

# IMPROVING REPRODUCIBILITY: BEST PRACTICES FOR CELL CULTURE

## INTRODUCTION

It has been reported that an estimated 15-20% of cell lines used worldwide are misidentified,<sup>1</sup> and unauthenticated cell lines have resulted in several retractions.<sup>2</sup> The problem of cell line misidentification has been known since the 1960s, when isozyme profiling revealed that 18 cell lines had been contaminated with HeLa Cells.<sup>3</sup> Despite published records of mislabeled cell lines, thousands of citations have accumulated for studies using or citing data generated from these cell lines.

Cell culture is also fraught with problems that confound reliable data, such as mycoplasma contamination. Unlike yeast, for example, the presence of mycoplasma is not apparent from visual inspection. However, mycoplasma contamination can alter the behavior of a cell in unpredictable ways.

Despite the myriad of problems and consequences researchers may come across while running experiments, steps can be taken to validate cell culture, improve transparency and reduce risk.

## CELL CULTURE

In 2008, a pediatric research lab at the University of Texas MD Anderson Cancer Center published in the journal *Clinical Cancer Research* exciting evidence of a therapeutic target to suppress invasion and metastasis in osteosarcoma. The survival rate of osteosarcoma patients had not improved for more than 20 years, which made the newly identified target particularly compelling because pharmacological inhibitors for it already existed.<sup>1</sup>

However, five years and nearly 80 citations later, the authors retracted the paper in 2013. DNA fingerprinting had revealed that the cell lines, OS187 and COL, were actually the widely used NCI60 colon cancer line HCT 15 and a human neuroblastoma cell line, respectively. Further investigation revealed that a vial of OS187 dating back to the lab's second passage of the cells in 2002 also was misidentified.<sup>2</sup>

This retraction is only one of many similarly heartbreaking setbacks. Cell line misidentification has plagued research for 50 years, dating back to the discovery of HeLa contamination of 18 cell lines.<sup>4</sup> Yet, the problem persists. A PubMed query of six of the lines identified in the 1960s as HeLa-contaminated (KB, HEP-2, Chang liver, Int-407, and WISH) identified nearly 400 citations between March 2009

and February 2014 (search term: "[name] cell"). Even though these publications underwent peer-review, the text of many still describes the cell lines as "normal" human cells, not cervical cancer cells.

How many labs unknowingly continue to use contaminated cell lines? Retraction notices often are noticeable only in the news or when the full-text is accessed directly on the journal's website, but not in PubMed or Google Scholar search results. Worse, how many of these experiments formed the basis for additional preclinical studies, drug discovery efforts and clinical trials?

The impact of contaminated cells extends beyond the lab bench. Promising results generated from the use of misidentified esophageal cell lines led to at least three NIH grants, more than 100 scientific publications, 11 US patents, and patient recruitment for clinical trials.<sup>3</sup> The ethical costs of such events also are considerable, damaging patients' and the public's trust in scientific research and discouraging researcher who enter careers expecting to discover new truths that can change lives for the better. To manage the integrity of cell lines and protect all stakeholders, organizations have formed to set and enforce standards for cell lines. These organizations include the American Type Culture Collection (ATCC), International Cell Line Authentication Committee (ICLAC), and European Collection of Cell Cultures (ECACC).

Misidentification of cell lines is not the only serious challenge to acquiring reliable, meaningful data. Microbial contamination poses common and recurring threats.

The most well known contaminants—bacteria, fungi, and viruses—are visually detectable, as their presence typically causes media to become turbid. Bacteria, fungi, and viruses typically kill the cells they affect. A plate of dead cells is a good indicator that they have been contaminated. Although the researcher must throw those specific cells away, the contamination usually can be contained to that batch alone.

Mycoplasma are far more dangerous microbial contaminants, as they cause no discernible change in turbidity or pH even at high concentrations. Mycoplasma can induce abnormal behavior in cultured cells, including altered growth rates, morphological changes, chromosomal aberrations, and altered cell metabolism. Since mycoplasma lack cell walls<sup>4</sup>, contaminated cells cannot be treated with most antibiotics. Thus, mycoplasma contamination is difficult to control. If left unchecked, mycoplasma can contaminate an entire operation or facility.

To maintain the integrity of cell lines and the experimental results they help produce, steps can be taken before acquiring cell lines. Vendors should be able to answer basic questions (See **Box 1: Six Questions to Ask When Selecting a Cell Line Vendor**) and evidence the upfront measures performed to validate cell line quality and health. As established cell repositories such as the ECCAC or ATCC have already performed extensive tests on the cell lines in their libraries, purchasing cells from these organizations is both efficient and more secure.

As cell lines are received, consistent application of validation methods (See **Box 2: Validation Techniques for Cell Lines**) and then proper laboratory techniques (See **Box 3: Tips for Storing and Using Cell Lines**) is necessary to maintain reliable cell cultures. Establishing a laboratory-wide plan of action for when contamination is detected also will offer peace of mind and minimize disruptions to research progress.

### **BOX 1: Six Questions to Ask When Selecting a Cell Line Vendor**

Asking a few simple questions of your vendor before purchasing cells can help ensure that cell lines are authentic and free of contamination. A reputable vendor will have adequately addressed all of the questions below.

1. Is the cell line authenticated?
2. Is the cell line found in the misidentified cell line database?
3. Is the short tandem repeat (STR) profile known? Does the vendor supply a Certificate of Analysis (CofA) showing the STR profile of a cell?
4. Has every batch been tested for mycoplasma?
5. Does the vendor supply references and journal citations for the cell lines?
6. Is technical support available and will they answer specific questions about cell line properties and identification?

### **BOX 2: Validation Techniques for Cell Lines**

Tests for mycoplasma should be performed weekly to monthly. Cell line authentication should be performed before the first use, every six months to one year, and when a mix-up is suspected. Though it is not recommended to do so, if cell lines are obtained from a colleague they must be tested for authenticity and mycoplasma before use. The more reliable route to acquire cell lines is to purchase them from a reputable repository, such as ECACC or ATCC.

#### **Mycoplasma Detection**

Low levels of contamination are difficult to detect may require two or more methods of detection.

#### **Mycoplasma Culture**

This is a standard method of detection. The FDA/European Pharmacopia-approved protocol is the most sensitive method. A selective and highly-enriched growth medium is used on standard agar plates. Colonies have a distinctive “fried egg” appearance. A positive result using this method is conclusive proof of mycoplasma contamination. However, because this method does not detect all species, such as *M. hyorhins*, DNA staining and/or PCR are required to assure the absence of contamination.

**Average time:** One month **Average cost:** \$250 per sample

#### **DNA Staining**

This method relies on the Hoechst 33258 stain, which binds to DNA and fluoresces in UV light. The stain causes DNA-rich nuclei and any mycoplasma in the cytoplasm to fluoresce. Mycoplasma and mitochondria are easily differentiated because the former has ten times the DNA content and thus are much brighter. If all the cells are healthy with nice distinct nuclei, detection is fairly unambiguous. Commercial kits, such as Sigma-Aldrich's MYC-1, are available.

False positives can be caused by cell detritus or cells undergoing apoptosis. False negatives are also possible as this is the least sensitive method, detecting contaminations with a titer of approximately  $10^5$  mycoplasma per ml or higher. However, unlike the culture method, it will detect all types of mycoplasma.

**Average time:** One hour **Average cost:** \$70 per sample

#### **PCR**

PCR-based mycoplasma detection can be very sensitive, capable of detecting a few as 20 copies of a mycoplasma genome within 2  $\mu$ L of sample. Mycoplasma detection is achieved by a primer/probe system that amplifies the highly conserved 16S rRNA operon coding region of the mycoplasma genome. PCR detection is effective for 19 species of mycoplasma, including *M. hyorhins* that is not detectable by the culture method.

If a lab routinely performs PCR, one of the commercial kits, such as the Sigma-Aldrich Lookout® Mycoplasma Test Kits, will work well. If not, a number of services are available to perform PCR-based mycoplasma testing for a fee.

**Average time:** One to two hours **Average cost:** \$15 per sample for DIY; \$120 for contracted services

#### **Cell Line Authentication**

##### **ICLAC Cross-Contaminated/Misidentified Cell Line Database Search**

Verify that all cell lines in use are not listed in any database of misidentified cell lines ([www.iclac.org](http://www.iclac.org)). The comprehensive ICLAC database is updated periodically, but retractions sadly occur more frequently. After searching the list, it is worth querying Google for the cell line name and “retraction”.

**Average time:** 3 minutes **Average cost:** \$0

##### **Short Tandem Repeat Profiling (DNA Fingerprinting)**

STRs are repeated two to six base sequences of DNA. The number of STRs at any locus is highly variable within the human population and these variations are heritable. ECACC, ATCC and other cell repositories use the ASN-0002-2011 Standard Method based on the Combined DNA Index System (CODIS), which analyzes STRs at 13 specific and several additional loci to determine human cell line authenticity. Kits for STR profiling are available commercially, but one can ensure conclusive identification by enlisting a repository such as ECACC or a core facility that specializes in this type of analysis and has access to other validated human cell lines.

No standardized method yet exists to validate the identity of non-human cell lines. Barcoding is used to determine the species of non-human samples.

Public Health England's ECACC division supplies authenticated cell lines as well as DNA barcoding and STR profiling services ([www.hpacultures.org.uk](http://www.hpacultures.org.uk)).

**Average time:** Four hours **Average cost:** \$300 per sample

## DNA Barcoding

DNA barcoding involves PCR amplification and DNA sequencing of specific regions of mitochondrial DNA. The cytochrome c oxidase subunit 1 mitochondrial region (COI) is the standard target for humans; the rbcL region is the standard target for plants. These regions' DNA sequence is the "barcode" that differentiates species, although there is some variation between individuals of the same species.

**Average time:** Two days **Average cost:** \$300 per sample

## BOX 3: Tips for Storing and Using Cell Lines

Proper storage and handling of cells and cell culture reagents, as well as good aseptic cell culture technique, can minimize contamination.

### Storage

- Create a reserve of cells at earlier passages to serve as a cell bank.
- If required, purchase media, sera, and other culture reagents that are endotoxin-free and manufactured under cGMP. Sera should be tested for mycoplasma and viruses.
- Each researcher should maintain a personal stock of reagents, if possible, to reduce bacterial and cross-contamination.
- Maintain a separate bottle of media for each cell line.
- Purchase fresh, validated cells only. Do not accept cells from other labs. The most common source of mycoplasma infection in cell culture research is another previously infected culture.
- Maintain a log of mycoplasma and validation testing. Retain CofAs for reference.
- Cell lines should be stored below the glass point of water (at least -150 °C). Monitor nitrogen storage temperature and maintain a temperature log. Program an alert to sound if the temperature exceeds a certain deviation from the set temperature.

### Usage

- Avoid distractions in order to prevent cross contamination when working with cells.
- Use antibiotic-free media unless undertaking primary culture. Overuse of antibiotics can lead to resistant bacteria strains.
- Take care when using two or more antibiotics in the same culture, as the cytotoxic concentrations for the combined treatments are lower than those listed for the individual antibiotics.
- Discard waste and spray hood with 70% ethanol after use. When working with multiple cell lines, work with one cell line at a time and clean the hood before moving to the next cell line.
- Do not allow cells to become fully confluent. Passage cells at 70–80% confluency or as advised by the ATCC or ECACC data sheet.
- Do not use incoming cell lines until testing has confirmed the absence of mycoplasma and the identity of the line.
- Document new cell line details upon acquisition. Record how many times a cell line has been passaged, as some cell lines exhibit different characteristics after multiple passages. Implement a standard for when to discard cells in culture and thaw a vial of stock.

## References

1. Burnett, E., Penn, L. & Finley, D. Contaminated cell lines are bad for your health. *Biofiles* **8** (2012).
2. Masters, J. R. Cell-line authentication: End the scandal of false cell lines. *Nature* **492**, 186, doi:10.1038/492186a (2012).
3. Gartler, S. M. Apparent HeLa cell contamination of human heteroploid cell lines. *Nature* **217**, 750–751 (1968).
4. Razin, S. in *Medical Microbiology* (ed S. Baron) (Galveston (TX), 1996).