Validation of the MycoTOOL Mycoplasma Real-Time PCR Kit

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Abstract

Mycoplasma contamination of biopharmaceuticals – as a consequence of cell culture contamination in the manufacturing process – poses a potential health risk to patients. To minimize this risk, regulatory agencies require manufacturers of biopharmaceuticals to ensure the absence of mycoplasmas in the manufacturing process and end product.1–3 To test for mycoplasmas, key regulatory guidelines such as EP6.7.1, USP<61>4 and JP5 have in the past recommended protocols that relied exclusively on the culture method or indicator cell culture methods. These protocols suffer from several limitations, such as lengthy overall testing time (28 days) and difficult-to-cultivate or non-cultivable mycoplasma species. Hence, many regulatory agencies now additionally accept rapid nucleic acid amplification techniques (NAT) such as real-time quantitative polymerase chain reaction (qPCR) for mycoplasma testing. The MycoTOOL Mycoplasma Real-Time PCR Kit (MycoTOOL RT) is a commercially available qPCR assay developed by Roche Pharma that has now been validated according to the EP6.7 NAT validation guidelines.6 In the following, we present the MycoTOOL RT work flow and validation results. The full validation report is available upon request and under confidential disclosure agreement.

Keywords:
Mycoplasma testing, qPCR assay, mycoplasma detection, biopharmaceuticals.

1. Materials and methods

1.1. Materials

1.1.1. Materials – Mammalian and Mycoplasma cells

For each sample, we used 5 × 10³ of a standard CHO cell culture that had been confirmed to be mycoplasma-free in fourfold determination. We obtained stocks of ten different mycoplasma reference strains from three different sources and determined their generic copy per colony forming units (CFU/CFU) ratio using an in-house method (Table 1). The mycoplasma strains chosen represent all strains required by the EP6.7 and additionally include M. orale, M. salivarium and M. hominis.

Validation design

The validation of MycoTOOL RT followed the criteria as mentioned in the EP6.7 and ICH-Q2 R1 guidelines:

1. Limit of detection (LOD)

We spiked CHO cell culture samples with each mycoplasma reference strain (Table 2) in a dilution series ranging from 10–0 CFU/ml. The MycoTOOL RT assay was run in eightfold determination and repeated on three different days, yielding 24 results per dilution. The LOD was defined as the lowest number of CFU that could be detected in 21 out of 24 samples.

Acceptance criteria by EP6.7 were met if:

• the LOD was ≤ 10 CFU/ml.

2. Specificity

We spiked CHO cell culture samples with three-gene-positive bacterial strains (Staphylococcus aureus, Lactobacillus acidophilus, Clostridium sordellii) in a tenfold dilution series ranging from 10–1 to 10⁴ CFU/ml. The MycoTOOL RT assay was run in eightfold determination and repeated on three different days, yielding 24 results per dilution. Acceptance criteria by EP6.7 were met if:

• the spiked samples showed a qPCR quantification cycle (Cq) value at or above the respective calculated LOD of Table 1.

3. Robustness

We spiked 10 CHO cell culture samples with M. orale at a concentration three times higher than the calculated LOD and performed the MycoTOOL RT assay using at least two different manufacturing batches of the kit.

Acceptance criteria by EP6.7 were met if:

• all spiked samples gave positive results for each analyst.

4. Precision

Three different analysts spiked 8 CHO cell culture samples with M. orale at a concentration of 100 times higher than the calculated LOD and prepared 11 unspiked CHO cell culture samples. We performed the MycoTOOL RT assay with all samples. The spiked and unspiked samples were placed alternatively on the microtiter plate of the MagNA Pure II for DNA isolation as well as on the multiwell plate of the LightCycler 480 II system for qPCR.

Acceptance criteria by EP6.7 were met if:

• all spiked samples gave positive results and all unspiked samples gave negative results.

5. Cross-contamination

We spiked 10 CHO cell culture samples with M. orale at a concentration 100 times higher than the calculated LOD and prepared 11 unspiked CHO cell culture samples. We performed the MycoTOOL RT assay with all samples. The spiked and unspiked samples were placed alternatively on the microtiter plate of the MagNA Pure II for DNA isolation as well as on the multiwell plate of the LightCycler 480 II system for qPCR.

Acceptance criteria by EP6.7 were met if:

• MycoTOOL RT was at least as sensitive as the compendial test methods.

Results

Determination of GC/CFU Ratio

To ensure the viability of the mycoplasma reference strains is sufficiently high, a GC/CFU ratio of ≤ 100 is recommended.4 At tested mycoplasma reference strains showed GC/CFU ratios ≤ 100 (Table 1).

Acceptance criteria by EP6.7 were met if:

• the mean of all samples was within a range of ± 3 Cq-values.

2. Specificity

Cross-detection was observed for E. coli and/or Staphylococcus aureus, respectively: fluorescein and/or rhodamine emit in the near-infrared (700–800 nm) range and the LightCycler 480 II system works in this range. Regulatory agencies require testing during the harvest, as indicated by the arrow.

3. Robustness

10 out of 10 M. orale dilutions were successfully detected for each MycoTOOL RT manufacturing batch. The ΔCq between runs was 0.04.

4. Precision

All M. orale dilutions gave a positive result and the ΔCq between runs was 1.93 (Table 3).

5. Cross-contamination

All M. orale spiked samples yielded positive results (10 of 10) and all unspiked samples yielded negative results (10 of 10). Thus, we detected no cross-contamination during the whole work flow.

6. Comparability

Results of the comparability study are summarized in Table 4. We concluded that all three methods are sensitive methods to detect mycoplasma contamination with a sensitivity ≤ 10 CFU/ml. In addition, MycoTOOL RT is able to detect strains that are non-cultivable.

Table 1: Determination of GC/CFU Ratio

<table>
<thead>
<tr>
<th>Species</th>
<th>LOD [CFU/ml]</th>
<th>GC/CFU Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. hominis</td>
<td>25.4</td>
<td>74.2</td>
</tr>
<tr>
<td>M. salivarium</td>
<td>26.4</td>
<td>74.2</td>
</tr>
<tr>
<td>A. laidlawii</td>
<td>22.8</td>
<td>74.2</td>
</tr>
<tr>
<td>M. hypoxia</td>
<td>61</td>
<td>10</td>
</tr>
<tr>
<td>M. genitalium</td>
<td>73</td>
<td>10</td>
</tr>
<tr>
<td>M. fermentans</td>
<td>72</td>
<td>74.2</td>
</tr>
<tr>
<td>T. citri</td>
<td>51.8</td>
<td>74.2</td>
</tr>
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Table 2: Summary of total detection limits across all laboratories.

Table 3: Testing results to determine the precision of the MycoTOOL RT assay.

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Table 4: Acceptance criteria for MycoTOOL RT assay.

Table 5: Mycoplasma reference strains.

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To ensure that the viability of the mollicute reference strains is sufficiently high, a GC/CFU ratio of ≤ 100 (Table 1).

Table 6: Comparison between the different mycoplasma detection methods.

Table 7: Comparison between the different mycoplasma detection methods.

Summary and discussion

The validation study demonstrates the compliance of MycoTOOL RT with the EP6.7 NAT validation guideline. The results demonstrate that MycoTOOL RT is sensitive, specific, robust, precise, and comparable to the compendial mycoplasma methods. Thus, it fulfills all requirements as given by EP6.7 to detect mycoplasma contamination during CHO manufacturing processes of biopharmaceuticals. Like Roche Pharma Biotech Penzberg in Germany many manufacturers of biopharmaceuticals are moving towards rapid NAT methods and we believe that this trend will continue in the future.

References

2) Guidance of European Pharmacopoeia on chapter 2.10 Mycoplasma Testing. Stuttgart, Germany; 2007
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4) EP 6.7.1, ed. EPCT, 2010
5) EDQM 2.0, ed. EDQM, 2005
7) FDA and USDA Agriculture Administration. Plants to control the contamination of seeds used to produce biopesticides. Rockville, MD, USA; 1993
8) Lundqvist G, Eriksson E. Validation of Analytical Procedures: Test of Methodology, Sweden; 2008

Regulatory disclaimer

It is the responsibility of the end user to ensure that the end-use product complies with the relevant regulatory requirements. Roche CustomBiotech GmbH, Penzberg, Germany (address correspondence to: marneheim.custombiotech@roche.com)