IMPROVING REPRODUCIBILITY: BEST PRACTICES FOR ANTIBODIES, CELL LINES, AND SMALL MOLECULES

INTRODUCTION

In the midst of beeping lab timers, presentations, and grant deadlines, it is easy to take for granted the quality of lab reagents. Recent headlines have highlighted the risks of not validating reagents prior to experimentation, with many stories about wasted years of work, false hopes of reproducing promising results, and the destabilization of partnerships arranged with commercial partners.

One disheartening example is of a research group in Toronto who suspected an antibody to study pancreatic cancer biomarkers was faulty. After two years, $500,000 spent, and thousands of patient samples wasted on additional characterization methods, the group found that what they thought was an antibody against CUZD1 was actually against CA125.1

An estimated 15–20% of cell lines used worldwide are misidentified2 and unauthenticated cell lines have resulted in several retractions.3 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.4 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 other 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.

Many of the small molecules used in bioassays and screening libraries are shipped with certificates of analysis verifying their purity, but factors such as structure are often overlooked. In one instance, the work of several research groups was invalidated when it was discovered that the wrong isomer of bosutinib, a pharmacologically active molecule was distributed. While HPLC analysis indicated high purity, the molecule was not subjected to quality control tests specific enough to distinguish the isomers.5,6 Furthermore, lack of attention to physical and chemical properties, such as melting temperature and light sensitivity, can cause a change in the phase or the chemical composition of the molecule that may affect its stability, as well as the results of downstream assays.

Despite the myriad examples of the consequences, many labs do not validate the identity of their cell lines and antibodies or the purity and composition of their small molecules. Perhaps for some, validation is too time-consuming for early studies. For others, where and when to begin are the key questions. What is clear, however, is that careers can suffer due to unvalidated reagents and cell lines in preliminary studies. Furthermore, pharmaceutical companies—which increasingly rely on academic discovery to develop candidates for their clinical trials—cite lack of a standard practice to validate reagents as a contributor to the irreproducibility of pre-clinical studies and the subsequent high failure rate of clinical trials.7

According to a survey conducted in conjunction with the Association for the Advancement of Science (AAAS), the translational research community standards and expectations are rising (see Figure 1).8 Turning a blind eye to validation—at any stage in research—is quickly ceasing to be an option. Journals and funding agencies are setting stricter requirements to increase reproducibility. As of mid-2013, manuscripts submitted to Nature journals must be accompanied by a reproducibility checklist, part of which requires researchers to provide proof of antibody validation, cell-line authentication, and testing for mycoplasma contamination. The AACR journals now require authors to either provide a citation for a previously characterized antibody or perform their own characterization for a less-studied antibody.

The NIH has initiated pilot studies to train their post-docs on ways to enhance reproducibility and transparency, and the use of a checklist for grant reviewers to ensure the validity of experimental design.9 It may soon require grant applicants to validate their results, protocols, and reagents for grant applications.

While vendors are responsible for reagent quality, researchers also share responsibility, as personal incentives for reproducible research are high. Publicity over a manuscript retraction—and the consequent loss of productivity—is much more damaging to an investigator’s career than a vendor’s bottom line. For example, one antibody company continues to operate despite retractions, negative publicity, and investigation by the Federal Trade Commission.10

Taking several small steps can reduce the risk researchers unknowingly take when running an experiment. Some of these steps are simply asking smart questions before purchasing products or recording product information as reagent boxes are opened. Other steps fit into toolboxes of validation strategies to ensure critical reagents, including as antibodies, cell lines, and small molecules, match experimental requirements for identity, function, and structure.
Q: Which of the following actions would you be willing to take to ensure that your research is perceived as reproducible? (Check all that apply.)

**Reagents**
- Use standardized/validated reagents - 55%
- Use Good Manufacturing Practices (GMP) materials - 42%

**Outside the Lab**
- Have another lab reproduce findings - 50%
- Obtain outside expert statistical analysis - 46%

**Methods**
- Perform rigorous quality controls, including repeats - 71%
- Ensure thorough documentation - 67%
- Follow Good Laboratory Practices (GLP) - 67%
- Increase sample sizes - 54%

**Unwilling**
- Unwilling to take any of the proposed actions - 3%

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Q: In your current lab, what is the minimum number of times an experiment is conducted before including the resultant data in a manuscript submission?

- Once - 4%
- Twice - 12%
- Three Times - 51%
- Four Times - 9%
- Five Times or More - 17%
- Not Sure - 7%

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Q: Do you believe it is necessary for individuals working in translational research to be held to a higher standard of laboratory practice/experimental design than those working in basic research? Why?

- Yes - 44%
- No - 45%
- Not Sure - 11%

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**Figure 1.** Results from a survey of translational researchers conducted by the AAAS and Sigma-Aldrich in 2013.
ANTIBODIES

Due to the nature of the mammalian immune system, antibodies are often imperfect biological products. This imperfection often impacts specificity, selectivity, and reproducibility. Today, the lack of uncharacterized antibodies and limited availability of application data has become a serious problem for the research community. While it is true that certain vendors knowingly sell mislabeled antibodies, many researchers have begun overlooking the need for supporting data and as a result, they have put quality and reproducibility of their research at risk. No matter the vendor, researchers should safeguard their experiments and careers by evaluating every antibody prior to conducting their experiments. This approach reduces the risk of losing precious samples while increasing the confidence in reproducibility.

While lack of antibody specificity and selectivity may result from improper storage conditions or unexpected intricacies of the biological target an antibody’s binding characteristics may also be attributed to a number of factors related to its production and purification.

Antibodies are produced by challenging a host animal with the target antigen. Serum from that host will contain the polyclonal antibodies produced by a number of different B cells, each raising an antibody to recognize a different epitope on the antigen. Isolating single B cells from the host’s spleen and fusing them one-by-one to immortalized myeloma cells allows production of a monoclonal antibody. The single-epitope specificity of a monoclonal antibody is a result of the antibody being produced by only one B cell. While monoclonal antibodies may be more specific, any minute change in epitope structure can markedly reduce binding affinity.

Specificity is also dependent on the method used for antibody purification. Purification by Protein A or Protein G yields a less homogenous product than purification by immunogen affinity. One can also expect that the antibody provided may vary between production lots, particularly if the antibody is polyclonal.

The first step a researcher can take to help ensure the purchase of a specific and robust antibody is to carefully select an antibody vendor. A vendor’s reliability can be estimated through the availability of specification sheets and other documentation that detail characteristics about the antibody and its production process (see Box 1: Eight factors to consider when selecting an antibody vendor). Also, one should be particularly mindful of this sort of information when viewing a relatively unknown vendor's catalog that promotes many niche antibodies that are not available from established vendors.

Since the number of applications and experiments that require quality antibodies is so vast, many vendors provide application validation data generated by third parties or external researchers (see Figures 2–5 for data examples). These third parties enhance the likelihood of successful use of the antibody since data as well as recommended titers are shared. Some of the most robust providers of this information include the Human Protein Atlas project (www.proteinatlas.org), Antibody Resource (www.antibodyresource.com), Biocompare (www.biocompare.com), 1degreebio (www.1degreebio.org), and the Human Antibody Initiative (HAI) at the Human Protein Organization (HUPO), which has generated the Antibodypedia (www.antibodypedia.org) catalog of validated antibodies against human proteins.

Because an antibody’s specificity, selectivity, and reproducibility cannot be assumed from vendor specifications or third-party data, confirmatory tests are necessary. Even reputable vendors cannot account for loss of integrity during shipping or handling in the lab. For these reasons, careful testing, storage, and consideration of the true utility of every antibody—both upon receipt and at regular intervals during its lifetime in the lab—is crucial to safeguard the integrity of experimental results (see Box 2: Validation Techniques for Antibodies and Box 3: Tips for Storing Antibodies).

Despite the availability of evaluation or application-based data, there are no universal guidelines for the community. As part of HUPO’s Proteomic Standards Initiative, community members published a proposal to formalize standards for validating antibodies and other protein affinity reagents. The proposed Minimum Information About A Protein Affinity Reagent (MIAPAR) defines a checklist of product information for use by manufacturers, vendors, QC labs, users, and various databases. This checklist enables a more defined approach to compare affinity reagents and select the one most appropriate for the application.
Antibodies have a higher batch-to-batch variation and therefore may only require testing before the first use. Polyclonal premium reagents that generate the most reproducible results, and thus may only require testing before the first use. If the end application is not a Western blot, the antibody must be tested for that application, whether it is ELISA, immunohistochemistry, immunoprecipitation, or another technique.

Performing a Western blot is the simplest first step to evaluate a new antibody before use. If the end application is not a Western blot, the antibody must be tested for that application, whether it is ELISA, immunohistochemistry, immunoprecipitation, or another technique. The frequency of antibody evaluation depends on the clonality of the antibody. Monoclonal antibodies are considered highly specific, premium reagents that generate the most reproducible results, and thus may only require testing before the first use. Polyclonal antibodies have a higher batch-to-batch variation and therefore require that every lot of material be evaluated. If a new lot of material fails to perform comparably to a previous lot, first reach out to the vendor to see if they have made any small changes, such as immunogen sequence, and for technical advice if necessary.

**Western Blot**
For Western blots, use a panel of positive and negative cell lines with variable expression levels of the protein of interest. If such lines do not exist, transfec the protein of interest in non-expressing cells to create a positive control or use RNAi to knock down the protein of interest to generate a negative control.

It is important to assess the final blot for multiple bands. A monoclonal antibody and a pure polyclonal antibody should ideally produce only one band for the protein of interest. In some cases, these antibodies will produce multiple lighter bands in addition to the band of interest. Compare these bands with the vendor’s full western blot image. If the band of interest is not present at several concentrations, consult vendor and/or discard antibody.

A pattern of bands is not necessarily the sign of a faulty antibody, but could indicate that the target protein is expressed in multiple isoforms or undergoes post-translational modifications that also interact with the antibody. Multiple lower molecular weight bands may also indicate cell lysate degradation. For more sensitive applications, multiple bands may indicate that a monoclonal or highly specific antibody is required.

**Average time:** 4 hours  **Average cost:** $100 to $400

**Mass Spectrometry & Capillary Electrophoresis**
When precision characterization is necessary for specific applications (e.g. biomarker validation or therapeutic development), liquid chromatography combined with mass spectrometry detection (LC-MS) can be used to analyze the structural composition of a monoclonal antibody. Features such as molecular weight, amino acid sequence, post-translational and other modifications, carbohydrate structure, and disulfide linkages can be interrogated via methods such as sub-unit mapping, peptide mapping, N-terminal sequencing and glycan profiling. Capillary electrophoresis based methods can also provide data detailing the purity, molecular weight, isoelectric point and charge heterogeneity of a purified antibody preparation.

For the average researcher, these techniques are usually not required.

**Average time:** 14–21 days (from a typical service provider)  **Average cost:** $1,500 to $3,500

**BOX 1: Eight Questions to Consider When Selecting an Antibody Vendor**

Asking a few simple questions of your vendor before purchasing an antibody can help differentiate reliable suppliers. A reputable vendor will be willing to answer the following:

1. **Can you provide documentation of the antibody titer, immunogen or epitope sequence, and various validated applications?**
2. **Are references and journal citations available for the antibody?**
3. **What are the names of positive and negative cell line controls?**
4. **How was the antibody purified?** While the choice of purification method does not necessarily correlate with the quality of the antibody, different methods are useful for purifying monoclonal and polyclonal antibodies. For monoclonal antibodies, Protein A/G purification is sufficient. For polyclonal antibodies, affinity purification may be used as an alternative to Protein A/G purification.
5. **Is the supporting antibody performance data reflective of a native or recombinant antigen?** For Western blot data, the vendor should show the entire gel, not just the band of interest. Ideally, multiple cell lines should be analyzed.
6. **Was the antibody raised in-house or brought in from an external supplier?** If the antibody was brought in from an external supplier, is the vendor willing to share the identity of their supplier or how externally-sourced antibodies are qualified?
7. **Are data for antibody performance for the indicated applications available?** If so, are the relevant protocols provided? If not, does the vendor have a guarantee program that gives researchers one year to self-validate the antibody and return it if there is an issue?
8. **Is technical support available to answer specific questions about antibody properties and purification?**

**BOX 2: Techniques for Evaluating Antibodies**

Performing a Western blot is the simplest first step to evaluate a new antibody before use. If the end application is not a Western blot, the antibody must be tested for that application, whether it is ELISA, immunohistochemistry, immunoprecipitation, or another technique.

The frequency of antibody evaluation depends on the clonality of the antibody. Monoclonal antibodies are considered highly specific, premium reagents that generate the most reproducible results, and thus may only require testing before the first use. Polyclonal antibodies have a higher batch-to-batch variation and therefore require that every lot of material be evaluated. If a new lot of material fails to perform comparably to a previous lot, first reach out to the vendor to see if they have made any small changes, such as immunogen sequence, and for technical advice if necessary.

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**BOX 3: Tips for Storing and Using Antibodies**

Antibody performance can degrade significantly over time and when subjected to improper storage conditions. In addition to recommendations from the vendor, the practices below help to ensure maximum antibody performance and longevity.

**Storage**
- Aliquot antibodies (e.g., 20 μL) to avoid contamination and minimize free/thaw cycles, particularly for extended storage. Some antibody formulations that contain glycerol, BSA, or other protein stabilizers may tolerate repeated freeze/thaws cycles, but it is best to avoid unnecessary temperature changes.
- Do not store antibodies in “frost-free” freezers.
- If slight turbidity occurs after prolonged storage, clarify the solution by centrifugation before use.
For continuous use, store antibodies at 2–8 °C for up to one month.
• Monitor freezer temperature and maintain a temperature log. Program an alert to sound if the temperature goes above or below desired thresholds.
• To maximize sample recovery, use low-binding tubes when working with or storing antibodies.
• When opening a new supply of antibodies, record the open date, lot/batch number, product number, expiry date, aliquot labels, and any special instructions in a lab notebook. Without this information, doubts or mistakes at a later cannot be addressed.

Usage
• To increase confidence in antibody performance, Sigma-Aldrich recommends lot-by-lot validation of polyclonal antibodies. Monoclonal antibodies exhibit greater lot-to-lot consistency, but validation should still be conducted for each lot.
• Keep antibodies on ice when working at the bench.
• To obtain the best results, first determine the optimal working concentration of the antibody for your application by a titration test.
• Working dilution samples should be discarded within 12 hours of preparation.

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.13

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.14

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.4 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.15 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 walls16, 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 4: 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 5: Validation Techniques for Cell Lines) and then proper laboratory techniques (See Box 6: 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 4: 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?
DIY; $120 for contracted services

$15 per sample for mycoplasma testing for a fee. If not, a number of services are available to perform PCR-based mycoplasma detection. The Sigma-Aldrich Lookout® Mycoplasma Test Kits, will work well. If a lab routinely performs PCR, one of the commercial kits, such as Sigma-Aldrich’s MYC-1, are available. 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

**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 μL 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. hyorhinis 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). 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).

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 5: 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. hyorhinis, 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

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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

**BOX 6: 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 and avoid using cells that exceed a certain deviation from the set temperature.
• 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.

SMALL MOLECULES

Small molecules are used as modulators for cellular processes, as substrates for and readouts of cellular activity, and as part of screening libraries to identify promising compounds. Though antibodies, cell lines, and cell culture conditions often come under scrutiny as key contributors to confounding variation between experiments, proper use and storage of small molecules are also important to ensure experiments can be reproduced.

Vendors typically test small molecules to confirm molecular structure and validate for purity. However, differences in stereochemistry can go unnoticed for years, as was the case with the aforementioned bosutinib, and significantly alter the outcome of an assay. Confirming the identity of a small molecule is an everyday practice for many chemists and material scientists, but can be a challenge for researchers without access to instruments to perform nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry, for example.

Quality vendors will supply information and data proving they have performed these analyses (See Box 7: Four Factors to Consider When Selecting a Small Molecule Vendor and Box 8: Validation Techniques for Small Molecules). The most important step a researcher can take to avoid problems with small molecules, aside from performing these tests in their own labs, is to educate themselves about the molecule’s characteristics, including its structure, concentration, and method of purification.

Proper handling and storage practices (See Box 9: Storage and Usage Tips for Small Molecules) are also crucial to ensure optimum performance of small molecules, whether solid or dissolved in solution. Sigma-Aldrich recommends that researchers be mindful of the three S's: solvent, solubility, and stability.

Solvent — The solvent used to dissolve a small molecule may be toxic to cultured cells. The solvent could also confound colorimetric and fluorescence assay results, for example by changing the absorbance maximum of a molecule or quenching fluorescence, respectively.

Solubility — When a molecule “crashes” out of solution, its concentration is no longer accurately known. Read reagent bottles, resources such as the CRC Handbook of Chemistry and Physics, or peer-reviewed papers to determine the solubility of the molecule. The solvent will also play a role in a molecule’s solubility. The old adage of “like dissolves like” is important here.

Stability — If a molecule is light or air-sensitive, any exposure can lead to decomposition or alteration of the functional groups of a molecule. Labeling information and/or the Material Safety Data Sheet (MSDS) should specify instructions for handling and storing the molecule. Expiration dates are often provided on reagents found in assay kits.

BOX 7: Four Questions to Consider When Selecting a Small Molecule Vendor

Asking a few simple questions of your vendor before purchasing a small molecule or reagent kit can help ensure quality. A reputable vendor will adequately address all of the questions below.
1. Does the vendor supply a CoA showing the analysis performed on the molecule?
2. Does the vendor supply references for the synthesis of the molecule?
3. If the molecule is used in a colorimetric or fluorescence, does the vendor supply the appropriate spectra?
4. Is technical support available and will they answer specific questions about purification and analysis?

BOX 8: Validation Techniques for Small Molecules

Synthetic and analytical chemists use the methods below to purify and identify the small molecules used as reagents or components in chemical libraries. While it is not practical for most life science researchers to use the methods below to validate their reagents, it is important to be familiar with those that may be listed on the product specification sheet and CoA.

Molecule purification/separation
Liquid chromatography (LC) and gas chromatography (GC) are used to separate molecules in solution. GC is used specifically on volatile molecules.

Molecular weight determination
Mass Spectrometry (MS) is a technique to measure the mass-to-charge ratio of a molecule, which is then used to determine its molecular weight. MS to determine molecular weight often follows LC or GC to separate molecules of interest.

Structure
There are two types of techniques commonly used to gain structural information about a molecule.
Nuclear Magnetic Resonance (NMR) spectroscopy measures the behavior of atomic nuclei in the presence of electromagnetic radiation, which is used to determine structure and location of atoms in a molecule. The two most common types of NMR are 1H NMR (“proton NMR”), which is used to determine the location and stereochemistry of hydrogen atoms, and 13C NMR (“carbon NMR”), which is used to determine the location, substitution of, and stereochemistry around carbon atoms.

Elemental analysis is used to determine the chemical formula of a molecule or the specific percentage of a certain element in a molecule. It is often used in conjunction with other techniques such as NMR and chromatography.

**BOX 9: Tips for Storing and Using Small Molecules**

Proper storage and handling of small molecules reagents will maintain the integrity and stability of the molecule, as well as reduce the incidence of contamination and mislabeling.

**Storage**

- Write the open date on the outside of the package. Record lot number, product number, and molecular structure in a lab notebook when a new product is opened. Without this information, doubts or mistakes at a later cannot be addressed.
- Keep an eye out for and adhere to printed expiration dates on reagents and reagent kits.
- Label all prepared solutions with chemical name, concentration, solvent, and date.

**Usage**

- Each researcher should maintain a stock of reagents, if possible, to reduce contamination.
- Vortex or mix reagents before use.
- Do not pipette directly from or stick a spatula into reagent containers.
- If research has an intended clinical application, GMP reagents/materials may be required. GMP materials are manufactured under high quality and regulated standards.

**References**