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# Development of a state-of-the-art Mycoplasma qPCR Assay

Designed to be compliant with European, Japanese and United States Pharmacopeia and comparison to cell culture based methods

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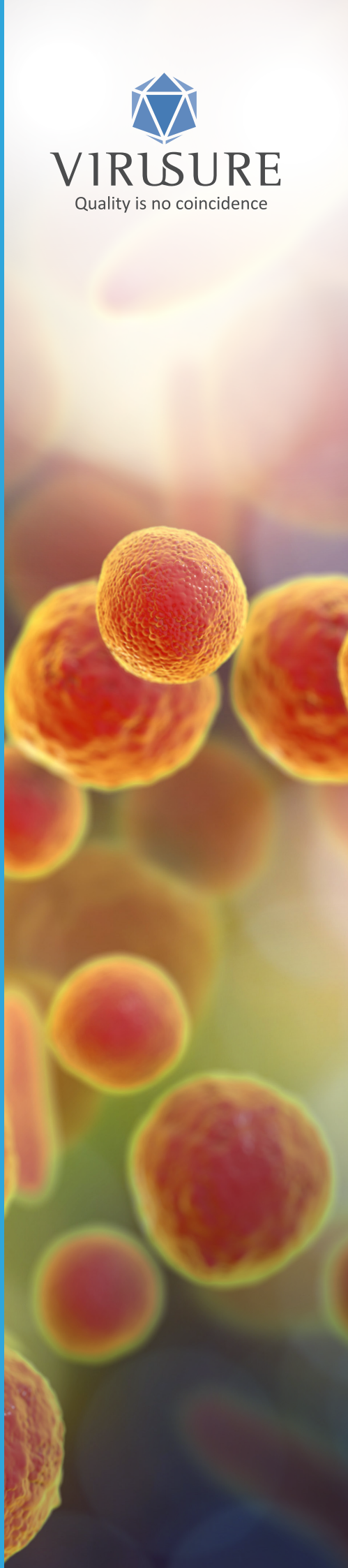

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# Background

Mycoplasmas (a generic name for Mollicutes including the families *Mycoplasma, Spiroplasma and Acholeplasma)* are a widely diverse group of more than 250 species, having a broad host range including humans, birds, insects, plants and more[1]. Because of a number of characteristics (e.g. their broad distribution in nature, covert growth in infected cells, and their small size, which enables them to pass through 0.2 µM filters), they are a frequent contaminant of cell culture systems[1-4]. The adversity of the effects on infected cells varies but can be very severe depending on the mycoplasma species infecting the cells, type of cells, the mycoplasma burden, and the duration of the infection[3]. Contaminations might result in drastic changes in the metabolism and growth of the cells, apoptosis, or DNA/RNA synthesis. They can affect signal transduction, induce chromosomal aberrations, or deplete media components all leading to compromised data during biochemical and biological assays. Also virus growth can be inhibited leading to reduced titers in virus stock productions[1][3][5]. Even more importantly than mycoplasma contaminations being a significant and costly problem for the entire biopharmaceutical industry[6], mycoplasmas can also act as pathogens for humans, animals and plants and therefore pose a risk for everyone receiving contaminated products.

In summary, mycoplasma contamination is a critical problem that can compromise both safety and efficacy of cell culture derived products. To avoid such problems it is requested by regulatory authorities to perform mycoplasma testing on various types of cell banks and products derived of these[7-9]. For many years, the gold standard methods for the detection of mycoplasma have been cell culture based. There are two assays recommended by the European (Ph. Eur), the Japanese (JP), and the United States pharmacopeia (USP): the culture method (broth/agar method), where test samples are either directly cultured on agar plates or previously inoculated into broth media, of which subcultures are transferred to agar plates on days 3, 7, and 14. This method will detect most organisms of the genera *Mycoplasma* and *Acholeplasma*. Cell culture-adapted mycoplasmas that are unable to grow on these conventional media are detected by the indicator cell method through DNA-specific staining of the intracellular mycoplasma and fluorescence microscopy. Which technique is applied depends on the type of materials tested: Master/working cell banks, virus seed lots, or control cells are tested with both methods, whereas for virus harvests, bulk vaccines, and final lots solely the culture method is prescribed. Although it has been shown over the last decades that these methods are suitable for the highly sensitive detection of mycoplasma contaminations, they also have major drawbacks owing to the nature of the assays. Besides the fact of the long assay duration (up to 28 days to provide sufficient incubation time for certain mycoplasma species), agar plates are hard to read, which make their evaluation highly subjective as the colony morphology and size of mycoplasma vary greatly (e.g. depending on the mycoplasma species, cultivation medium, concentration of the culture, and the stage of growth). Therefore, evaluation by experienced operators is crucial for the accurate interpretation of the results[10].

More recently, according to Ph. Eur., JP and USP, nucleic acid amplification (NAT) assays can also be used for the performance of mycoplasma testing, providing that a minimum sensitivity of 10 CFU/ml can be reached. A prerequisite is a suitable validation of the method demonstrating specificity, robustness, and equivalent sensitivity to the cell culture-based methods. The major advantages of PCR-based assays are better cost-efficiency and the delivery of results within one day instead of 28. With the rapidly increasing number of biopharmaceuticals, some of which with very short shelf lives, the quick delivery of reliable results is an important topic in release testing. Furthermore, a well-validated qPCR assay delivers data in a standardized way, making interpretation of the results independent of experienced operators, and generates therefore more consistent and comparable results than culture-based methods.

**The following chapters will therefore outline in more detail the requirements specified in the Ph. Eur., the JP, and the USP regarding NAT-based detection of mycoplasma, as well as the efforts undertaken by ViruSure to develop and validate a qPCR assay in compliance with these requirements.**

# Requirements of Ph. Eur, JP, and USP regarding the development of a qPCR assay for mycoplasma detection

Although the wording and the mycoplasma strains requested for NAT validation differ slightly in the pharmacopeial guidelines (see Table 1), the principal demands on the assay to substitute culture-based assays are the same: *NAT-based methods can be used as an alternative for both culture methods after suitable validation.*

1

| Ph. Eur. | JP | USP |
|---|---|---|
| *A. laidlawii* | *A. laidlawii* | *A. laidlawii* |
| *M. fermentans* | *M. fermentans* | *M. fermentans* |
| *M. hyorhinis* | *M. hyorhinis* | *M. hyorhinis* |
| *M. orale* | *M. orale* | *M. orale* |
| *M. pneumoniae* | *M. pneumoniae* | *M. pneumoniae* |
| *M. arginini* | *M. arginini* | - |
| *M. synoviae* [A] | *M. synoviae* [A] | *M. synoviae* |
| *S. citri* [B] | *S. citri* [B] | - |
| - | *M. salivarium* | - |
| - | - | *M. gallispeticum* |

**Table 1** List of strains for NAT-based mycoplasma detection required by the respective pharmacopeia.
[A] where there is use of or exposure to avian material during production
[B] where there is use of or exposure to insect or plant material during production

More precisely, validation should focus on the demonstration of the specificity, sensitivity (limit of detection (LOD)) and robustness of the assay.
In terms of specificity, no false positive results should be obtained from possible contaminations of closely related bacteria (e.g. *Clostridium, Lactobacillus and Streptococcus).*
For the LOD, a sensitivity of 10 CFU/ml should be reached for 95% of all tested replicates in order to be considered suitable to replace the culture method or the indicator cell method, respectively. This must be demonstrated in a comparability study using viable strains. The assay should not be impacted by minor variations in method parameters to be considered as robust to indicate its reliability during normal usage.

Additionally, in chapter 2.6.21. of the Ph. Eur., it is recommended to use the ICH Q2(R1) guideline as reference for any PCR validations.
Besides Specificity, LOD and Robustness, ICH adds the validation of Linearity, Range, Repeatability, Intermediate precision, Accuracy, and the Limit of Quantitation (LOQ) to the parameters to cover.

# Development and validation of a Ph. Eur., JP and USP compliant TaqMan™-based qPCR assay for mycoplasma detection

## Primer Design

For the primer design, sequences of the highly conserved 16S or 23S rRNA genes of the strains mentioned in Table 1 were retrieved from the National Center for Biotechnology Information (NCBI) database and an alignment was created. In the end, three primer/probe sets were generated to cover all strains: one primer set covering most of the mycoplasma strains, and, due to the lack of sufficient sequence homologies, two specific sets for *M. gallisepticum* and *M. pneumoniae*. All three assays were designed utilising a TaqMan™-labelled probe to increase the specificity the assays. The primer and probe sequences were subject to an extensive in silico analysis to test for their specificity against closely related bacteria. For this analysis, each primer/probe sequence was blasted against the *Clostridium, Lactobacillus* and *Streptococcus* sequences of the NCBI database. The resulting hits for each primer and probe were compared to check if any bacterial sequence might be detected by an entire set. This was not the case indicating that the designed sets should be specific for mycoplasma detection only. Further experimental confirmation was not necessary as all samples submitted to ViruSure undergo an initial sterility testing. Samples are only further processed upon a negative outcome making it highly unlikely that a positive signal in the qPCR experiment would be derived from bacterial contamination. This, together with the extensive *in silico* analysis, reduces the risk for false positive results significantly.

A number of papers with primers designed for mycoplasma NAT testing have been published in recent years[12-15]. A summary of the primer binding regions for the 16S rRNA is shown in Figure 1. Many of the published primer sequences are located in the same region targeted by the ViruSure qPCR method. This confirms the general applicability of designing primers in this region for broad-ranging detection of mycoplasma by NAT. The specificity of these primer designs was attested within these publications, further strengthening the results obtained by the *in silico* specificity evaluation.

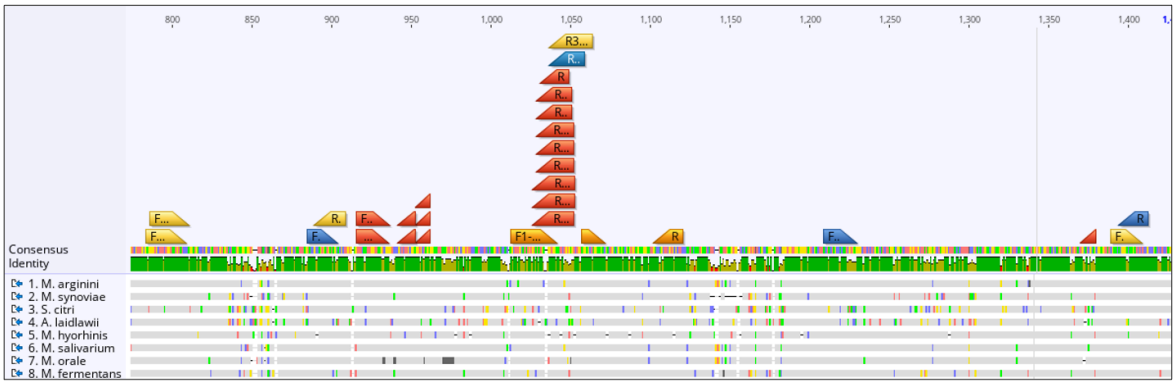



**Figure 1** Primer binding region of various published primer/probe sets. They are located in the same region of the 16S rRNA gene as the primers developed at ViruSure. Primers derived from one publication were coloured the same way: Red - Sung et al.[12]; Blue - Salling et al.[13]; Orange - Janetzko et al.[14]; Yellow - Vega-Orellana et al.[15]

## Performance of Comparability Study

For the comparability study the strains recommended by the Ph. Eur., JP and USP were obtained from recognised mycoplasma reference laboratories. The CFU/ml of these strains are stated in Table 2.

2

| Strain | $log_{10}$ | CFU/ml Final $log_{10}$ dilution (≙ $log_{10}$ CFU/ml) |
|---|---|---|
| *A. laidlawii PG8* | 8.85 | -7.75 (≙ 0.73) |
| *M. fermentans PG18* | 9.00 | -8.00 (≙ 0.63) |
| *M. hyorhinis BTS-7* | 8.94 | -7.94 (≙ 0.63) |
| *M. orale CH19299* | 9.89 | -8.90 (≙ 0.62) |
| *M. gallisepticum PG31* | 8.34 | -7.34 (≙ 0.63) |
| *M. salivarium* | 9.87 | -8.87 (≙ 0.63) |
| *M. arginini G230* | 3.22 | -2.20 (≙ 0.65) |
| *M. synoviae WVU1853* | 2.75 | -1.75 (≙ 0.63) |
| *S. citri* | 3.00 | -2.00 (≙ 0.63) |
| *M. pneumoniae* | 2.98 | -2.00 (≙ 0.61) |

**Table 2** Mycoplasma strains used for the comparability study between cell culture-based methods and qPCR. Stated are the different mycoplasma strains including their CFU/ml, the final dilution tested by qPCR and the corresponding CFU/ml concentration. The samples were diluted down to 10 CFU/ml (1.0 $log_{10}$ CFU/ml). As 140 µl sample are loaded on the column, but eluted in 60 µl, the extraction procedure includes a concentration step of 0.37 $log_{10}$ which in turn decreases the LOD additionally by this factor.

Most published qPCR assays were developed for the detection of Mycoplasmas 16S rDNA encoding for the 16S subunit of the ribosome.
Assuming that one CFU is related to one genome copy of DNA, it is a difficult goal to detect an LOD of 10 CFU/ml (equaling 10 mycoplasmas) with LODs of qPCR assays generally at approximately 1000 copies/ml. Several approaches are mentioned by the Parenteral Drug Association (PDA) to increase the sensitivity of NAT-based mycoplasma assays[19].

These approaches include biological enrichment (culture for two to three days prior to PCR) or physical enrichment of mycoplasma (increase mycoplasma nucleic acids by pre-treatment steps e.g. magnetic capture hybridization, centrifugation, filtration etc.) and are used by other companies. ViruSure used the third approach described in this document to circumvent this limitation, which is the extraction of RNA for the detection of the mycoplasmas.

The resulting sensitivity increase is based on the higher copy number of 16S rRNA relative to 16S rDNA; depending on the mycoplasma strain and its respective growth phase, 200 to 1000 copies of rRNA copies are available per cell[16-18]. The same PDA document mentions that the usage of rRNA enhances the likelihood of viable mycoplasma detection due to RNA's natural instability and rapid degradation in dead bacterial cells[20]. Hence, this design also results in fewer false positive results, thereby reducing the risk of costly deviations and potential disposal of batches of biopharmaceutical product whilst the timely culture-based methods are used for follow-up investigations. For validation of the NAT assay, dilution series of each mycoplasma strain were generated to a final concentration of 10 CFU/ml or lower prior to extraction and analysis. 24 replicates of each mycoplasma 10 CFU/ml dilution were tested in multiple qPCR experiments and demonstrated that 95% of all replicates could be detected using the respective primer/probe sets. For each of the qPCR assays, a synthetic DNA standard with defined genome copies/µl was used as a positive internal control. During the validation, 24 replicates from each standard curve dilutions were generated. These replicates were evaluated to define the dilution which corresponds to 10 CFU/ml of the extracted samples so that it can be used as reference marker for 10 CFU/ml of mycoplasma contamination in the validated qPCR assay. As defined in the Ph. Eur., JP, and USP, the NAT assay should be capable of detecting mycoplasma at or below the defined LOD of 10 CFU/ml in at least 95% of the replicates. Table 3 lists the number of detected replicates for each 10 CFU/ml dilution and the corresponding percentage of detection. Based on these experiments it can clearly be stated that the validated approach established at ViruSure is capable of detecting 10 CFU/ml of each of the required mycoplasma strains.

3

| Sample | Number of replicates detected | % detected |
|---|---|---|
| *A. laidlawii* | *24/24* | *100%* |
| *M. fermentans* | *24/24* | *100%* |
| *M. hyorhinis* | *24/24* | *100%* |
| *M. orale* | *24/24* | *100%* |
| *M. arginini* | *24/24* | *100%* |
| *M. synoviae* | *23/24* | *95.8%* |
| *S. citri* | *24/24* | *100%* |
| *M. salivarium* | *24/24* | *100%* |
| *M. gallisepticum* | *24/24* | *100%* |
| *M. pneumoniae* | *24/24* | *100%* |

**Table 3** The number of replicates of the 10 CFU/ml dilution detected and the corresponding percentage for each of the respective reference strains.

Table 3 shows that nearly 100% of the replicates at 10 CFU/ml were detected, indicating that the sensitivity of the assay lies below 10 CFU/ml.
To investigate the actual LOD further, extracted 10 CFU/ml samples of *M. orale, A. laidlawii, S. citri, M. gallisepticum and M. pneumoniae* were diluted in a 2-fold dilution series down to 0.63 CFU/ml before usage in the qPCR assay.
6 replicates of each dilution were tested and the results in Table 4 show good sensitivity of the qPCR assay even below 5 CFU/ml. As also the extraction efficiency of the RNA is important for the sensitivity of the assay, a similar 2-fold dilution series was performed prior to extraction for three strains (*S. citri, M. gallisepticum and M. pneumoniae)*. The results of this experiment are listed in Table 5 and show good sensitivity down to below 5 CFU/ml. Below that mycoplasma are detected in less than 100% of the replicates.

4

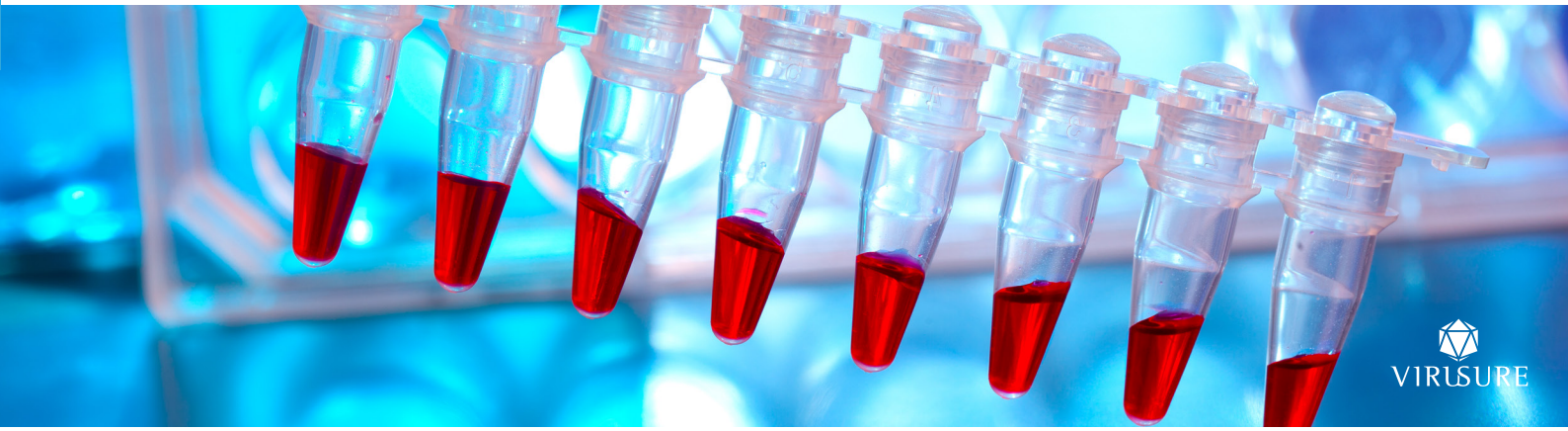
| CFU/ml | M. orale | A. laidlawii | S. citri | M. gallisepticum | M. pneumoniae |
|---|---|---|---|---|---|
| *5.00* | *6/6* | *6/6* | *5/6* | *6/6* | *6/6* |
| *2.50* | *6/6* | *6/6* | *6/6* | *6/6* | *6/6* |
| *1.25* | *6/6* | *6/6* | *6/6* | *5/6* | *6/6* |
| *0.63* | *3/6* | *6/6* | *6/6* | *4/6* | *6/6* |

**Table 4** The number of detected replicates of each dilution below 10 CFU/ml can be observed. The samples used were extracted at 10 CFU/ml and diluted in 1:2 steps before the qPCR assay.

5

| CFU/ml | S. citri | M. gallisepticum | M. pneumoniae |
|---|---|---|---|
| *5.00* | *6/6 (0.58)* | *6/6 (0.47)* | *6/6 (0.19)* |
| *2.50* | *5/6 (0.13)* | *4/6 (0.68)* | *6/6 (0.54)* |
| *1.25* | *3/6 (0.44)* | *3/6 (0.40)* | *1/6* |
| *0.63* | *3/6 (0.17)* | *1/6* | *2/6 (0.15)* |

**Table 5** The number of replicates detected of each dilution below 10 CFU/ml. The 1:2 dilution series was performed before the extraction and 6 replicates of each extracted dilution were tested in the qPCR assay. In brackets the standard deviation of the Ct-values for each dilution displays little variation within all of the detected replicates.



## Validation of the PCR assays according to ICH Q2(R1) [11]

Additionally to the evaluation of the sensitivity of the assays, the Ph. Eur. and JP request the validation of the specificity and the robustness of the assay. Furthermore, the Ph. Eur. requests in chapter 2.6.21. the validation of qPCR assays according to the ICH Q2 (R1) guidelines, which besides Specificity, Robustness and LOD also includes validation of Linearity, Range, Repeatability, Intermediate Precision, Accuracy and Limit of Quantitation (LOQ). The results from these evaluations are summarized in the following sections.

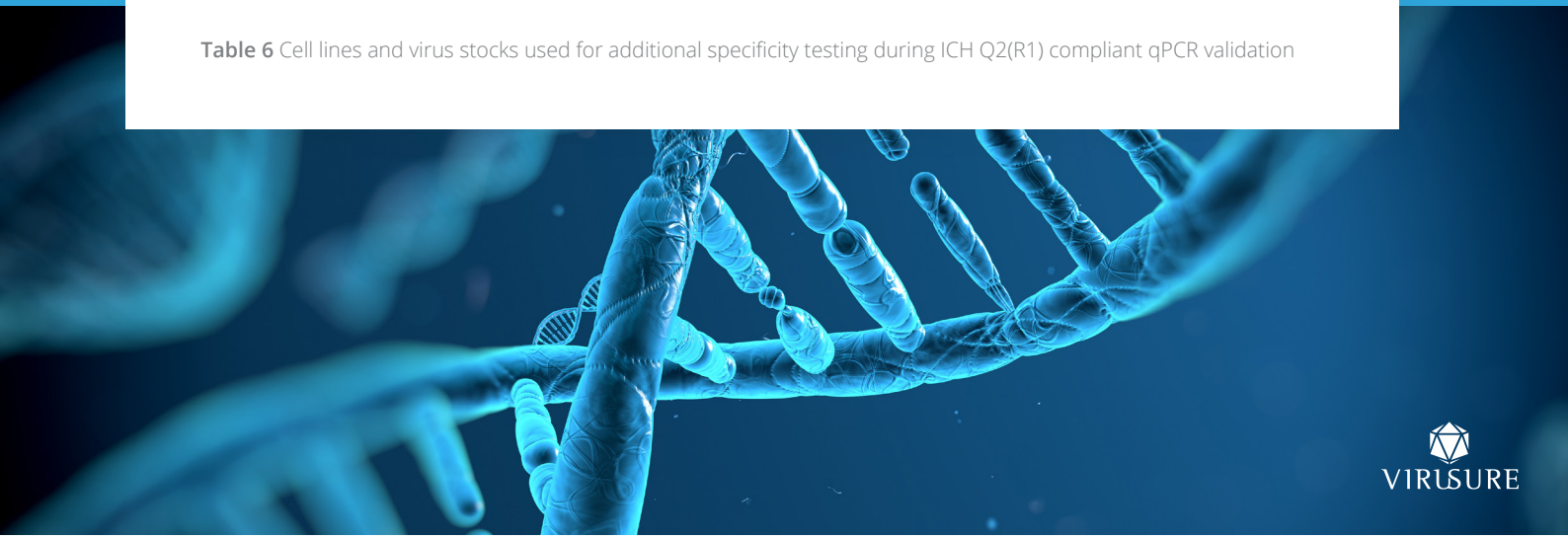
### Specificity

As mentioned previously, the designed primer sets underwent a thorough *in silico* analysis for unspecific detection of bacteria using the NCBI database. This analysis verifies the specificity of the assay, which is also affirmed by various published primers binding in the same regions. Additional internal specificity testing was performed by testing 10 cell lines and 10 virus stocks commonly used and likely to be tested at ViruSure to exclude the possibility of unspecific binding in these. The cell lines and virus stocks used are displayed in Table 6. None of the three qPCR assays showed signs of unspecific amplification; therefore, all three assays are considered as specific for the detection of mycoplasma only.

6

| Cell lines | Virus Stocks |
|---|---|
| *A9* | *Bovine Viral Diarrhoea Virus (BVDV)* |
| *BHK21* | *Encephalomyocarditis Virus (EMCV)* |
| *CHO-K1* | *Human Adenovirus (HAdV)* |
| *HeLa* | *Minute Virus of Mice (MMV)* |
| *MDBK* | *Porcine Circovirus (PCV)* |
| *MDCK* | *Porcine Parvovirus (PPV)* |
| *MRC-5* | *Pseudorabies Virus PRV)* |
| *PK-13* | *Mammalian Reovirus 3 (Reo-3)* |
| *Vero* | *Simian Virus 40 (SV-40)* |
| *PG-4* | *Xenotropic Murine Leukaemia Virus (x-MuLV)* |

**Table 6** Cell lines and virus stocks used for additional specificity testing during ICH Q2(R1) compliant qPCR validation



## Robustness

To show the robustness of the three assays, parameters identified as critical for performance of the assay were evaluated for the impact on the assay performance. This included the increasing/decreasing of the primer and probe concentrations and adjusting the annealing/extension temperature by ± 1°C compared to the standard temperature.

To evaluate the impact of these changes, one dilution of the synthetic DNA positive control was compared for variations in the Ct-value. A summary of the results can be found in Table 7. Less variation than 0.27 Ct-values could be observed for all three assays confirming their overall robustness.

7

| Assay | Effects Primer/Probe conc. | Effects Temperature | Effects combined |
|---|---|---|---|
| *Mycoplasma general* | *-0.20* | *-0.16* | *-0.08* |
| *M. Gallisepticum* | *0.05* | *0.06* | *-0.02* |
| *M. Pneumoniae* | *0.27* | *0.15* | *0.01* |

**Table 7** Summary of the Robustness testing results. Displayed are the Ct-value changes for the testing of a single dilution that resulted from the changes to the assay set-up. First the effects of the change in primer/probe concentration, second the effects of temperature changes in the qPCR run, and third the combined effect.

## Linearity, Range, Repeatability, Intermediate Precision

For the validation of linearity, range, repeatability and intermediate precisions, the performance of the assay was compared with two independent operators on three different days. The resulting standard curves were compared and should not differ by more than ± 0.5 $\log_{10}$ for each of the tested dilutions. The comparison of each value for each dilution can be found in Figure 2. Linear amplification could be observed for all three assays within a range of 6.08 to 1.58 $\log_{10}$ copies/PCR in the general assay, 5.08 to 0.58 $\log_{10}$ copies/PCR for *M. gallisepticum* and 5.08 to 0.58 $\log_{10}$ copies/PCR for *M. pneumoniae.* The assays were repeatable over all three days of performance and showed no variation independent of the operator who performed the assays.

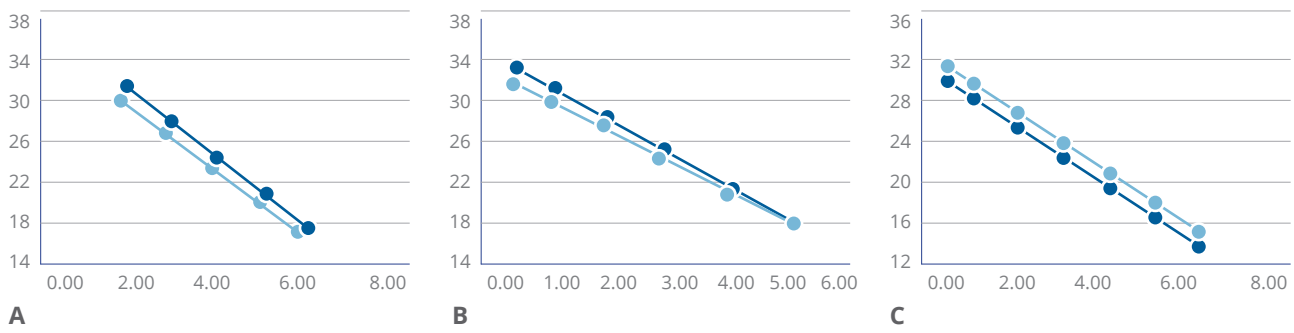



**Figure 2** Standard curves obtained during the Linearity, Range, Repeatability, and Intermediate Precision testing (A: general mycoplasma assay, B: *M. gallisepticum*, C: *M. pneumoniae).*

## Accuracy

The determination of the accuracy is not really applicable for this assay set-up. As RNA was used as template, the CFU/ml cannot be directly related to the GC/ml in the reference samples.
However, as the synthetic DNA standard, used as positive control for the qPCR assay, has a precisely defined concentration, this was used to evaluate the accuracy of the assay. Over the whole validation period the Ct-values of the respective dilutions were comparable. Further on, the Ct-values of the 10 CFU/ml dilution of the synthetic DNA standards are tracked in a trending database to eliminate the possibility of a loss in accuracy.

## Limit of Quantitation (LOQ) and Limit of Detection (LOD)

According to ICH Q2(R1), the LOQ is the dilution where at least 95% of all replicates are detected, and the LOD is the dilution at which 66.6% of all replicates should be detected. According to the Ph. Eur. for the LOD, 95% of the replicates should be detected. As more than 95% of the extracted 10 CFU/ml dilutions and the corresponding dilutions of the synthetic DNA standard curves were detected, the required specifications by both, Ph. Eur. and ICH Q2(R1), are met.

# Conclusions

This paper outlines the implementation of a qPCR approach for the broad ranging detection of mycoplasma contamination compliant to Ph. Eur, JP and USP. Three primer/probe sets were designed to cover all strains required by the guidelines, one that was designed into the 23S rRNA region of *M. gallisepticum*, one in the 16S rRNA region of *M. pneumoniae* and a general one in the 16S rRNA region to detect the remaining strains *(A. laidlawii, M. fermentans, M. hyorhinis, M. orale, M. arginini, M. synoviae, M. salivarium, S. citri)*.
These strains, which were obtained from recognised reference laboratories, were diluted to 10 CFU/ml. In accordance with PDA recommendations, it was decided to use RNA as template for the qPCR assay to first increase the sensitivity of the assay and second minimize the risk of false positive results. 24 replicates of each strain were tested by qPCR to demonstrate that 95% of all tested replicates can be detected in accordance with the guidelines. Additionally, it was demonstrated that the sensitivity of the qPCR assay lies below 5 CFU/ml, nearly all replicates of 2.50 CFU/ml could be detected and even below that signals were obtainable, showing that detected Ct-values below the 10 CFU/ml reference marker of the synthetic internal control are indicating very low-level mycoplasma contaminations.
To demonstrate the specificity of the assay, an *in silico* approach was used by BLASTing the primer/probe sequences against all *Clostridium, Lactobacillus and Streptococcus* sequences of the NCBI database. No hit was obtained for an entire primer/probe set showing specific binding of the primers to the mycoplasma sequences only.
Additional experimental specificity testing was not necessary as samples submitted to ViruSure are routinely subject to sterility testing, guaranteeing the absence of bacterial contaminations. Together with the results of the *in silico* analysis, this is sufficient with respect to specificity requirements. Still, the primer binding regions of published primer sets that bind in the same regions (16S or 23S rRNA gene respectively) were compared. These have demonstrated their specificity experimentally, strengthening the already extensively performed specificity analysis at ViruSure. Upon changing assay parameters, no impact could be observed on the results showing the robustness of the newly developed assays. Further to the validation of the three parameters in compliance with the Ph.Eur., JP and USP, the qPCR assays were validated according to ICH Q2(R1) in terms of Linearity, Range, Repeatability, Intermediate Precision and Accuracy.
The successful conduct of the comparability study and the validation experiments showed that the implemented and validated qPCR assays at ViruSure are capable of directly replacing the indicator cell line method and the culture method without any necessity to enrichment first in culture, and are fully compliant with the Ph. Eur., JP and USP.

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