Mycoplasma testing of Biopharmaceuticals: Current regulations, challenges and trends

Preface

Mycoplasma contamination of biopharmaceutical products (also known as biologics or large molecules) resulting from cell culture contamination in the manufacturing process poses a potential health risk to patients. Mycoplasmas can affect virtually every cell culture parameter with often only minor visible effects, creating an uncontrollable environment that is undesirable in the pharmaceutical industry. Therefore, regulatory agencies require manufacturers to test their biopharmaceutical products and to ensure the absence of mycoplasmas in released products. Most regulatory agencies have issued guidelines that provide protocols for mycoplasma testing, and some give recommendations for the validation of rapid NAT (nucleic acid amplification techniques) testing methods. These recommendations for rapid mycoplasma testing, however, are not harmonized, making establishment of such tests a challenge for manufacturing companies.

January 2018
Sebastian Machado Weber, Dr. Andreas Rohwer, Alexander Bartes, Christina Krause, Raphael Greiner
Roche CustomBiotech
Dr. Carl-Ulrich Zimmermann, Dr. Martina Sauert, Martin Vronka, Dr. Sandra Buczolits, Prof. Dr. Renate Rosengarten
Mycoplasma Biosafety Services GmbH

Technical report

From traditional culture methods to automated high-throughput NAT-based methods
This document aims to provide a guideline with recommendations on how to validate and implement the Roche CustomBiotech MycoTOOL Mycoplasma Real-Time PCR (polymerase chain reaction) Assay (MycotooL qPCR – http://go.roche.com/mycotool) in combination with the Roche MagNA Pure 96 or MagNA Pure 24 Sample Preparation System as a rapid, automated, NAT-based mycoplasma testing method for biopharmaceutical products.

Chapter Overview

1. A brief introduction about mycoplasmas is given. Furthermore, the relevance of mycoplasmas in biopharmaceutical manufacturing processes is discussed.

2. An overview, and examples, of existing non-compendial and compendial mycoplasma testing methods are provided. The two compendial methods, the Culture Method and the Indicator Cell Culture Method are handled in more detail, and the rapid NAT-based method is introduced as a compendial testing method.

3. Regulatory aspects to be considered during the implementation of a rapid mycoplasma testing method are addressed. Regulatory guidelines such as the European Pharmacopoeia (EP 2.6.7.), the United States Pharmacopeia (USP <63>), and the Japanese Pharmacopoeia (JP 17th Ed.) are discussed.

4. The fourth chapter addresses the potential design of a product-specific validation and implementation project and the recommended step-by-step approach. It touches upon each step in the process of implementation, from supplier due diligence and conducting a feasibility and validation study, to performing routine testing.

5. A summary and outlook is provided in the last chapter addressing long-term prospects offered by NAT-based rapid mycoplasma testing methods. The competitive advantages of these rapid methods when implemented as early warning systems and for lot release testing, as well as the revolutionary opportunities offered by these alternative testing systems for the biopharmaceutical industry, are discussed.

Table of contents

Introduction ................................................................. 4
Mycoplasmas in Cell Cultures ............................................. 5
Mycoplasmas in Biopharmaceutical Manufacturing Processes ................................................................................. 6
Mycoplasma Testing Methods Overview ........................................ 8
Non-compendial Testing Methods ....................................... 8
Compendial Testing Methods ............................................ 9
NAT-based Methods ..................................................... 13
Regulatory Overview ................................................... 17
Step-by-step Validation and Implementation of MycoTOOL qPCR ....................................................... 19
Summary and Outlook .................................................. 22
Appendix ............................................................................. 23
Glossary ............................................................................. 24
References .......................................................................... 26

About us

Roche CustomBiotech
Leveraging the know-how of Roche Diagnostics and Roche Pharmaceuticals, CustomBiotech delivers high quality raw materials, instrumentation and services for biopharmaceutical, cell therapy, or in vitro diagnostics companies, customized to your unique quality and regulatory needs.

For more information: (http://go.roche.com/custombiotech)

Mycoplasma Biosafety Services GmbH
Mycoplasma Biosafety is a contract research organization and service provider for GMP-compliant mycoplasma testing of biopharmaceutical products. As technological market leader in Industrial Mycoplasmology.

Mycoplasma Biosafety offers the most comprehensive range of mycoplasma testing services based on conventional culture methods and new rapid PCR-based assays. The company also develops and produces mycoplasma culture media and reference standard products for mycoplasma testing and test validations.

For more information: (http://go.roche.com/mbs)
**Introduction**

The term ‘mycoplasmas’ is often used as a trivial name for all members of the bacterial class Mollicutes (lat. mollis = “soft”, cutis = “skin”) (Figure 1). Mollicutes are characterized by the lack of a cell wall and a small genome size (0.5–2.2 megabase pair) with low GC (guanine-cytosine) content (20–40 mol%). Due to their small genome, mycoplasmas are host-dependent and live as commensals or infectious agents in or on a variety of hosts, including humans, other vertebrates, plants, and insects. These microorganisms can multiply under aerobic or anaerobic conditions. They have a pleomorphic cell morphology, with the exception of spiroplasmas, which have a spiral shape, and some mycoplasmas of the genus Mycoplasma, which have a flask-like shape due to a terminal (tip) structure (Mycoplasma gallisepticum, Mycoplasma pneumoniae).

Depending on species, mycoplasmas can grow in liquid media, either as single cells (Mycoplasma arthritidis) or in aggregates (Acholeplasma laidlawii, Mycoplasma pneumoniae, Mycoplasma fermentans). The lack of a cell wall makes mycoplasmas resistant to cell wall-targeting antibiotics such as penicillin. Furthermore, mycoplasmas can form biofilms on solid surfaces in liquid media, such as glass or plastic surfaces, which provides another level of resistance, namely to disinfecting agents and environmental stress conditions. The first mycoplasma species was cultured at the Institut Pasteur in 1896; it was isolated from cattle with pleuropneumonia and much later described as Mycoplasma mycoides subsp. mycoides SC (small colony type). Since mycoplasmas are not visible with standard light microscopy and usually barely affect the obvious state of the cell culture, they often remain undetected. Nevertheless, they impact cell growth and metabolism, and consequently, the therapeutic proteins expressed by host cells.

For a long time, mycoplasmas were largely underestimated as pathogens. For that reason, there was a lack of suitable molecular diagnostic approaches. This initial situation has changed considerably in recent years, and there has been greater acceptance and improvement of culture-based and molecular methods for mycoplasma detection.

**Mycoplasmas in Cell Cultures**

In addition to their growing clinical significance, mycoplasmas have gained great attention in the context of cell cultures. As they naturally reside in plant and animal tissues, every cell culture medium containing plant- or animal-derived supplements is prone to contamination by mycoplasmas. Due to their small size of only 0.1–0.8 μm on average (Figure 2), and their variable shape resulting from the missing cell wall, mycoplasmas can pass through standard sterilizing filters and enter cell cultures with culture media or raw material-derived additives.

The two most common sources of contamination are, laboratory personnel and already contaminated cell cultures, from which contaminant is passed on by cross-contamination. Since mycoplasmas are not visible with standard light microscopy setups and usually barely affect the obvious state of the cell culture, they often remain undetected. Nevertheless, they impact cell growth and metabolism, and consequently, the therapeutic proteins expressed by host cells.

**Biopharmaceuticals**

Biopharmaceuticals (also known as biologics) are medicinal products manufactured in and extracted from biological sources such as bacteria, yeast, mammalian cell lines, or mammals. Vaccines, blood components, recombinant proteins, gene therapies, tissues, and cells for cell therapy fall into this category. They can consist of nucleic acids, proteins, sugars and complex combinations of these and are either identical or similar to molecules naturally occurring in the human body. In contrast to chemically synthesized drugs (often referred to as small molecules), biopharmaceuticals are much larger with a molecular weight 100 times that of small molecules. Biopharmaceutical manufacturing in mammalian cell lines typically involves the development of a genetically engineered eukaryotic cell line (such as CHO or HEK293) to express the biopharmaceutical, and subsequent harvesting, purification, and drug formulation. Also refer to Figure 3.

Due to the complex manufacturing process, biopharmaceuticals face unique manufacturing and product release challenges. Firstly, cell lines may be contaminated with mycoplasmas, requiring mycoplasma testing prior to lot release. Secondly, they are sterilized through filtering, which has the potential risk that mycoplasmas or viruses pass through the filter. Thirdly, cell culture contamination may be introduced by raw materials. This is why mycoplasma testing methods, especially early warning systems (also known as in-process control), are essential to detect a contamination as fast and as early as possible.

Due to their genome reduction, mycoplasmas lack several metabolic pathways either completely or partially, forcing them to acquire necessary nutrients (amino acids, nucleobases, and fatty acids) from the environment and to exert a parasitic life style.

Figure 1: Taxonomy of the bacterial class Mollicutes. The red boxes indicate genera with relevant species in biopharmaceutical manufacturing processes.

*Genus containing mycoplasma species that are prevalent in humans. Source: Authors, 2017.

Figure 2: Relative size of different microorganisms. Source: Authors, 2017. This figure is a graphical illustration by the authors of this application note and provided under the terms of the Creative Commons Public License CC BY 3.0 (http://creativecommons.org/licenses/by/3.0/), and can be used under the terms of such license notwithstanding any rights that may exist with respect to the document it is embedded in.
Despite the negative effects of mycoplasmal contamination, cell cultures are rarely monitored, even though testing for mycoplasmal contamination is a necessary quality control procedure. Studies have shown a contamination rate of about 5-35% of existing cell lines available worldwide. The only meaningful safety precaution to maintain mycoplasmal-free cell cultures is to regularly test for mycoplasmas.

Only well-established routine mycoplasmal testing during the ongoing process can minimize the risk of a concealed contamination that can lead to serious problems. Outsourcing mycoplasmal testing to a trustworthy and experienced partner, such as Mycoplasma Biosafety, is an alternative that brings several advantages. Firstly, qualified results can be obtained in the shortest time possible. Secondly, an in-house testing facility does not need to be maintained, which frees resources to concentrate on the core business. Last but not least, the potential risk of mycoplasmal contamination associated with introducing necessary positive controls into a facility are avoided.

Mycoplasmas in Biopharmaceutical Manufacturing Processes

Especially in the biopharmaceutical industry, the effects of mycoplasmal contamination are devastating, as entire production batches must be discarded and the manufacturing plant must stop production. International regulatory authorities have published guidelines to demonstrate that biopharmaceuticals products must be free of mycoplasmases to ensure product safety, purity and potency. Therefore, early detection of mycoplasmases is essential for smooth processes in manufacturing of biopharmaceutical products. Figure 3 depicts common testing points. Given these multiple check points, numerous different methods for mycoplasmal testing have been developed and will be covered in the following chapter.

Cell Therapy

Advanced therapy medicinal products (ATMP) are a new class of therapeutics that are based on genes (gene therapy), somatic cells (cell therapy), or tissue (tissue engineering). These advanced therapies herald novel treatments of a number of diseases and thus, a huge potential for patients is expected. Cell therapy typically involves cell dissociation from a patient, cell culture outside the human body, and subsequent injection of cells back into the patient.

Compared to large molecule biopharmaceuticals, cell therapy products face additional manufacturing and release challenges. Firstly, most of them cannot be sterilized at all. Secondly, storage may be challenging as cell therapy products sometimes face a short shelf life and need to be injected into patients immediately. Thirdly, the batch size often consists of one dose rate and volumes are usually very small. In such cases, rapid mycoplasmal testing methods are favorable over conventional mycoplasmal testing methods because they require smaller volumes and release manufacturing batches much faster than conventional mycoplasmal testing methods.

Figure 3: Testing points for mycoplasmal contamination in the manufacturing process of biopharmaceuticals. After raw materials have been tested for contamination (grey loop), and solutions like buffer and media have been applied to working cultures, it is necessary to check for contamination (blue loop), as mycoplasmal contamination can also be introduced by the cell line and lab staff. It is also recommended to carry out in-process controls during the seed culture and the actual fermentation (light purple loops). The final and prescribed test point is the endpoint of the fermentation, the harvest (purple loop). Once the mycoplasmal-free state of the harvest has been proven, further test points are usually no longer necessary since the purification of the products is carried out without living organisms. Source: Roche CustomBiotech, 2017.

Mycoplasmal species frequently or potentially detected as contaminants in cell cultures and in biopharmaceutical processes are listed in Table 1. The effects of contamination in the manufacturing process can lead not only to reduced product quality, but also to lower expression levels and consequently reduced production yields. In addition, poor quality and contamination with mycoplasmases may trigger serious side effects in patients. The only meaningful safety precaution to maintain mycoplasmal-free cell cultures is to regularly test for mycoplasmas.

Table 1: Mycoplasmal species which are frequently, occasionally or potentially detected in cell cultures and in biopharmaceutical processes.

<table>
<thead>
<tr>
<th>Mycoplasmal species</th>
<th>Primary isolation source (relevant for products where raw materials of the following origins are used)</th>
<th>Frequent cell culture contaminant based on published reports</th>
<th>Potential contamination source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acholeplasma laidlawii</td>
<td>Bovine, porcine, avian, plant</td>
<td>Yes</td>
<td>Other cell line, bovine sera, nutrient broth powders</td>
</tr>
<tr>
<td>Mycoplasmal arginini</td>
<td>Bovine, ovine, caprine, porcine</td>
<td>Yes</td>
<td>Other cell line, bovine sera</td>
</tr>
<tr>
<td>Mycoplasmal bovis</td>
<td>Bovine</td>
<td>Yes</td>
<td>Other cell line, bovine sera</td>
</tr>
</tbody>
</table>
| Mycoplasmal fermentans | Human                                      | Yes                                                         | Other cell line, personnel ├──
| Mycoplasmal gallisepticum | Avian                                      | No                                                          | Other cell line, embryonated eggs |
| Mycoplasmal hyorhinis | Porcine                                      | Yes                                                         | Other cell line, porcine trypsin |
| Mycoplasmal orale     | Human                                        | Yes                                                         | Other cell line, personnel ├──
| Mycoplasmal salivarium | Human                                      | Yes                                                         | Other cell line, embryonated eggs |
| Mycoplasmal synoviae  | Plant                                         | No                                                          | Other cell line               ├──
| Spiroplasmal citri    |                                               |                                                             |                               ├──

Technical report | 7
Mycoplasma Testing Methods Overview

Detection of mycoplasmas presents a challenge for quality control of cell cultures and biopharmaceuticals because especially low-grade contaminations can only be identified through expertise and experience. Two classical methods have been used for regulatory mycoplasma testing in recent decades, as they have proven to be sensitive and reliable: (i) the Culture Method (also referred to as agar and broth method) and (ii) the Indicator Cell Culture Method. Non-compendial methods, such as enzyme-based and immunology-based assays, are easy and fast to apply but most do not reach the level of sensitivity of culture methods. Therefore, pharmacopoeial monographs do not consider these tests acceptable substitutes for regulatory testing. The necessity for fast yet sensitive and robust detection of mycoplasmas has increased over the past years.

Non-compendial Testing Methods

Non-compendial tests for mycoplasma detection often lack the sensitivity to detect the level of contamination in a sample that is required by regulatory monographs. Moreover, results are sometimes difficult to interpret if a contamination is low level. These tests are prone to giving false-negative results.

**Direct DNA Staining**
Direct staining of cultures with a DNA (Deoxyribonucleic acid)-specific fluorescent dye is sensitive, but not recommended for the purpose of detecting mycoplasma contaminations. Although the test reliably detects heavily contaminated cultures, interpretation of low-grade contaminations is often difficult because DNA from the cell culture may give rise to small points of fluorescence that can mimic mycoplasmas. An example of an enzymatic assay is the luciferase-based mycoplasma detection assay. Although the assay is fast (<20 min), relatively easy to handle (two luminescence readings), and the interpretation of results is easy, no such test has yet been shown to reach the limit of detection that is required from a compendial test (<10 CFU/ml colony forming unit)). Most mycoplasma species are detected at a titer of 10^4 to 10^5 CFU/ml. Mycoplasma PCR-ELISA

An application that combines PCR with a subsequent ELISA (enzyme-linked immunosorbent assay) is the mycoplasma PCR-ELISA, a photometric enzyme immunoassay that detects PCR-amplified mycoplasma DNA in cell culture. During the PCR reaction, digoxigenin-labeled nucleotides are incorporated into the amplicons, allowing their detection in a subsequent ELISA assay. The mycoplasma PCR-ELISA test is claimed to have a detection sensitivity of 1–3 mycoplasma “particles” for particular mycoplasma species (e.g., M. fermentans and A. laidlawii). However, since for others the Limit of Detection (LOD) was 1000 “particles” per ml sample, the test does not fulfill the requirements of the EP regulatory guideline as compendial test for mycoplasma detection.

Table 2: Advantages and disadvantages of compendial mycoplasma detection methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture Method</td>
<td>• Sensitive</td>
<td>• Up to 28 days incubation period</td>
</tr>
<tr>
<td></td>
<td>• Detects 0.1 CFU/ml</td>
<td>• Requires more than one growth medium for the cultivation of different mycoplasma species</td>
</tr>
<tr>
<td>Indicator Cell Culture Method</td>
<td>• Inexpensive</td>
<td>• Highly fastidious</td>
</tr>
<tr>
<td>NAT-based Method</td>
<td>• Sensitive</td>
<td>• Requires more than one growth medium for the cultivation of different mycoplasma species</td>
</tr>
<tr>
<td></td>
<td>• Detects &lt; 10 CFU/ml</td>
<td>• Highly fastidious</td>
</tr>
</tbody>
</table>

Compendial Testing Methods

These methods are, on one hand, based on classical microbiological culture procedures using liquid media and solid agar media and, on the other hand, based on rapid molecular techniques. The methods are summarized in this chapter and table 2.

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture Method</td>
<td>Traditionally, culture methods were used well before today’s molecular techniques and are still found in regulatory and compendial protocols throughout the world (formulated in the EP, USP, and JP regulatory guidelines).</td>
<td>Traditional culture methods were used well before today’s molecular techniques and are still found in regulatory and compendial protocols throughout the world (formulated in the EP, USP, and JP regulatory guidelines). The Culture Method is based on the targeted cultivation of mycoplasmas in culture media that promote mycoplasma growth. A sample to be tested is inoculated into the liquid mycoplasma culture media and onto agar, and mycoplasma growth is promoted by micro-aerophilic incubation conditions at 36 ± 1°C, 5.5 ± 0.5% CO2.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture Method</td>
<td>• Requires validation to substitute cell culture and indicator cell methods</td>
<td>• Requires DNA extraction kit and costly equipment/instruments</td>
</tr>
<tr>
<td>Indicator Cell Culture Method</td>
<td>• Requires more than one growth medium for the cultivation of different mycoplasma species</td>
<td>• Requires DNA extraction kit and costly equipment/instruments</td>
</tr>
</tbody>
</table>

Mycoplasma colony morphologies can vary from the typical fried-egg shape to more irregular shapes (Figure 4). As mycoplasma colonies can be very small, colony counting under the microscope requires some experience. A schematic illustration of the Culture Method is shown in Figure 5.
The relatively large sample volume (10 ml) and the long incubation period (28 days in total) render the Culture Method one of the most sensitive tests, with a theoretical and experimentally proven detection limit of 0.1 CFU/ml, corresponding to 1 CFU/10 ml sample. The method fulfills the EP 2.6.7 requirement of detecting 10 CFU/ml, which is why this method is still the reference method in regulatory documents worldwide.

The Culture Method has, however, a few disadvantages. The predominant drawbacks come from the lengthy cultivation period of 28 days. This time factor poses major challenges for many companies, including product release delays that entail higher storage costs, as well as increased personnel costs for logistics during testing of raw materials, the cell line, and the process controls in the up- and downstream process.

Another limitation is that the Culture Method requires usage of several different growth media. Not all mycoplasma species thrive in the same culture medium. Thus, depending on the source of the sample, different mycoplasma culture media are used in parallel to increase the detection spectrum of possible contaminating mycoplasma species. For this reason, the use of at least two standard mycoplasma culture media is recommended in EP 2.6.7: FRIIS medium for the detection of non-avian mycoplasmas and FREY medium for the detection of the avian mycoplasma species *M. synoviae*. The perhaps greatest disadvantage of the Culture Method is, however, that highly fastidious mycoplasma strains, such as the *M. hyorhinis* cultivar alpha strains (with *M. hyorhinis* DBS 1050 as reference strain), do not grow in the standard culture media due to growth inhibition by peptones and yeast products. Growth of these cell-culture adapted strains is dependent on their habitat, the cell culture. In order to detect these cultivar alpha strains, an additional test has to be performed in parallel, using the Indicator Cell Culture Method.
Indicator Cell Culture Method

The Indicator Cell Culture Method is normally carried out with Vero or 3T3 cell lines, although the use of a production cell line that is equivalent in effectiveness for detecting mycoplasmas is also accepted by the EP regulatory guideline. The indicator cell culture is inoculated with the sample and incubated at 35–38°C until grown to confluence. For positive controls, the indicator cell line is also inoculated with the type strain of M. orale and the M. hyorhinis cultivar alpha reference strain and with the absence of the test sample. Before staining, the subculture is fixed with a suitable fixing solution and stained with a fluorescent dye that binds to DNA. The presence of mycoplasmas is characterized by a spherical fluorescence pattern on the cell surface and by strong fluorescence in the surrounding areas. Mitochondria in the cytoplasm are also stained, but are easily distinguished from mycoplasmas. The test is invalid if the positive controls do not show fluorescence typical for mycoplasmas or if the negative control shows fluorescence typical for mycoplasmas. A schematic illustration of the Indicator Culture Method is shown in Figure 6.

10 ml sample
- Positive control: Vero cells
- Positive control: M. orale ≤ 100 CFU
- Negative control: Vero cells

Figure 6: Indicator Culture Method as carried out by Mycoplasma Biosafety. A freshly prepared Vero indicator cell culture is inoculated with 1 ml sample. Four positive controls are prepared. Two positive controls consist of Vero cells inoculated with 1 ml sample spiked with not more than 100 CFU M. orale and M. hyorhinis, respectively. The other two positive controls are Vero cells inoculated with not more than 100 CFU M. hyorhinis and M. orale without the sample. The two positive control strains are plated onto agar medium to check for viability. The negative control is a freshly prepared Vero cell culture that is left uninoculated. All cell cultures are incubated in a CO₂ incubator until the cell density of 100% is reached. The cell layer is then washed with buffer and trypsinated. The detached cells are resuspended in cell culture medium, transferred to chamber slide flasks and incubated in a CO₂ incubator until the cell density is approximately 50%. The cell layer is fixed twice with a freshly prepared fixing solution, allowed to air dry and stained with a Hoechst Stain.

Fluorescence Microscopic Evaluation

- Positive control: Vero cells + M. hyorhinis
- Positive control: M. orale ≤ 100 CFU
- Negative control: Vero cells

Mycoplasma Biosafety

Both the Culture Method and the Indicator Cell Culture Method take a long time to results (up to 28 days for the Culture Method and 7 days for the Indicator Cell Culture Method), and carry the intrinsic risk of introducing a mycoplasma contamination into the facility due to the required handling of viable mycoplasma cells as positive control organisms.

NAT-based Methods

NAT-based methods include all tests based on nucleic acid detection, often performed by PCR.

PCR

PCR is a molecular biological method used in many areas such as food and environmental analysis, forensics, and medical diagnostics. The underlying principle is the specific amplification of DNA to a level that can be detected. This amplification is carried out by the enzyme DNA polymerase in repeated amplification cycles that are automated by thermocyclers. One cycle consists of three main steps:

1. Double-stranded DNA (dsDNA) is denatured into single-stranded DNA (ssDNA) by heat
2. PCR primers bind to specific ssDNA sites (e.g. to specific target genes)
3. DNA polymerase elongates the annealed primers according to the sequence of the ssDNA

For more information about the PCR procedure in detail:
(http://go.roche.com/dnacopy)

Usually after 30–50 PCR cycles sufficient DNA is amplified for detection by gel electrophoresis and staining with fluorescent dyes.

PCR temperature protocols have been optimized with the goal to develop more sensitive, specific, or rapid PCR assays. Touchdown PCR (TD-PCR), for example, is very commonly used to make PCR assays more specific to a targeted gene. Primers bind with high specificity to a targeted DNA sequence at high annealing temperatures during the first few PCR cycles. This ensures the exclusive amplification of a specific DNA sequence. The annealing temperature is then gradually decreased to reach highest PCR efficiency. TD-PCR protocols reduce the amount of nonspecific DNA amplified by PCR.24 MycoTOOL qPCR leverages TD-PCR for highly specific mycoplasma detection.

You will find a summary and more information about the history and evolution from the conventional PCR to the qPCR here:
(http://go.roche.com/storypcr)

In contrast to PCR, Real-Time PCR (qPCR) reports the amplification of DNA in real time. Thus, there is no need for post-PCR DNA detection such as gel electrophoresis. This reduces the risk of contamination in the laboratory dramatically and facilitates the interpretation of end results. qPCR uses probes consisting of a fluorescent dye attached to a short DNA sequence (18–30 base pairs) that is added to the PCR. The probe is incorporated into the new strands of DNA produced in each amplification cycle. There are a variety of different probe designs on the market, but one of the most common ones are the hydrolysis probes used in MycoTOOL qPCR. This probe reports the amount of total DNA as fluorescence intensity after each PCR cycle. The fluorescent signal increases proportionally to the amplification of the target sequence. The fluorescence intensity is plotted over time and forms a typical sigmoid qPCR curve (see Figure 7).
Both, PCR and qPCR are sensitive methods and thus prone to DNA contamination. The most common sources of contamination are the DNA template itself and amplified DNA from post-PCR reactions. DNA molecules may be spread around the lab via air conditioning systems or laboratory staff. The most effective strategy to eliminate these contamination sources is a unidirectional workflow from sample drawing to DNA detection in separate work areas or separate rooms.\(^{14}\)

**Mycoplasma Testing Methods Overview**

The MycoTOOL Real-Time PCR Kit is a qPCR assay optimized for the detection of mycoplasmas in cell culture. It fulfills all EP 2.6.7 requirements for NAT-based assays for mycoplasma detection with respect to sensitivity (i.e., detection limit of ≤10 CFU), specificity, robustness and comparability. It does not require a mycoplasma enrichment or pre-incubation step. The kit uses primers and probes that are highly specific to the mycoplasma 16S ribosomal DNA gene. This allows the detection of more than 150 cultivable and non-cultivable mycoplasma species. It includes all frequently or potentially occurring cell culture contaminants, namely *M. arginini*, *M. fermentans*, *M. gallisepticum*, *M. hyorhinis*, *M. orale*, *M. salivarius*, *M. syngeniae*, and *S. citri* (Table 1), as well as the pathogenic human mycoplasma species *M. pneumoniae* and *M. hominis*.

Automated DNA extraction may be conducted with either a MagNa Pure 24 or a MagNa Pure 96 instrument, followed by subsequent qPCR performed on the LightCycler 480 II real-time PCR instrument. Manual DNA extraction may be done with the Roche QC Preparation Kit for samples with cell densities up to 5×10\(^6\) cells/ml, or with MycoTOOL Mycoplasma Detection Prep Kit, High Cell Density for samples with a cell density range of 5 × 10\(^6\) cells/ml to 1 × 10\(^7\) cells/ml. Carrier DNA is available for analysis of cell-free samples and may be added to the biological sample prior to nucleic acid extraction and purification. The entire workflow from sampling to result takes 4 to 6 hours, depending on the level of automation and the number of samples. The complete workflow is depicted in Figure 8.

**Mycoplasma Testing Methods Overview**

### MycoTOOL Mycoplasma Real-Time PCR Kit (MycoTOOL qPCR)

The MycoTOOL Real-Time PCR Kit is a qPCR assay optimized for the detection of mycoplasmas in cell culture. It fulfills all EP 2.6.7 requirements for NAT-based assays for mycoplasma detection with respect to sensitivity (i.e., detection limit of ≤10 CFU), specificity, robustness and comparability. It does not require a mycoplasma enrichment or pre-incubation step. The kit uses primers and probes that are highly specific to the mycoplasma 16S ribosomal DNA gene. This allows the detection of more than 150 cultivable and non-cultivable mycoplasma species. It includes all frequently or potentially occurring cell culture contaminants, namely *M. arginini*, *M. fermentans*, *M. gallisepticum*, *M. hyorhinis*, *M. orale*, *M. salivarius*, *M. syngeniae*, and *S. citri* (Table 1), as well as the pathogenic human mycoplasma species *M. pneumoniae* and *M. hominis*.

Automated DNA extraction may be conducted with either a MagNa Pure 24 or a MagNa Pure 96 instrument, followed by subsequent qPCR performed on the LightCycler 480 II real-time PCR instrument. Manual DNA extraction may be done with the Roche QC Preparation Kit for samples with cell densities up to 5×10\(^6\) cells/ml, or with MycoTOOL Mycoplasma Detection Prep Kit, High Cell Density for samples with a cell density range of 5 × 10\(^6\) cells/ml to 1 × 10\(^7\) cells/ml. Carrier DNA is available for analysis of cell-free samples and may be added to the biological sample prior to nucleic acid extraction and purification. The entire workflow from sampling to result takes 4 to 6 hours, depending on the level of automation and the number of samples. The complete workflow is depicted in Figure 8.

**Mycoplasma Testing Methods Overview**

### MycoTOOL Mycoplasma Real-Time PCR Kit (MycoTOOL qPCR)

The MycoTOOL Real-Time PCR Kit is a qPCR assay optimized for the detection of mycoplasmas in cell culture. It fulfills all EP 2.6.7 requirements for NAT-based assays for mycoplasma detection with respect to sensitivity (i.e., detection limit of ≤10 CFU), specificity, robustness and comparability. It does not require a mycoplasma enrichment or pre-incubation step. The kit uses primers and probes that are highly specific to the mycoplasma 16S ribosomal DNA gene. This allows the detection of more than 150 cultivable and non-cultivable mycoplasma species. It includes all frequently or potentially occurring cell culture contaminants, namely *M. arginini*, *M. fermentans*, *M. gallisepticum*, *M. hyorhinis*, *M. orale*, *M. salivarius*, *M. syngeniae*, and *S. citri* (Table 1), as well as the pathogenic human mycoplasma species *M. pneumoniae* and *M. hominis*.

Automated DNA extraction may be conducted with either a MagNa Pure 24 or a MagNa Pure 96 instrument, followed by subsequent qPCR performed on the LightCycler 480 II real-time PCR instrument. Manual DNA extraction may be done with the Roche QC Preparation Kit for samples with cell densities up to 5×10\(^6\) cells/ml, or with MycoTOOL Mycoplasma Detection Prep Kit, High Cell Density for samples with a cell density range of 5 × 10\(^6\) cells/ml to 1 × 10\(^7\) cells/ml. Carrier DNA is available for analysis of cell-free samples and may be added to the biological sample prior to nucleic acid extraction and purification. The entire workflow from sampling to result takes 4 to 6 hours, depending on the level of automation and the number of samples. The complete workflow is depicted in Figure 8.

The MagNa Pure 24 and 96 instruments purify nucleic acids from a wide range of starting materials (e.g. whole blood, plasma, cell culture) using magnetic glass particle technology. For more information use the QR-Code for our MagNa Pure 96 and for our MagNa Pure 24 System.

MycoTOOL qPCR includes three controls to ensure validity of results. To verify the integrity of all reagents used during qPCR, a plasmid-based positive control is added to each experiment. The third control used is a plasmid-based recovery control (RC; also known as an exogenous internal control) that is added to each sample prior to DNA isolation. It is co-amplified using a second set of primers and probes in a separate vial. The RC verifies the integrity of the complete workflow, from DNA isolation to PCR. Because the RC is spiked into the sample as an exogenous control, MycoTOOL qPCR is not limited to a specific cell line and may be used across the spectrum of cell lines commonly used in biopharmaceutical manufacturing.

The MagNa Pure 24 and 96 instruments purify nucleic acids from a wide range of starting materials (e.g. whole blood, plasma, cell culture) using magnetic glass particle technology. For more information use the QR-Code for our MagNa Pure 96 and for our MagNa Pure 24 System.

**Example of mycoplasma testing by MycoTOOL qPCR analysis.** In the first 15 cycles of the qPCR, the baseline describes the initial signal at which little change is seen in the fluorescence intensity. This signal can also be defined as background fluorescence of the reaction. The threshold cycle (Cq) is the cycle number at which the fluorescence signal of the sample exceeds the background signal. The lower the Cq value the higher the amount of DNA in the sample. Source: Roche CustomBiotech, 2017.

**Figure 7:** Example of mycoplasma testing by MycoTOOL qPCR analysis. In the first 15 cycles of the qPCR, the baseline describes the initial signal at which little change is seen in the fluorescence intensity. This signal can also be defined as background fluorescence of the reaction. The threshold cycle (Cq) is the cycle number at which the fluorescence signal of the sample exceeds the background signal. The lower the Cq value the higher the amount of DNA in the sample. Source: Roche CustomBiotech, 2017.

**Figure 8:** MycoTOOL qPCR workflow. Unprocessed sample (1 ml) with a cell density of 5×10\(^6\) cells/ml is prepared using a manual or automated workflow. For manual sample preparation, nucleic acids are isolated with the Roche QC Preparation Kit. With the automated nucleic acid isolation system, the sample DNA is purified with one of the MagNA Pure systems. Subsequently, a mycoplasma specific qPCR reaction is performed on the LightCycler 480 II Real-Time PCR system. The automated workflow based on the MagNA Pure 96 and LightCycler 480 II system, shown as the purple marked procedure, has been fully validated by Roche Pharma Biotech and according to EP chapter 2.6.7. The generic validation report is available on request under confidential disclosure agreement. A summary of the study design and results is available under http://go.roche.com/MycoTOOLqPCR.

**Source:** Roche CustomBiotech, 2017.

MycoTOOL qPCR includes three controls to ensure validity of results. To verify the integrity of all reagents used during qPCR, a plasmid-based positive control is added to each experiment. False negative results are controlled by a H\(_2\)O negative control. The third control used is a plasmid-based recovery control (RC; also known as an exogenous internal control) that is added to each sample prior to DNA isolation. It is co-amplified using a second set of primers and probes in a separate vial. The RC verifies the integrity of the complete workflow, from DNA isolation to PCR. Because the RC is spiked into the sample as an exogenous control, MycoTOOL qPCR is not limited to a specific cell line and may be used across the spectrum of cell lines commonly used in biopharmaceutical manufacturing.
All amplification reactions are carried out in technical replicates (two or four for the negative control and positive control/RC - sample, respectively). Figure 9 shows a typical pipetting scheme of a 96 well qPCR plate.

Acceptance criteria for a plate to pass evaluation are: all negative controls must give a negative result, and the positive control reactions as well as all RC reactions of a sample must give a positive result.

---

**Mycoplasma Master Mix**

**Recovery Control Mix**

---

The regulatory approval system

The health authorities are responsible for the scientific evaluation, supervision and safety monitoring of medicinal products developed by pharmaceutical companies. Regulatory approval ensures that all medicinal products available on the market are safe, effective and of high quality. Pharmaceutical approval and market authorization of medicines for humans and animals requires manufacturers to meet official quality standards. This is controlled by different regulatory agencies and committees. The standards that manufacturers have to meet are defined and published in the pharmacopoeias. The pharmacopoeia lists all tests to be carried out on medicines, intermediates and raw materials, and is legally binding for a country or all member states of a union.

---

**Table 3:** Overview of the health authorities in the EU, the USA, Japan, China, Brazil and Argentina, their regulatory agencies, legally binding documents and raw materials, and is legally binding for a country or all member states of a union.

<table>
<thead>
<tr>
<th>Health Authority</th>
<th>UK</th>
<th>USA</th>
<th>Japan</th>
<th>China</th>
<th>Brazil</th>
<th>Argentina</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Regulatory Agency for Pharmaceutical Approval</strong></td>
<td>Committee for Medicinal products for Human Use (CHMP)</td>
<td>National Approval by MHRA</td>
<td>Food and Drug Administration (FDA)</td>
<td>Ministry of Health, Labour and Welfare (MHLW)</td>
<td>Ministry of Health (MOH)</td>
<td>Brazilian Ministry of Health</td>
</tr>
<tr>
<td><strong>Publisher Pharmacopoeia</strong></td>
<td>European Directorate for the Quality of Medicines and Healthcare Products (EDQM)</td>
<td>British Pharmacopoeia Commission</td>
<td>US Pharmacopeial Convention</td>
<td>Pharmaceutical and Medical Devices Agency (PMDA)</td>
<td>Chinese Food and Drug Administration (CFDA)</td>
<td>National Sanitary Surveillance Agency (ANVISA)</td>
</tr>
<tr>
<td><strong>NAT Acceptance for Mycoplasma Testing</strong></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>?</td>
<td>Not described</td>
</tr>
<tr>
<td><strong>Specification of NAT Validation Requirements</strong></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>?</td>
<td>✓</td>
</tr>
</tbody>
</table>

---

**Regulatory Overview**

Since mycoplasma contamination evidently affects cell cultures, testing for mycoplasmas has increasingly become regulated by authorities. Today, mycoplasma testing in biopharmaceutical manufacturing is controlled by law in almost all countries around the world. Regulatory authorities have published legally binding documents with national pharmacopoeias for mycoplasma testing. These documents define the methods used and products to be tested. However, recommended methods for mycoplasma testing and their detailed test protocols may differ among countries. In general, one must differentiate between conventional culture-based and alternative NAT-based mycoplasma testing methods.

The traditional compendial methods, such as the Culture Method and the Indicator Cell Culture Method (see chapter 2.2.), are considered the long-standing gold standard and thus, are widely recommended in all pharmacopoeias. Although individual methodological steps may slightly vary from one national pharmacopoeia to the other, the protocols for these culture-based tests are largely harmonized across the countries. Please refer to Table 3 for a summary of regulatory authorities and pharmacopoeias containing regulatory guidelines relevant for mycoplasma testing in some countries.
The situation is very different for rapid mycoplasma testing methods, like NAT. Even though many national pharmacopoeias mention NAT as a valid mycoplasma testing method, there is little harmonization across countries regarding protocols or validation requirements. Some pharmacopoeias such as the EP and JP mention detailed validation guidelines, whereas others merely point out that NAT is a valid testing method after validation. However, all countries require an appropriate validation and comparison with conventional mycoplasma testing methods.

Validation Requirements

### Limit of Detection (LOD)

To define the detection limit, a positive cut-off point should be determined for each species (the chapter provides a list of mycoplasma species to be used as test organisms). For each strain, a minimum of three independent 10-fold dilution series should be tested, with a sufficient number of replicates at each dilution to give a total number of 24 test results for each dilution. The positive cut-off point is defined as the concentration of mycoplasmas that can be detected in 95 percent of test runs, thus in at least 23 test results.

### Specificity

It is important to use PCR primers that are specific for a wide range of mycoplasmas. However, it is likely that PCR primers will also detect other bacterial species. This potential cross-detection should be documented by testing related bacterial genera such as gram-positive bacteria with close phylogenetic relation to mycoplasmas (the chapter provides a list of bacterial genera to be tested).

### Robustness

The measure of the NAT method’s capacity to remain unaffected by small but deliberate variations in method parameters and test method modifications needs to be demonstrated. The chapter provides examples of variations and test modifications that may be tested.

### Comparability

The comparability should include a comparison of the LODs between NAT and the compendial methods. The chapter defines the following acceptance criteria:
1. Culture Method replacement by NAT: A detection limit of at least ≤10 CFU/mL needs to be demonstrated.
2. Indicator Cell Culture Method replacement by NAT: A detection limit of at least ≤100 CFU/mL needs to be demonstrated for each mycoplasma test species.
3. In both cases the NAT alternative method needs to be performed in parallel to both conventional methods to evaluate simultaneously the LOD of both methods using the same samples of CFU-calibrated mycoplasma test strains.

| Table 4: Summary of validation requirements as detailed in EP 2.6.7. For more detailed information, refer to the EP mycoplasma NAT validation guideline |

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limit of Detection (LOD)</td>
<td>To define the detection limit, a positive cut-off point should be determined for each species (the chapter provides a list of mycoplasma species to be used as test organisms). For each strain, a minimum of three independent 10-fold dilution series should be tested, with a sufficient number of replicates at each dilution to give a total number of 24 test results for each dilution. The positive cut-off point is defined as the concentration of mycoplasmas that can be detected in 95 percent of test runs, thus in at least 23 test results.</td>
</tr>
<tr>
<td>Specificity</td>
<td>It is important to use PCR primers that are specific for a wide range of mycoplasmas. However, it is likely that PCR primers will also detect other bacterial species. This potential cross-detection should be documented by testing related bacterial genera such as gram-positive bacteria with close phylogenetic relation to mycoplasmas (the chapter provides a list of bacterial genera to be tested).</td>
</tr>
<tr>
<td>Robustness</td>
<td>The measure of the NAT method’s capacity to remain unaffected by small but deliberate variations in method parameters and test method modifications needs to be demonstrated. The chapter provides examples of variations and test modifications that may be tested.</td>
</tr>
<tr>
<td>Comparability</td>
<td>The comparability should include a comparison of the LODs between NAT and the compendial methods. The chapter defines the following acceptance criteria: 1. Culture Method replacement by NAT: A detection limit of at least ≤10 CFU/mL needs to be demonstrated. 2. Indicator Cell Culture Method replacement by NAT: A detection limit of at least ≤100 CFU/mL needs to be demonstrated for each mycoplasma test species. 3. In both cases the NAT alternative method needs to be performed in parallel to both conventional methods to evaluate simultaneously the LOD of both methods using the same samples of CFU-calibrated mycoplasma test strains.</td>
</tr>
</tbody>
</table>

There are other non-legally binding documents that may be considered as a reference for implementation of NAT mycoplasma testing methods:

(i) The Parenteral Drug Association (PDA) published a technical report named “Alternative Methods for Mycoplasma Testing” in 2010. It guides new users of rapid mycoplasma tests and describes assay procedure, assay validation, and potential applications for alternative mycoplasma testing methods.\(^{(i)}\)

(ii) Roche Pharma uses the MycoTOOL qPCR assay to release new products to the market. The assay has been validated according to EP chapter 2.6.7. and the generic validation report is available on request under confidential disclosure agreement. A summary of the study design and results is available under http://go.roche.com/MycoTOOLqPCR.\(^{(ii)}\)

In addition to the USP, theme-specific guidelines are published by FDA departments. The CBER of the FDA published a guideline in 1993 called “Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals” (PTC), and a “Guidance for Industry” in 2010 that provide additional information on mycoplasma testing. Neither is legally binding.\(^{(i)}\)\(^{(ii)}\)

Bioanalytical Method Validation is another non-legally binding document published by the FDA that helps gain an overview of best practices to validate an alternative method for mycoplasma testing.\(^{(i)}\)

Figure 10: Flowchart for the implementation of MycoTOOL qPCR. The left hand side of the flowchart depicts validation carried out by leveraging Roche’s generic validation report (implementation for CHO manufacturing processes). Implementing MycoTOOL qPCR for a non-CHO process or changing the method used in Roche’s generic validation requires revalidation of the MycoTOOL qPCR method. This process, presented on the right hand side of the flowchart, is more time consuming. For either validation procedure as well as routine testing Mycoplasma Biosafety may be a preferred contract research organization to outsource most of the steps involved. Source: Authors, 2017.
Supplier Due Diligence
This step may take several days to a few months. It involves a due diligence of marketed mycoplasma detection kits and CRO to carry out the study. Product specifications must be checked against testing requirements and the capabilities of the mycoplasma detection kit suppliers and CROs have to be assessed. Before moving forward with the validation, several points should be clarified:

• Does the supplier provide appropriate documentation with regards to design and manufacture of instruments and reagents?
• Does the supplier have change control systems in place?
• Does the supplier provide quality and supply agreements if required?
• Does the supplier deliver in-time and reliably?
• Are references of previous validation studies available?
• Do the supplier and CRO respond to questionnaires and allow physical audits at their testing facility?
• Does the supplier provide end-user training programs, on-site technical service, installation and operational qualification services, preventive maintenance service, and a technical support hotline?
• Does the CRO have sufficient experience with product-specific NAT validation studies and implementation of NAT-based routine testing?
• Does the CRO provide a validation report, protocols or similar documentation?
• Does the CRO provide solutions for technology transfers?

During this phase, it may also be required to prepare an economic assessment or financial justification (i.e., write a business case) to apply for budget internally. Thus, associated costs, such as one-time costs (requirements for laboratory space, instrumentation costs, installation costs, etc.) and operating costs (maintenance, cost per sample, etc.) should be clarified with the contract service provider during this step.

Feasibility Study
This step may take from two to several months. Mycoplasma Biosafety accomplished a feasibility study for a European customer within four weeks). The validation study demonstrates that MycoTOOL qPCR is capable of consistently detecting mycoplasmas according to regulatory requirements. It may be performed internally using the respective instrumentation, or it may be outsourced to a CRO such as Mycoplasma Biosafety.

Roche Pharma Generic Validation Report
Roche Pharma conducted a full generic validation according to EP 2.6.7. (NAT validation guideline) for MycoTOOL qPCR with defined instrumentation (LightCycler® 480 Instrument II and MagNA Pure 96, see Figure 10) and specifically for processes using Chinese hamster ovary (CHO) cell lines. Typically, regulatory agencies such as FDA or EMA do not require users to fully revalidate MycoTOOL qPCR as long as the main process, including instrumentation, is unchanged. Thus, a product-specific validation study using the unchanged workflow will require testing fewer samples and hence less time. However, this option should be discussed with the relevant regulatory agency before making a decision.

This validation report is available on request and under confidential disclosure agreement. Please find a scientific poster with a summary of the data under this link.

(https://go.roche.com/MycoToolqPCR)

During this phase, it may also be required to prepare a feasibility study to verify the technical feasibility of the approach. This includes assessing the feasibility of the instrumentation, the laboratory space, and the personnel required. The feasibility study should also consider the number of samples to be tested and the overall cost of the study.

Validation Study
This step may take from one to several months (Mycoplasma Biosafety accomplished a feasibility study for a European customer within one week). It may be accomplished by using rental equipment on site or, more efficiently, by collaborating with a CRO who conducts the study. A feasibility study is a technical proof-of-concept testing to reach the target LOD. Such adjustments can be a larger PCR reaction volumes or an increase in PCR cycles.

Validation Strategy
This step may take from a few weeks to several months. The validation strategy provides a roadmap for all experiments that will be conducted during the MycoTOOL qPCR validation study (e.g., LOD testing, robustness testing, specificity analysis). The strategy includes a timeline, responsible parties, planned experiments, and relevant acceptance criteria. The generic validation conducted by Roche can be taken into account. Usually, a few criteria of the generic validation must be re-confirmed (such as the LOD for a selected number of mycoplasma reference strains). Generally, using the generic validation report as basis for a validation study reduces the number of samples to be tested and the overall cost of the study. It is important to always discuss the strategy with the respective regulatory agency, especially if the Roche generic validation may streamline your project.

Submission & Routine Testing
After successful submission and approval of the validation report by the regulatory authority, routine testing may be carried out. Depending on the customer and the project, routine testing may be carried out by internal quality control experts or the complete process remains outsourced to a CRO such as Mycoplasma Biosafety.
Automated NAT-based rapid methods for mycoplasma testing offer revolutionary opportunities and competitive advantages for the biopharmaceutical industry. Latest sample preparation and mycoplasma detection kit improvements and recent advances addressing product-specific validation challenges to meet the regulatory requirements will certainly push these new test systems towards being the superior long-term alternative in comparison to compendial culture methods. Based on recent developments and trends, it is expected that in the next few years regulatory bodies like the EMA and the US FDA will increasingly approve routine mycoplasma testing of biopharmaceuticals by rapid NAT-based methods, using commercial tools such as the Roche CustomBiotech MycoTOOL qPCR assay in combination with the Roche MagNA Pure Sample Preparation System. This guideline gives a general overview and specific recommendations on how to validate and implement MycoTOOL qPCR as a rapid automated NAT-based mycoplasma testing method for in-process control and lot release of biopharmaceutical products.

Current users of MycoTOOL qPCR appreciate the benefits of this highly automated analytical system. Easy handling, rapid workflow for same-day results, high-throughput sample processing, cost-effectiveness, lack of product matrix and PCR inhibition effects even with “worst case products” of high cell densities, and the ability to test cell-free samples are convincing advantages of the system. Straightforward validation designs for this automated mycoplasma testing system can be individually tailored for any biopharmaceutical product. The implementation of MycoTOOL qPCR as an early warning system for raw material testing, in-process control and for final lot release increases efficiency and product quality by reliably excluding the risks of mycoplasma contaminations during manufacturing stages.

A rise in the application of automated NAT-based MycoTOOL qPCR method for routine mycoplasma testing can be expected in the future as it has significant advantages compared to compendial culture methods and customized product-specific validation plans from experienced experts are available from contract service laboratories. This will ultimately lead to a change in quality control and lot release standards throughout the biopharmaceutical industry and will certainly impact the market.
### Glossary

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ANMAT</td>
<td>Drug Regulatory Authority (Argentina)</td>
</tr>
<tr>
<td>ANVISA</td>
<td>National Sanitary Surveillance Agency (Brazil)</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CBER</td>
<td>Center for Biologics Evaluation and Research (US FDA)</td>
</tr>
<tr>
<td>CDER</td>
<td>Center for Drug Evaluation and Research (US FDA)</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit/s</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary (cells)</td>
</tr>
<tr>
<td>ChP</td>
<td>Chinese Pharmacopia</td>
</tr>
<tr>
<td>ChPC</td>
<td>Chinese Pharmacopia Commission</td>
</tr>
<tr>
<td>Cq</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CRO</td>
<td>Contract Research Organization</td>
</tr>
<tr>
<td>CFDA</td>
<td>Chinese (State) FDA</td>
</tr>
<tr>
<td>CVMA</td>
<td>Center for Veterinary Medicine (US FDA)</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>d(s)DNA</td>
<td>Double-stranded DNA</td>
</tr>
<tr>
<td>EDQM</td>
<td>European Directorate for the Quality of Medicines</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>EP</td>
<td>European Pharmacopia</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
</tr>
<tr>
<td>G+C</td>
<td>Guanine-cytosine content</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
</tbody>
</table>

### Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>ICH</td>
<td>International Committee on Harmonization</td>
</tr>
<tr>
<td>JP</td>
<td>Japanese Pharmacopia</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>MHLW</td>
<td>Japanese Ministry of Health, Labor and Welfare</td>
</tr>
<tr>
<td>MHRA</td>
<td>Medicines and Healthcare Products Regulatory Agency (UK)</td>
</tr>
<tr>
<td>MOH</td>
<td>Chinese Ministry of Health</td>
</tr>
<tr>
<td>MycoTOOL gPCR</td>
<td>MycoTOOL Mycoplasma Real-Time PCR Assay/Kit</td>
</tr>
<tr>
<td>NAT</td>
<td>Nucleic acid amplification technique/s</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDA</td>
<td>Parenteral Drug Association</td>
</tr>
<tr>
<td>PMDA</td>
<td>Pharmaceutical and Medical Devices Agency</td>
</tr>
<tr>
<td>PTC</td>
<td>Points to Consider (FDA/CBER Guideline 1993)</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Real-time PCR</td>
</tr>
<tr>
<td>RC</td>
<td>Recovery control DNA</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SC</td>
<td>Small colony type</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>TD-PCR</td>
<td>Touchdown PCR</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>US</td>
<td>United States (of America)</td>
</tr>
<tr>
<td>USP</td>
<td>United States Pharmacopia</td>
</tr>
</tbody>
</table>
References

1) European Directorate for the Quality of Medicines (EDQM), European Pharmacopoeia (EP), 9th Ed., Chapter 2.6.7: Mycoplasmas; 2016
5) Mackuski L et al., Biofilm formation by mycoplasma species and its role in environmental persistence and survival, Microbiology 152: 313-322; 2006
7) Yu X et al., Impact of genome reduction on bacterial metabolism and its regulation, Science 320: 1268-1269; 2008
8) Rosegarand R et al., The changing image of mycoplasmas: from innocent bystanders to emerging and reemerging pathogens in human and animal diseases. In: Mühldorfer I and Schäfer KP (Eds), Emerging Bacterial Pathogens, Contributions to Microbiology, Vol 8, pp 166-185, Karger, Basel; 2001
9) Uphoff CC and Drexler HG, Prevention of mycoplasma contamination in leukaemia/lymphoma cell lines. Human Cell, 14: 244-247; 2001
11) Pawar, V et al., Trends in the incidence and distribution of mycoplasma contamination detected in cell lines and their products. IOM Let 3: 77; 1994
13) McGarry G. Mycoplasma Infection of Cell Cultures. Springer; 2012
16) Zinkowicz S et al., Mycoplasma contamination revised: mesenchymal stromal cell cultures harboring Mycoplasma hyorhinis potently inhibit lymphocyte proliferation in vitro. PLOS One 6: e16035; 2011
18) Pitt A et al., Assay for detecting mycoplasma by measuring acetate kinase or carbama kinase activity. WO2004094856 A1; 2004
19) Slater KJ et al., Mycoplasma detecting methods and materials. US7585644 B2; 2009
21) Boche, Mycoplasma PCR ELISA. REF 11 663 929 010
22) Wirth M et al., Mycoplasma detection by the mycoplasma PCR ELISA. Biochimica 3: 33-35; 1995
24) Metzger ML and Caskey CT, Polymerase chain reaction (PCR). eLS; 2009
25) Dörfner Schmidt and Dewsker GS, Setting up a PCR laboratory. Genetec Research, 5: 52-57; 1993
26) Containing all countries inside the Schengen Agreement: EU member states and countries of the European Economic Area
27) European Directorate for the Quality of Medicines (EDQM), European Pharmacopoeia (EP), 9th Ed.; 2016
28) British Pharmacopoeia Commission, British Pharmacopoeia; 2016
31) Chinese Pharmacopoeia Commission (ChPC), Chinese Pharmacopoeia (ChP); 2015
32) National Sanitary Surveillance Agency (ANVISA), Brazilian Pharmacopoeia, 5th Ed.; 2010
33) Drug Regulatory Authority (ANMAT), Argentine Pharmacopoeia 7th Ed.; 2013
35) Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER), Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals (PTC), Attachment 2, Recommended Procedures for Detection of Mycoplasma Contamination in Biological Products Produced in Cell Substrates: 1993
36) Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER), Guidance for Industry: Characterization and Qualification of Cell Substrates and other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications; 2010
37) Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), and Center for Veterinary Medicine (CVM), Guidance for Industry, Bioanalytical Method Validation; 2011
39) Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER), Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals (PTC), Attachment 2, Recommended Procedures for Detection of Mycoplasma Contamination in Biological Products Produced in Cell Substrates: 1993
40) Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER), Guidance for Industry: Characterization and Qualification of Cell Substrates and other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications; 2010
41) Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), and Center for Veterinary Medicine (CVM), Guidance for Industry, Bioanalytical Method Validation; 2011
43) Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER), Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals (PTC), Attachment 2, Recommended Procedures for Detection of Mycoplasma Contamination in Biological Products Produced in Cell Substrates: 1993
44) Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER), Guidance for Industry: Characterization and Qualification of Cell Substrates and other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications; 2010
45) Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), and Center for Veterinary Medicine (CVM), Guidance for Industry, Bioanalytical Method Validation; 2011