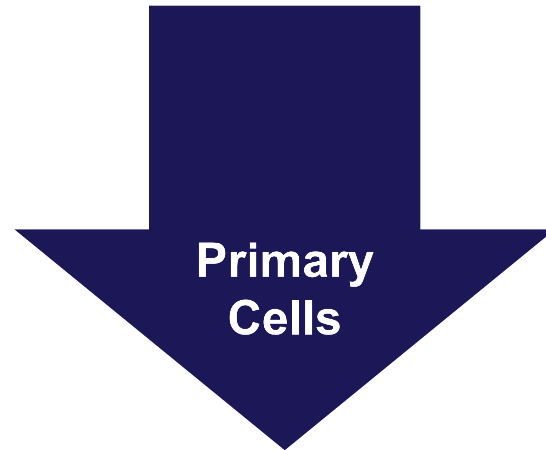
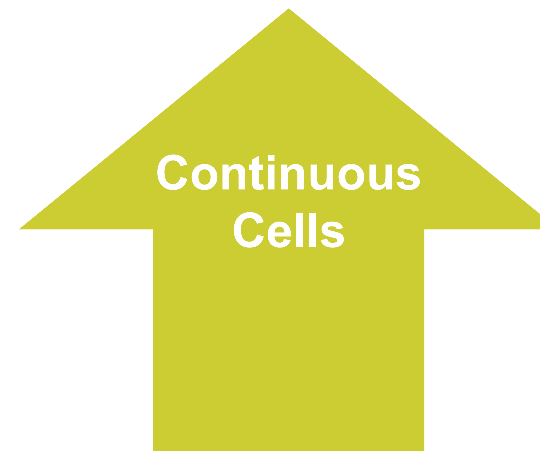
# Primary cells versus continuous cells




- 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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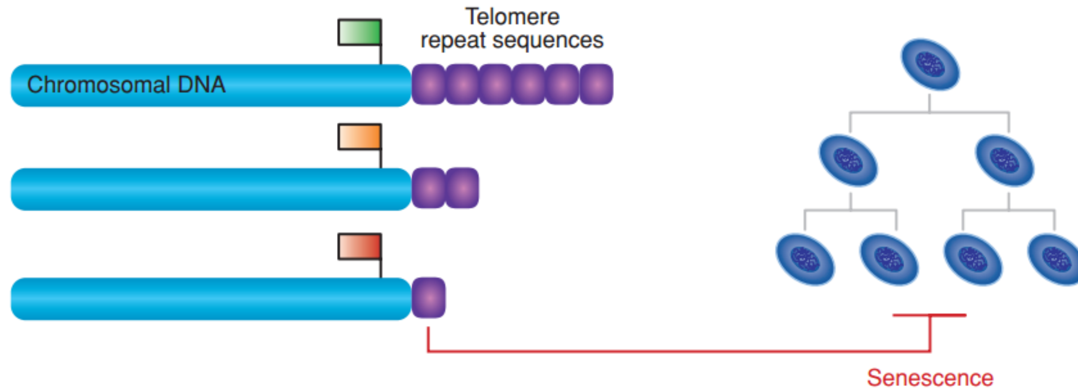
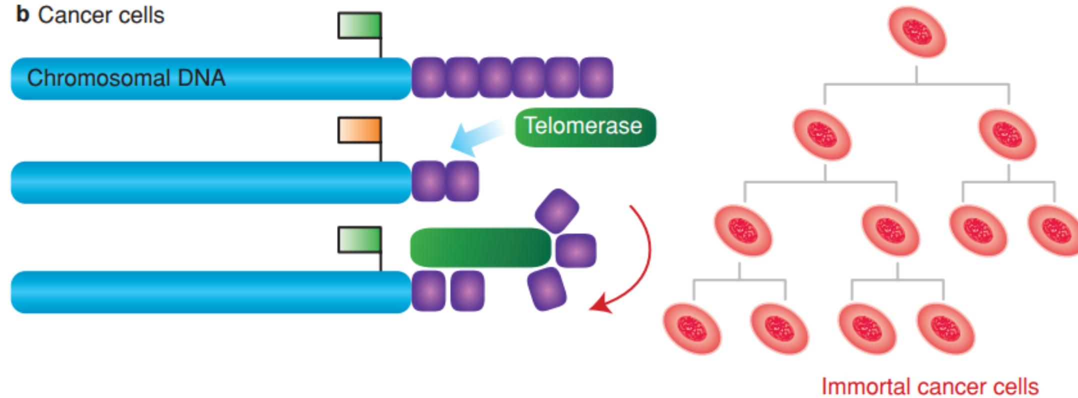
# hTERT-immortalized primary cells




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

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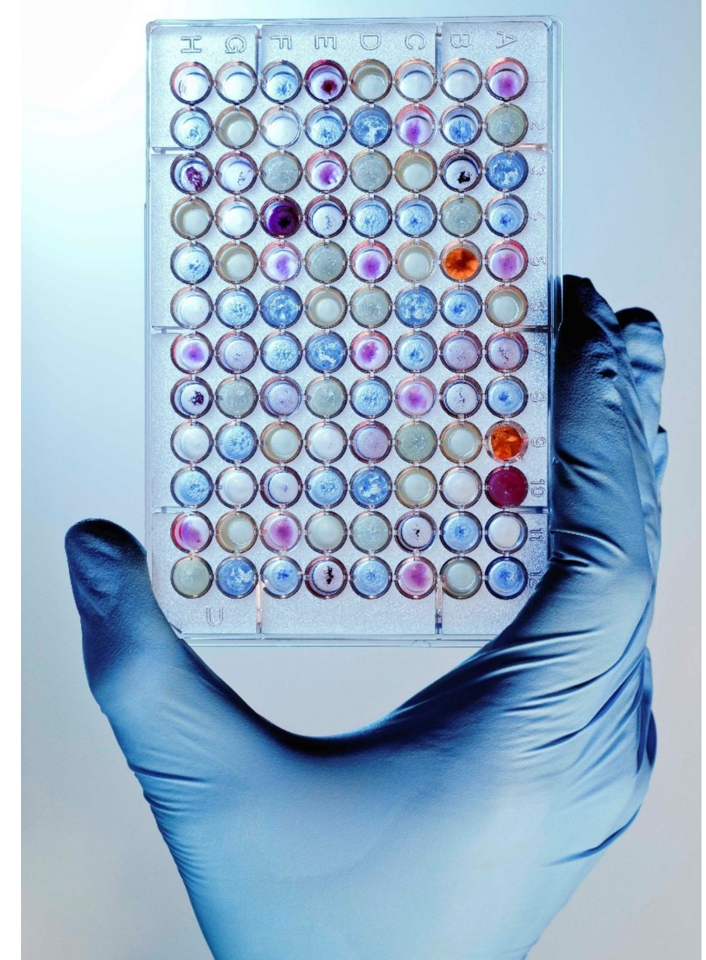
# Potential workflow situations: High-throughput screening

**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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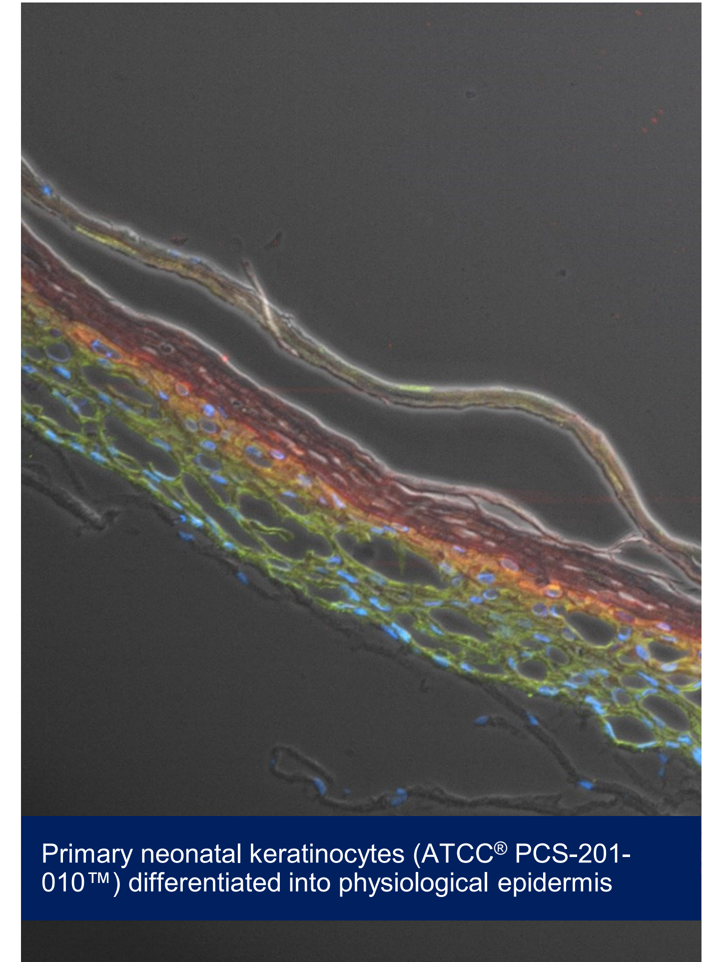
# Primary cells as a control

**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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# Cell handling/media handling

# 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

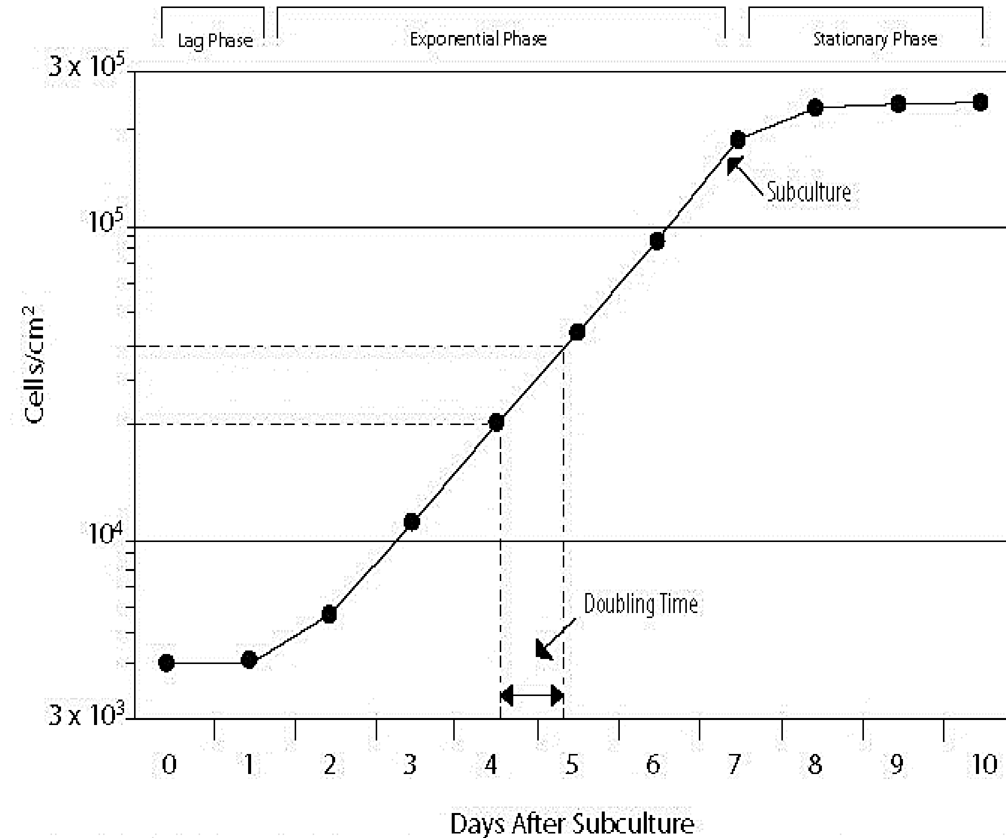


**Figure 1**. Growth curve for cells grown in culture. Cells should be subcultured while still in the exponential phase.

*ATCC Animal Cell Culture Guide, 2014.*



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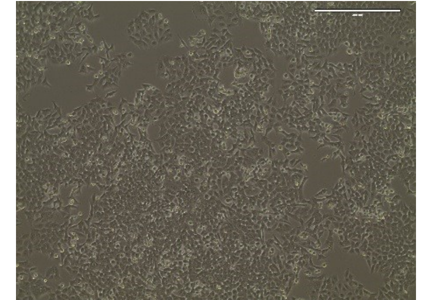
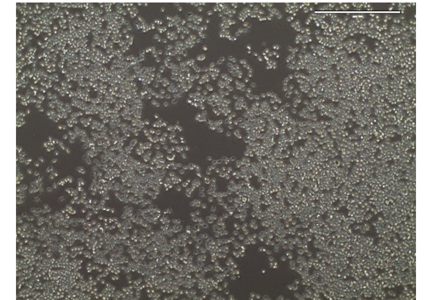
# Trypsinization

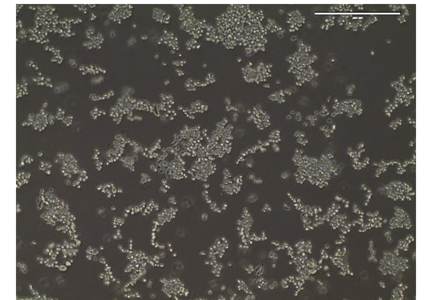
**At 80% confluency (primary cells), cells can be passed using Trypsin-EDTA**

- 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



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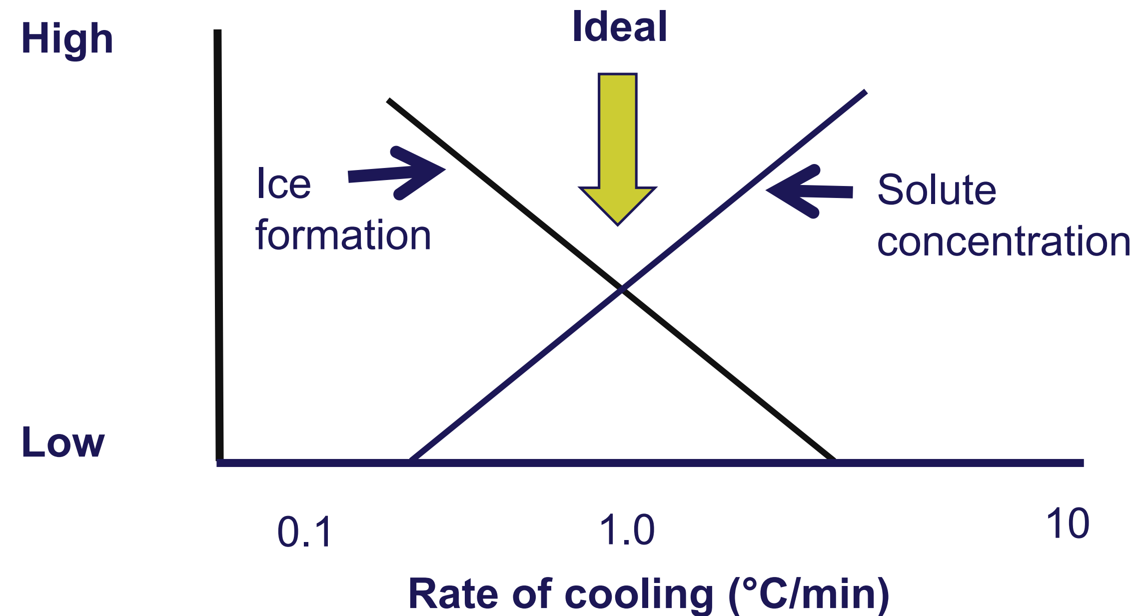
# Cryopreservation




- 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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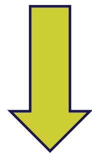
# Freezing down cells

**-70°C**

Controlled-rate freeze chamber

-1°C/min cooling rate

A few hours to 24 hours



**-140°C**

Liquid nitrogen tank

# Low temperature storage

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

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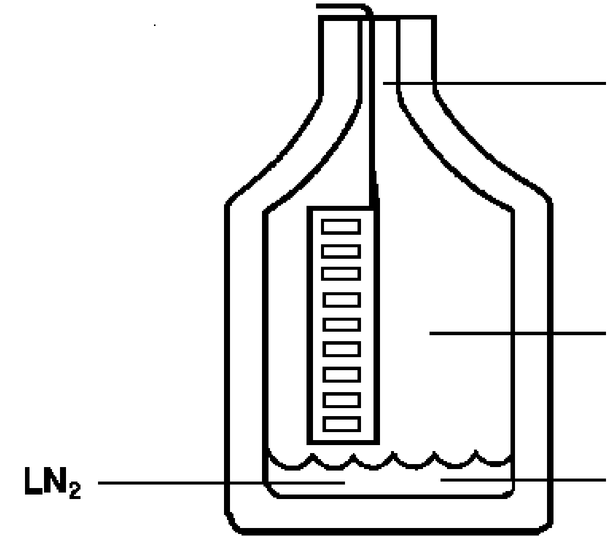
# Low temperature storage

**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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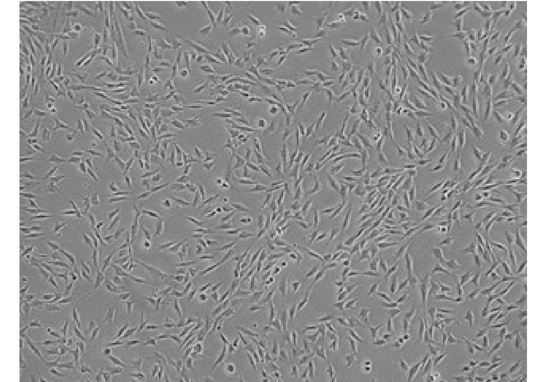
# Cell characterization

**Characterizing cells**

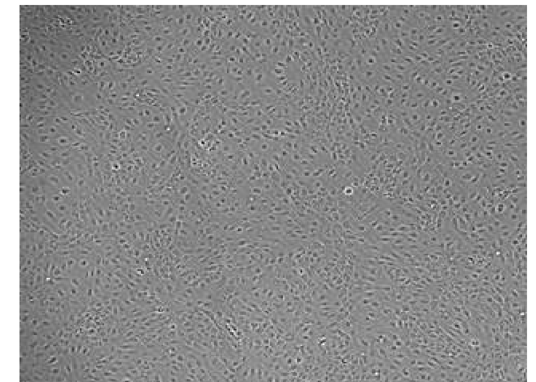
- Cell count before plating
  - Calculating % viability

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

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



Fibroblasts



HUVEC

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# Contamination

**Sources**

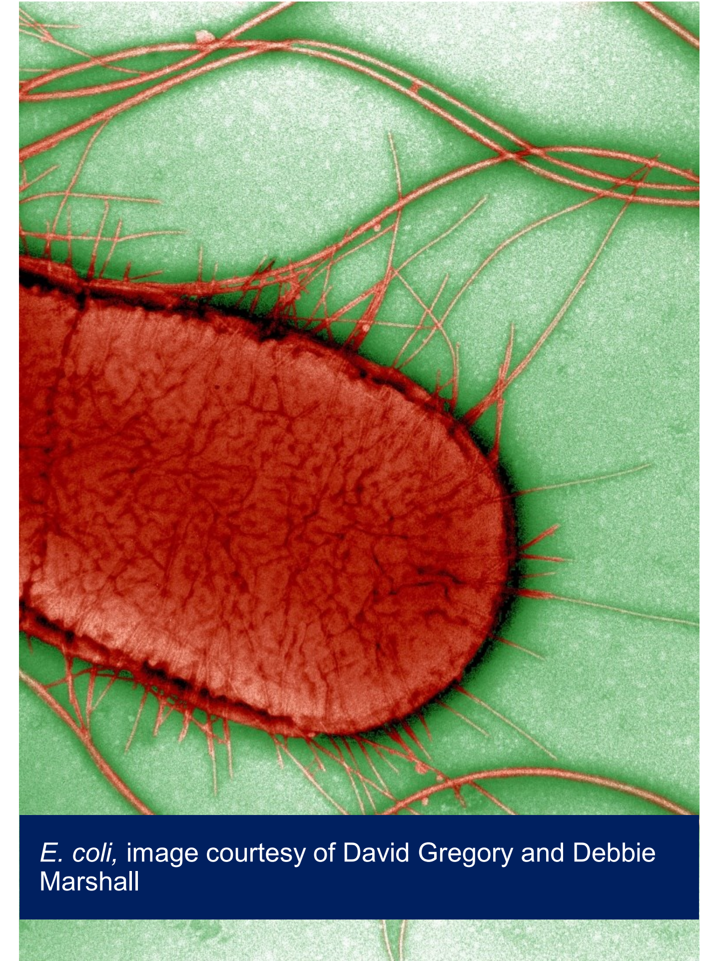
- Contaminated cell lines
- Improper aseptic technique

**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



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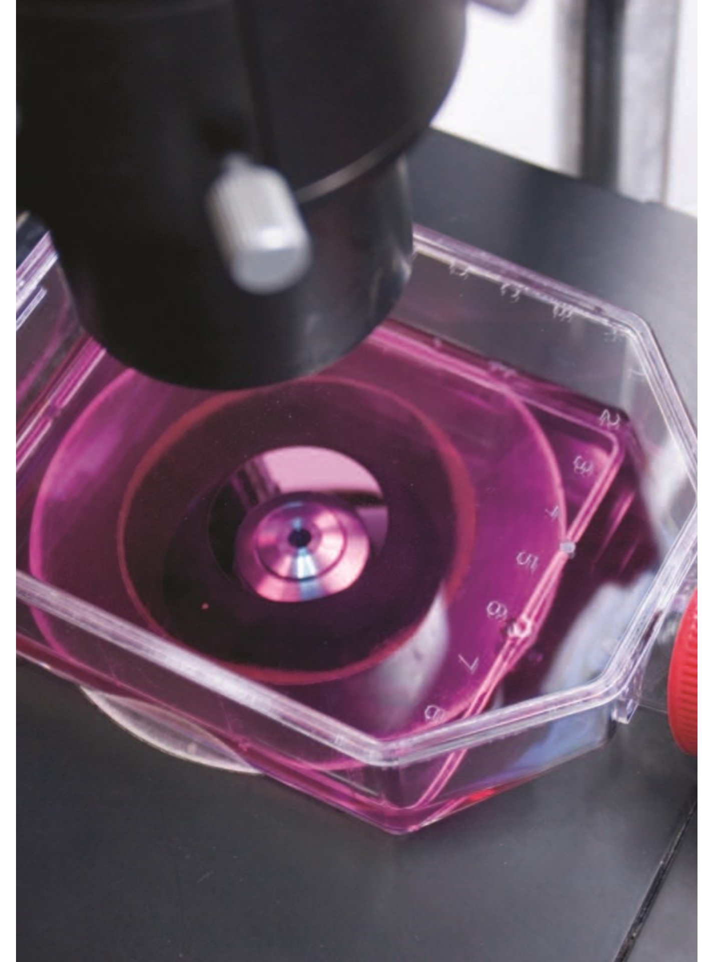
# Mycoplasma contamination

**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**

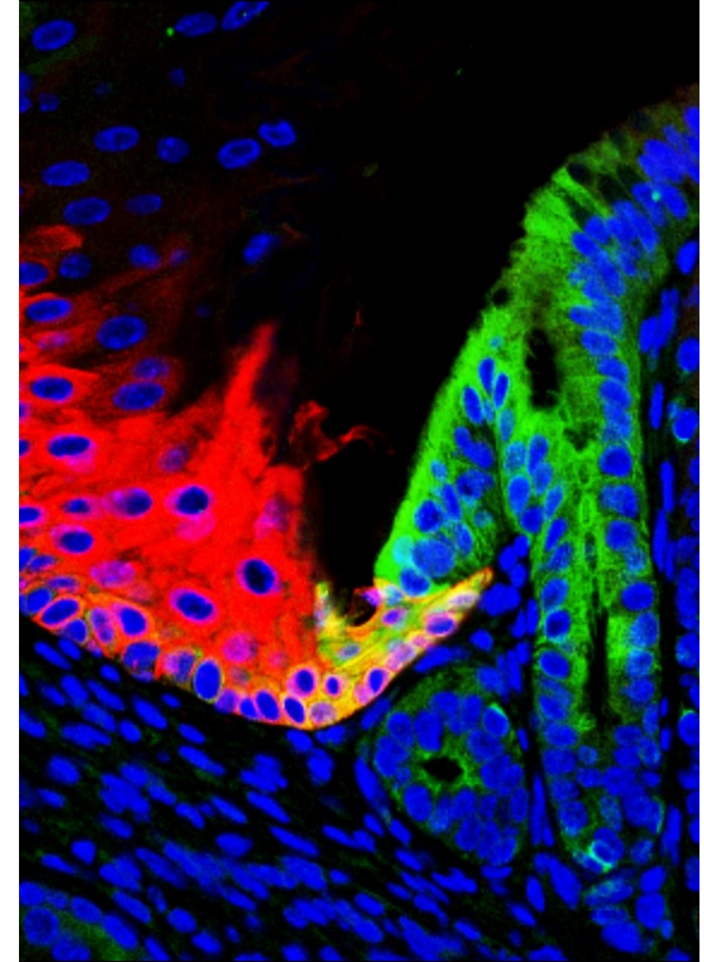
**Leads to the replacement of the original cell line with the contaminant**

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**



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



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# Cell characterization

**Universal Mycoplasma Detection Kit**

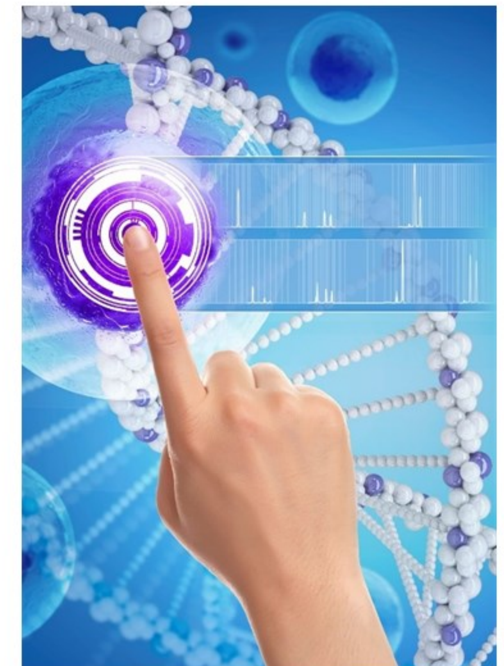
PCR-based kit (ATCC® 30-1012K™)

Detects any of the 60 most common mycoplasmas

**ATCC STR Profiling (Human and Mouse)**

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**

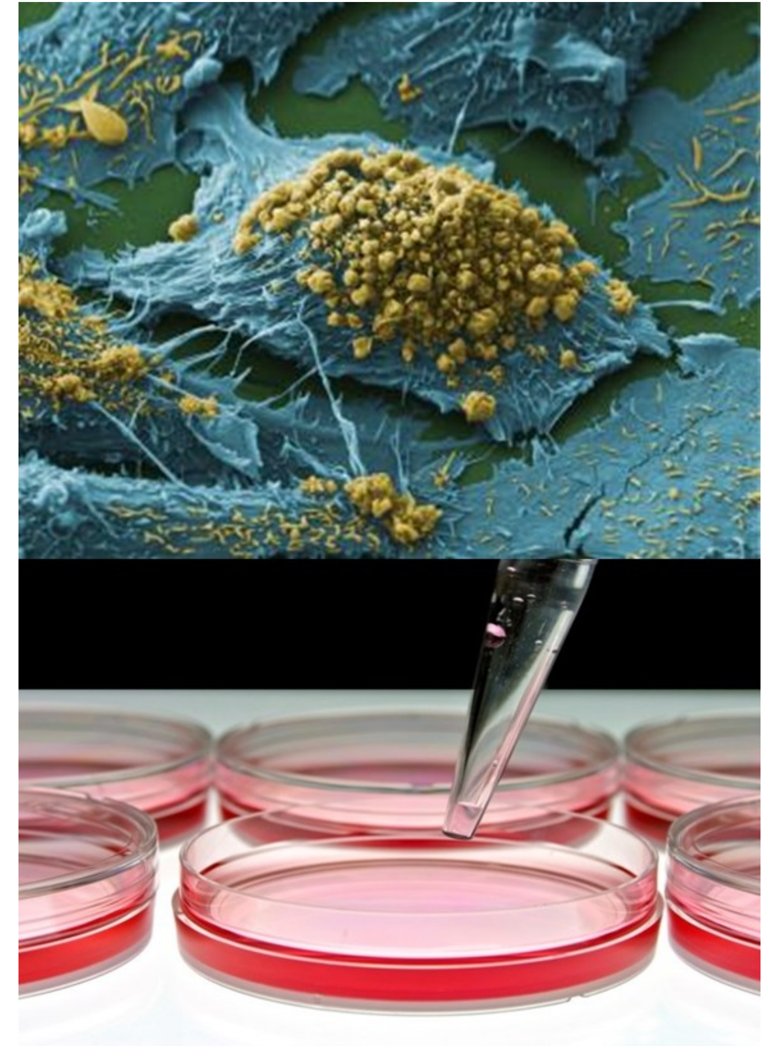
- Poor culturing practices
- Dust and aerosol

**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

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# 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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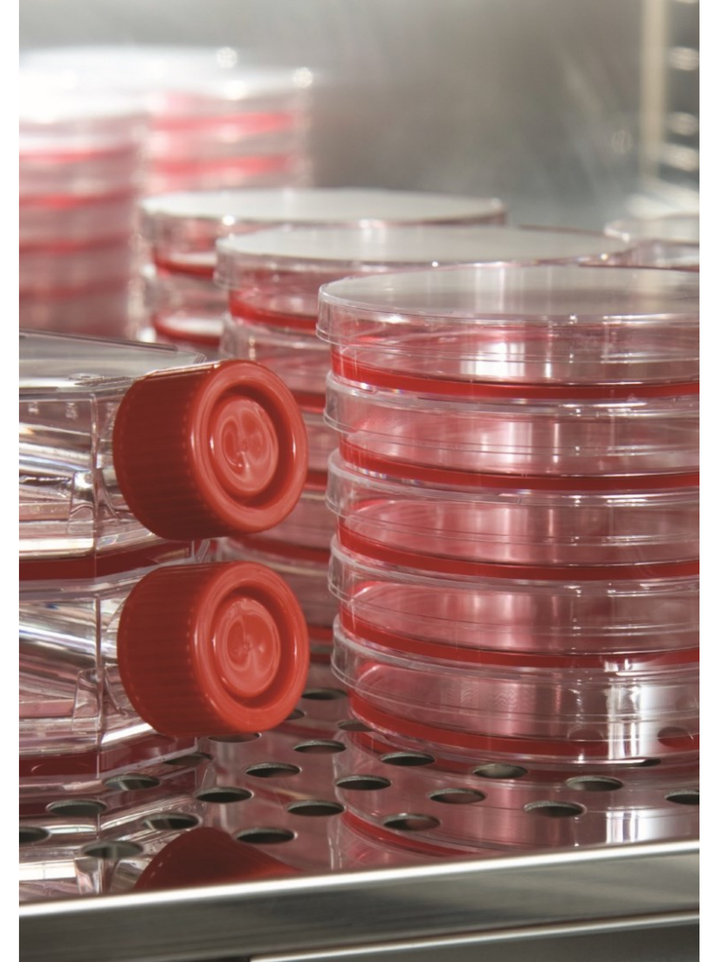
# Media choices

**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





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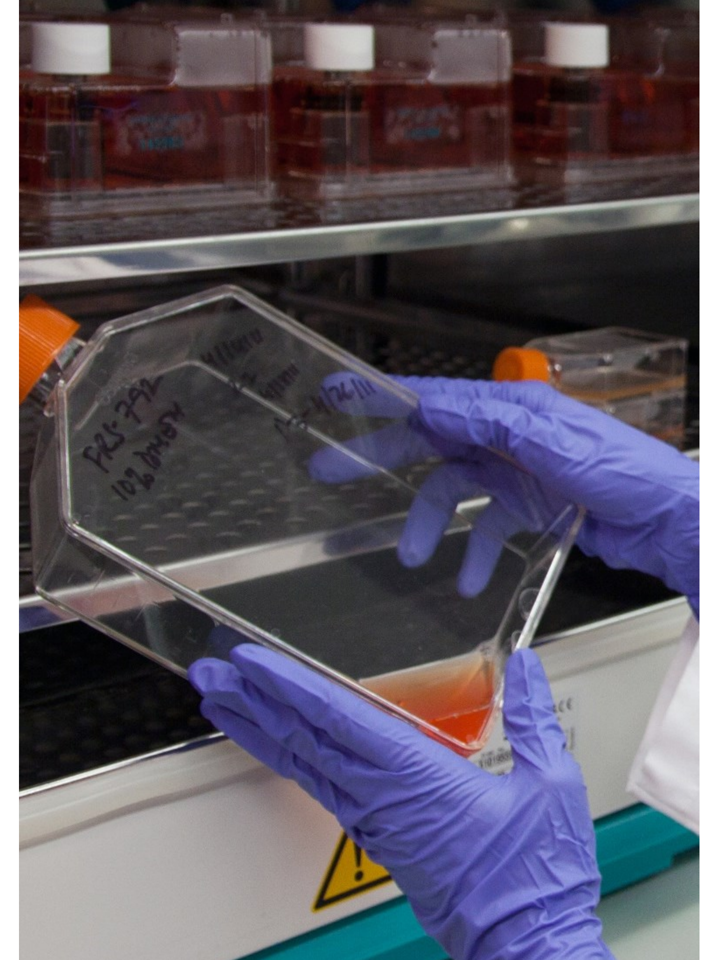
# Media choices

**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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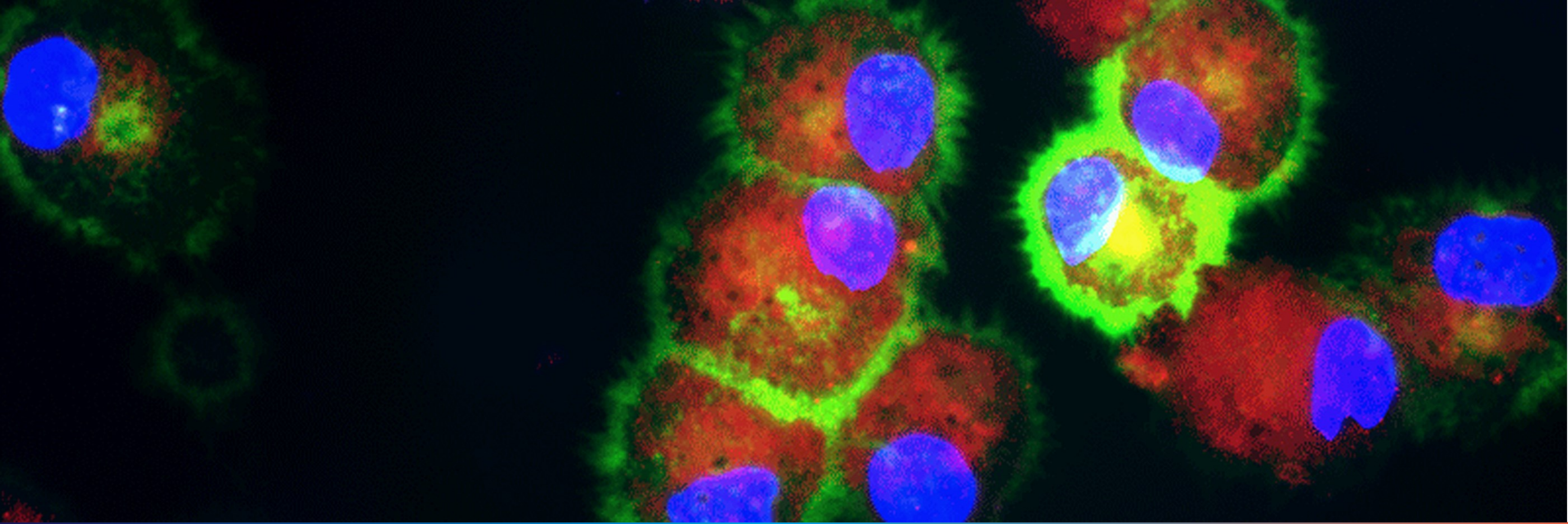

# Viability assays



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# 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



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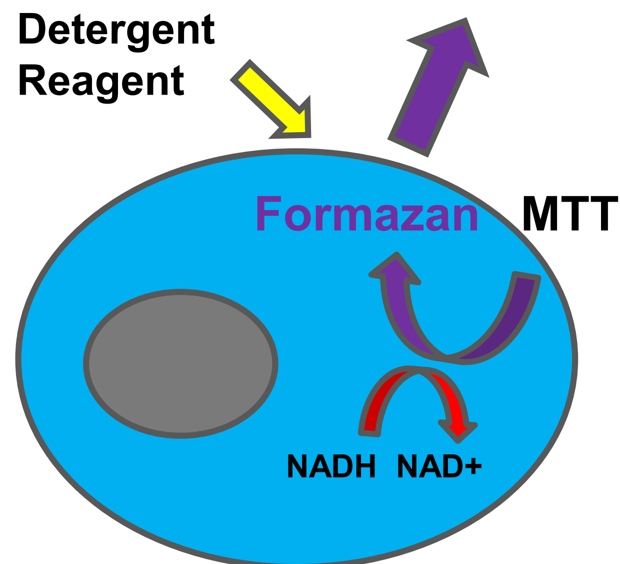
# Viability assays

### MTT Reaction

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

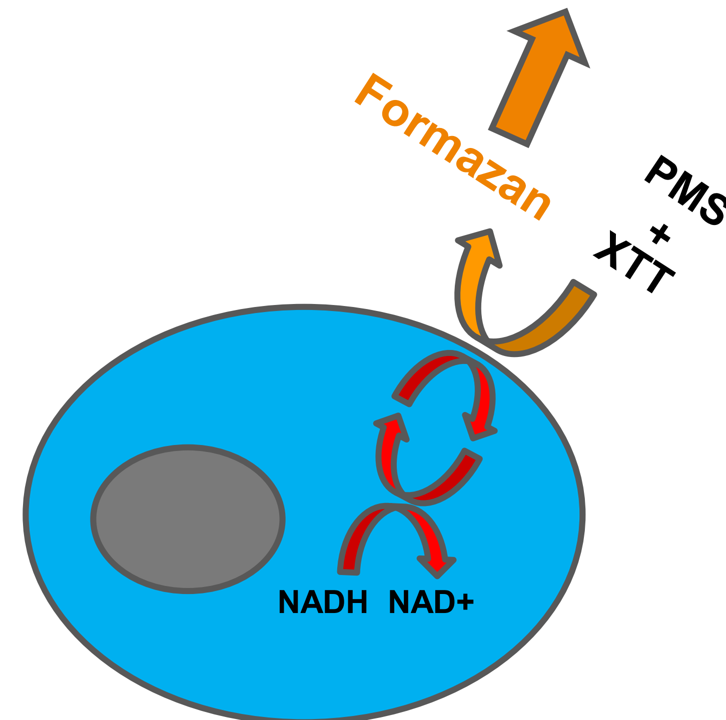
Media turns **PURPLE**




### XTT Reaction

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

Media turns **ORANGE**



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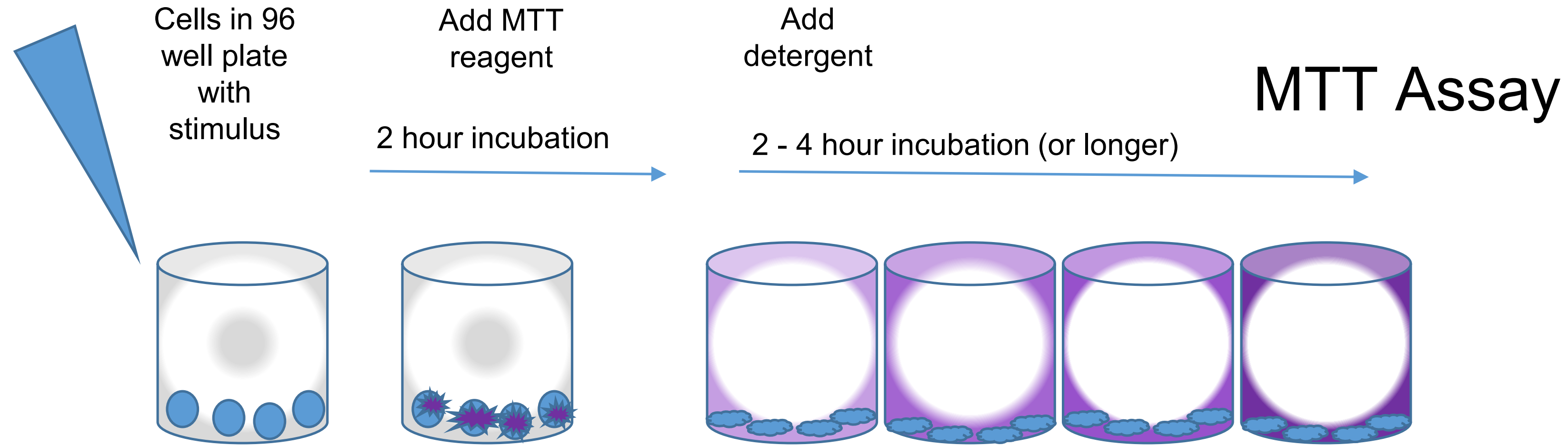
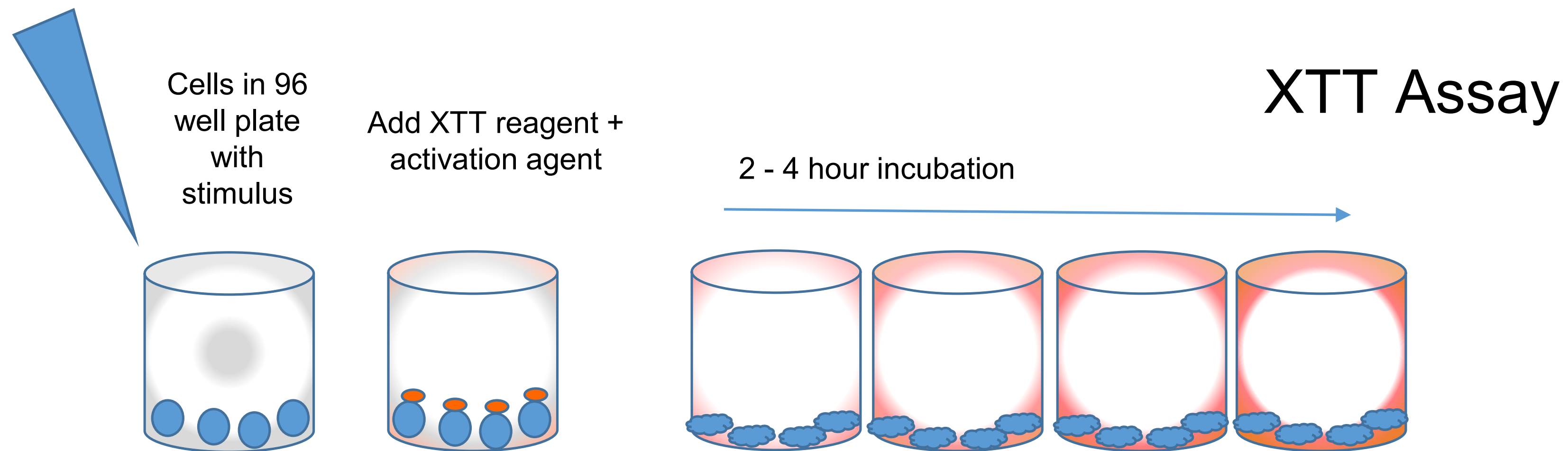
# Viability assays



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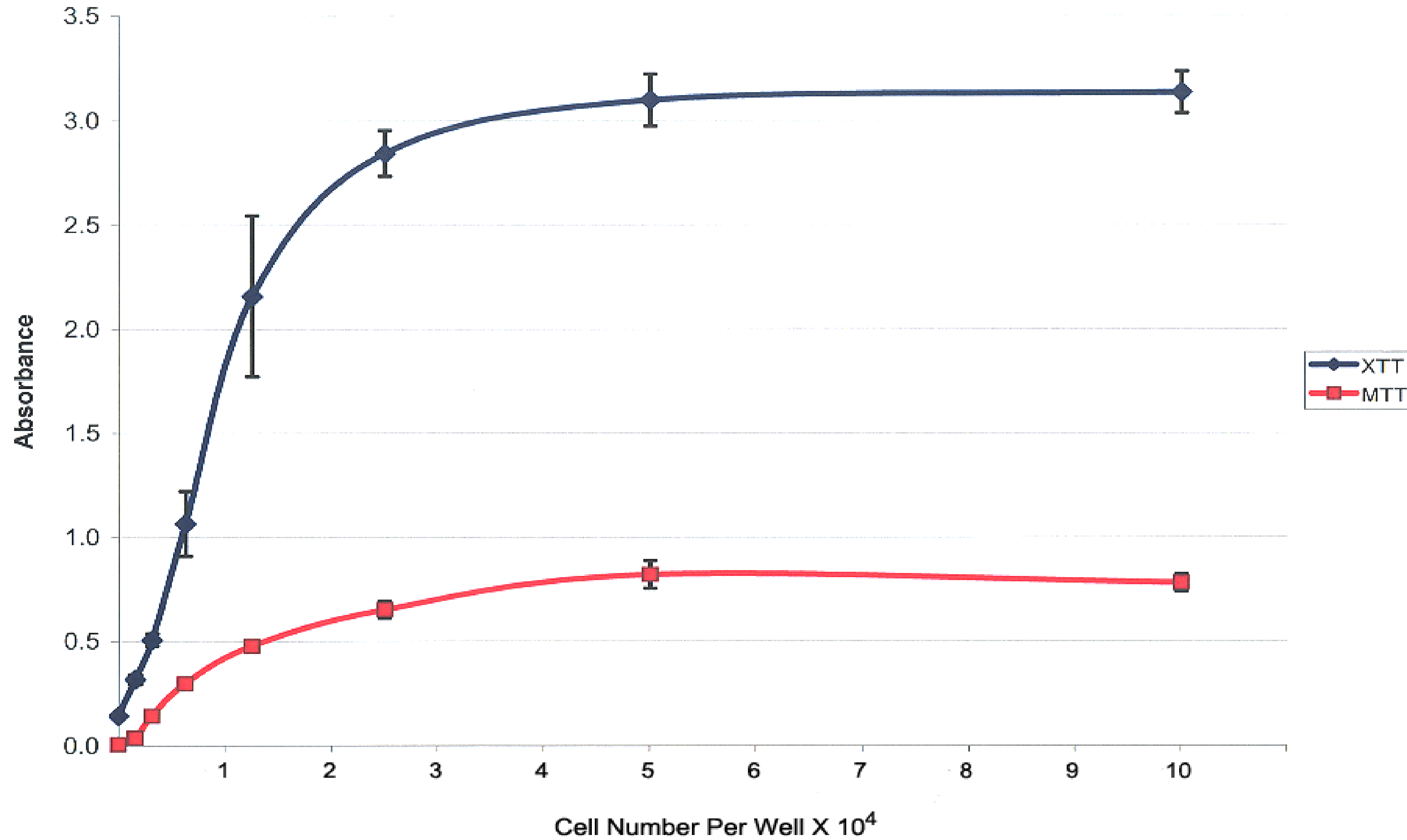
# Viability assays

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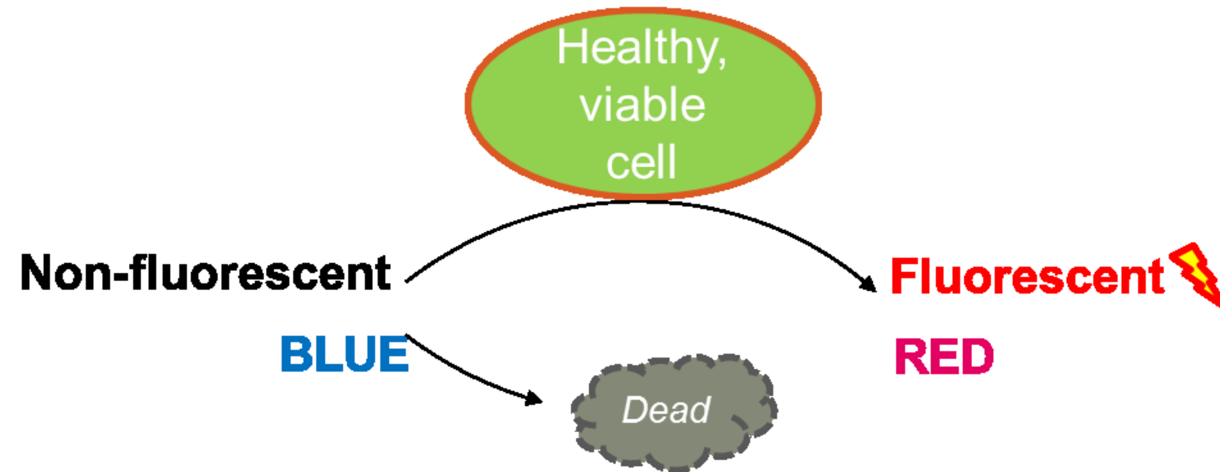
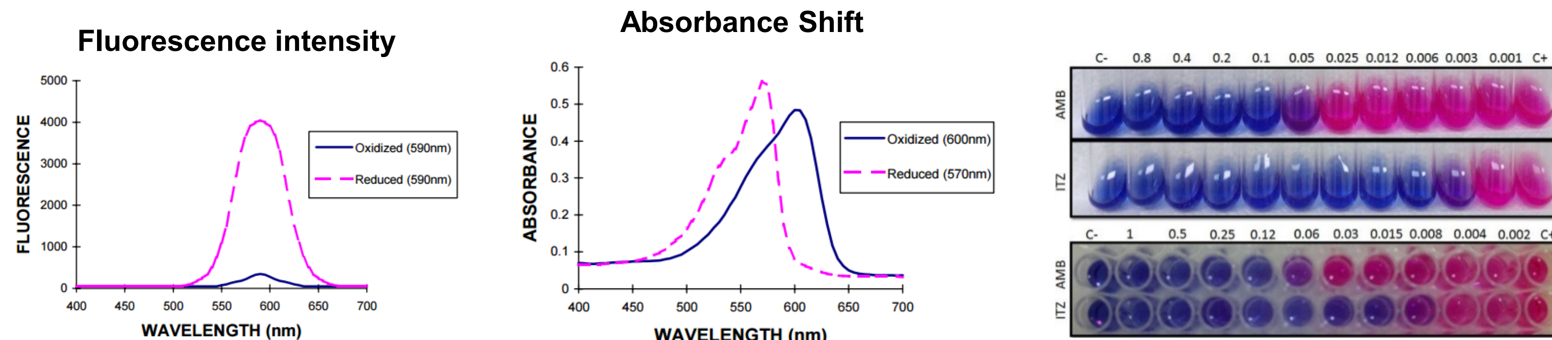
# Reliablue™ Cell Viability Reagent

Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a blue dye that is weakly fluorescent until reduced (redox) at which point it becomes pink and highly fluorescent.




Resazurin is cell permeable but non-toxic and is metabolically reduced by living cells (but not dead cells or in the culture media) resulting in a change in absorbance and increase in fluorescence.



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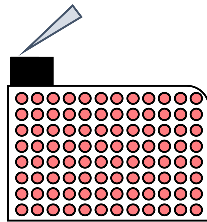
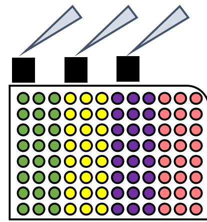
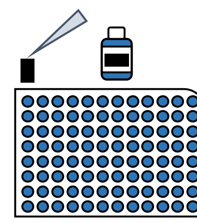
# Reliablue™ Cell Viability Reagent

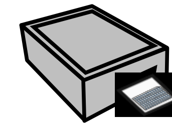
Reliablue™ Reagent is supplied in a 10X ready-to-use format that can be added directly to cells, typically in multiwell plates. An overview of the workflow is shown below.

**Basic 4-Step Assay Workflow**





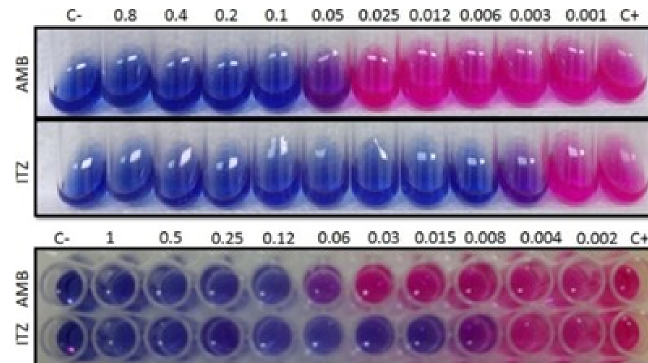




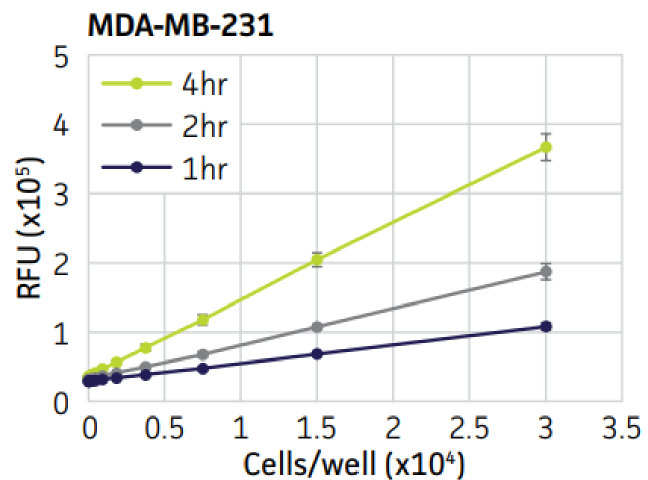
**① Grow cells** **② Add treatments** **③ Add Reliablue** **④ Read on plate-reader**

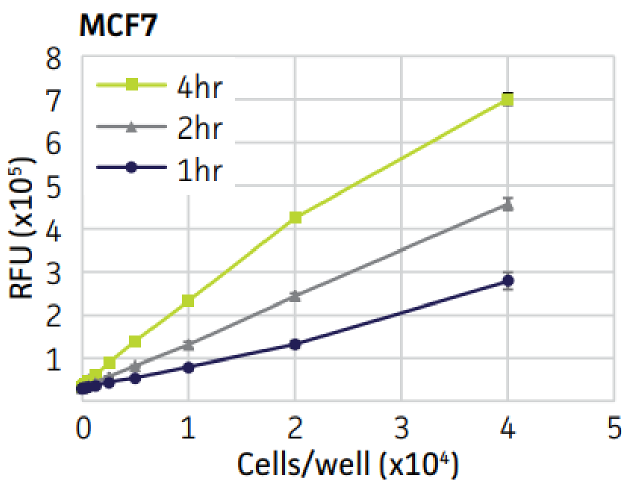
Blue = Dead (or control)

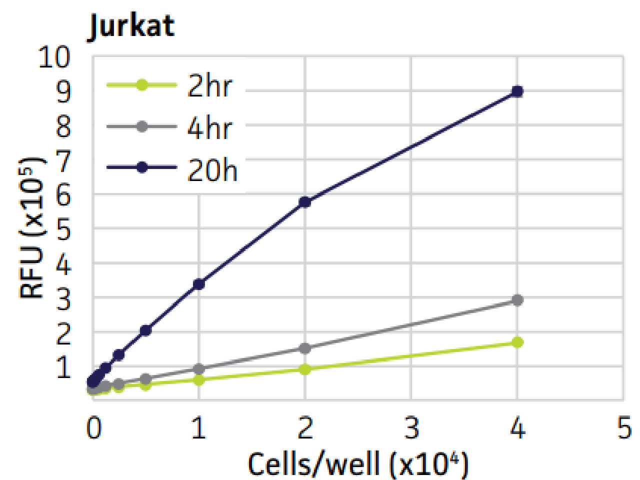
Red = Alive and Actively Growing

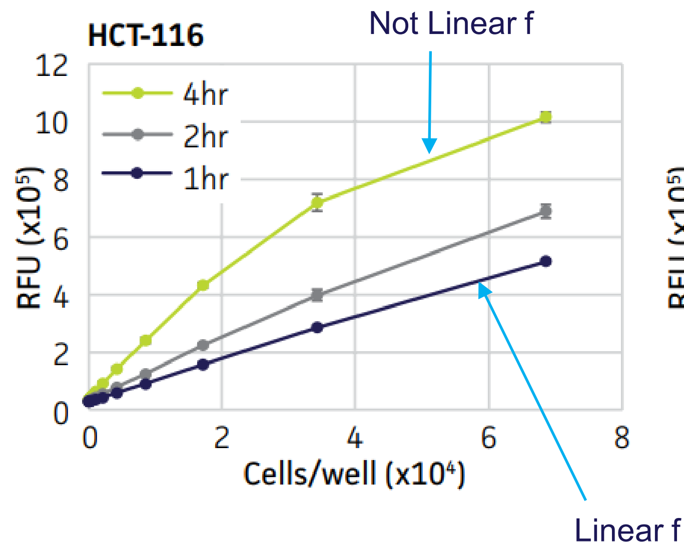


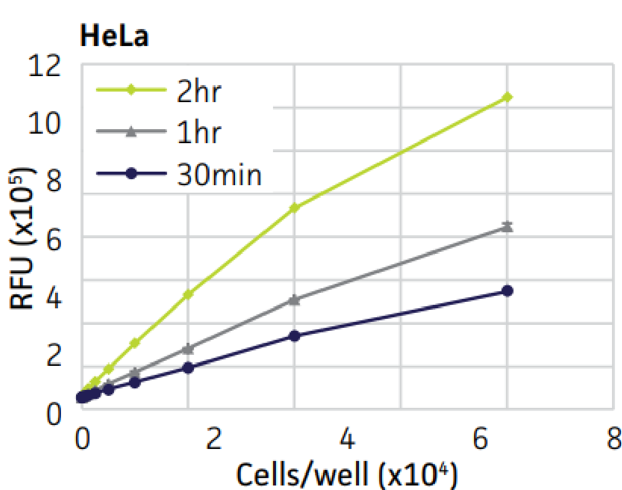
# Reliablue™-derived viability curves

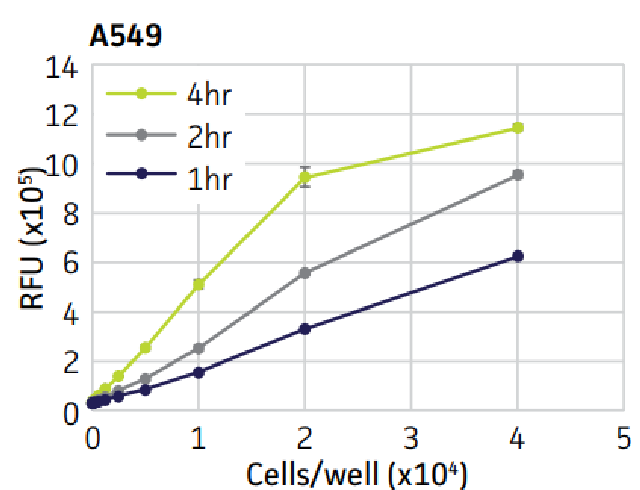


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# Summary



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# Summary

### Cell culture workflow

- Use cell lines for standardization and confirmation of each experiment
- Use primary cells after standardization to further validate the results
- hTERT-immortalized cells are the best of both worlds

### Cell Handling/media handling

- Be sure to employ best practices to eliminate contamination and ensure optimal growth and storage

### Viability assays

- MTT, XTT, and Reliablue can confirm cell growth characteristics

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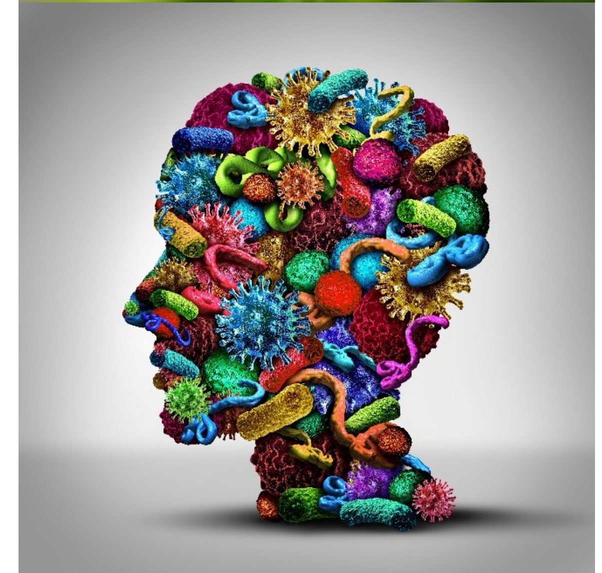
# Cultivating collaboration to support global health

**Upcoming webinars:**

- Tick-borne Diseases: Developing Molecular Approaches to Detect Multiple Pathogens | August 15, 12:00 ET
- Making Sense Out of Microbiome Data – The Importance of Standards | August 22, 12:00 ET

**www.atcc.org/webinars**



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