

Validation of the MycoTOOL Mycoplasma Real-Time PCR Kit



Sebastian Weber, Raphael Greiner
Roche CustomBiotech, Mannheim, Germany (address correspondence to: mannheim.custombiotech@roche.com)

Alexander Bartes, Holger Kavermann, Yvonne Knack, Sven Deutschmann
Roche Pharma Biotech, Penzberg, Germany

Abstract

Mycoplasma contamination of biopharmaceuticals – as a consequence of cell culture contamination in the manufacturing process – poses a potential health risk to patients.¹ To minimize this risk, regulatory agencies require manufacturers of biopharmaceuticals to ensure the absence of mycoplasmas in the manufacturing process and end product.²⁻⁶ To test for mycoplasmas, key regulatory guidelines such as EP2.6.7², USP<63>³ and JP⁴, have in the past recommended protocols that relied exclusively on the culture method or indicator cell culture method. These protocols suffer from several limitations, such as lengthy overall testing time (28 days) and difficult-to-cultivable 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 in-house by Roche Pharma that has now been validated according to the EP2.6.7 NAT validation guidelines.² 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.

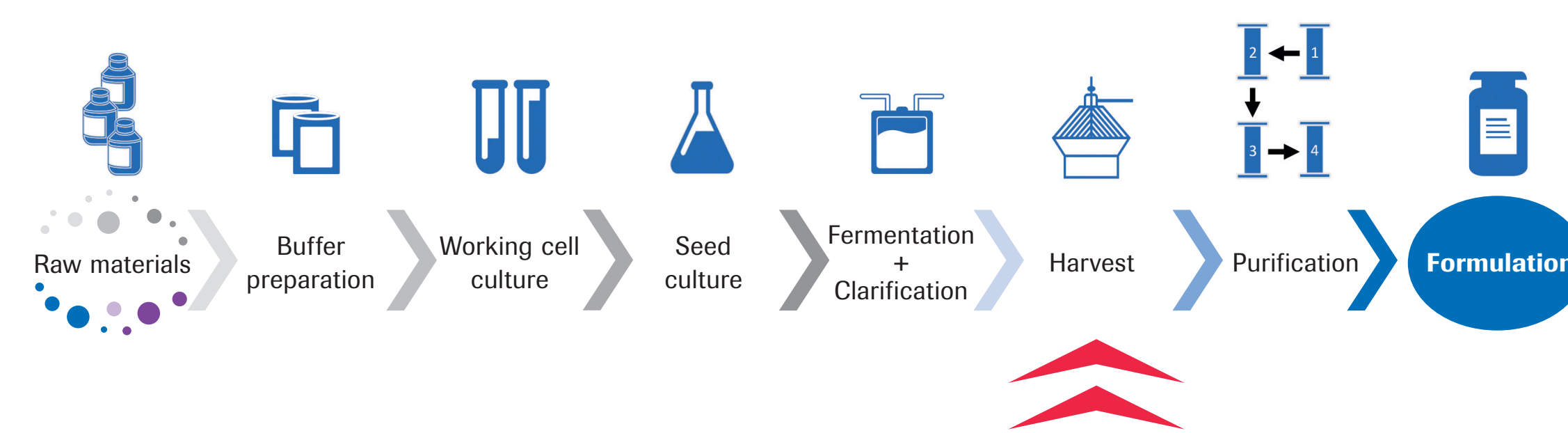


Figure 1: The MycoTOOL RT assay allows mycoplasma testing anytime during the production process, which may then be aborted to save costs in case of contamination. Regulatory agencies require testing during the harvest, as indicated by the arrow.

MycoTOOL RT Kit Design

Work Flow

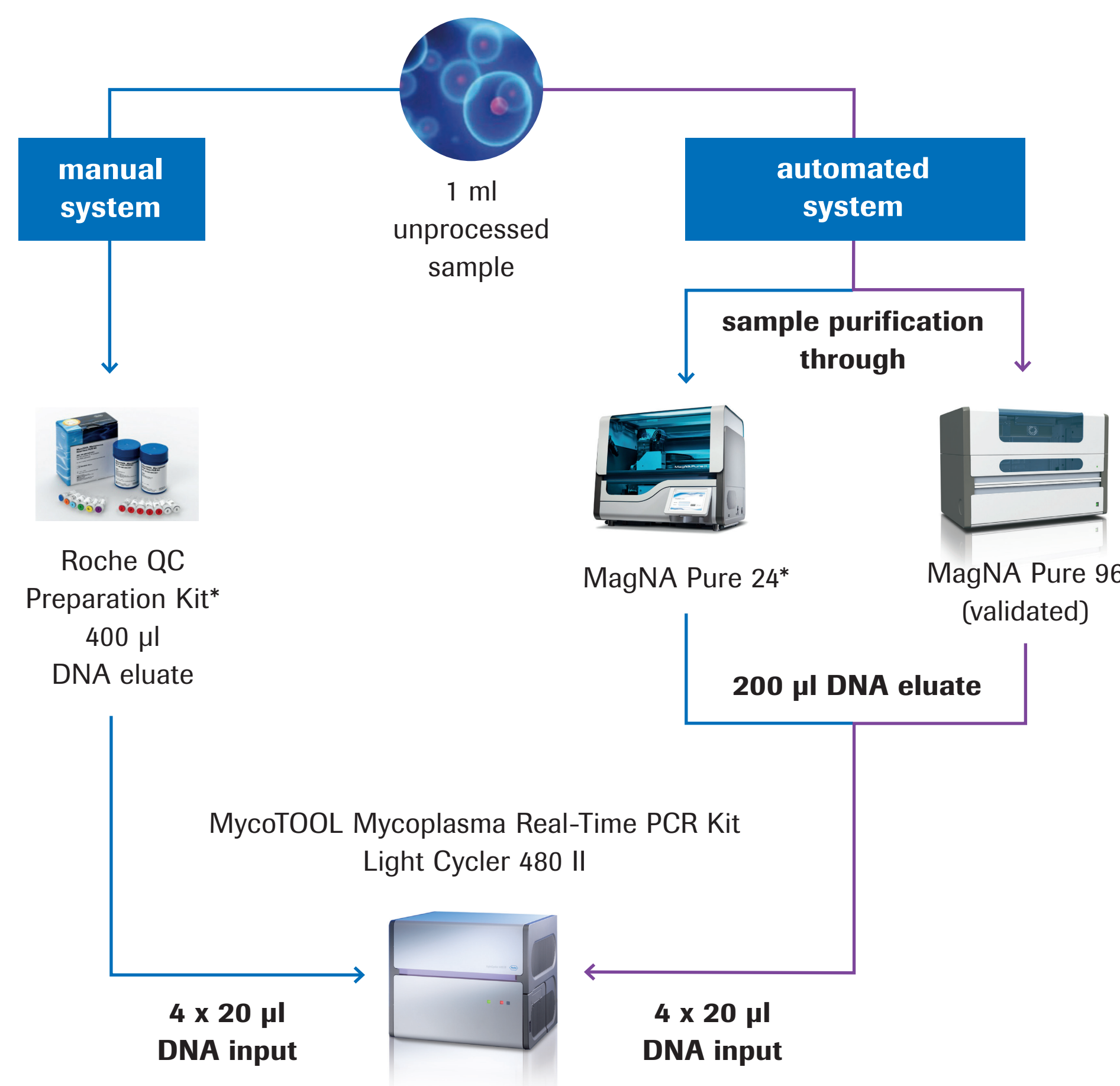


Figure 2: MycoTOOL RT work flow using either a manual or automated DNA extraction method. The automated work flow based on the MagNA Pure 96 and LightCycler 480 II systems shown as the red marked process procedure has been fully validated by Roche Pharma Biotech as it is presented in this poster.

* Both, the MagNA Pure 24 and the QC Sample Preparation Kit, are functionally tested, but not validated.

Control concept

MycoTOOL RT uses the following fluorescent dyes to detect mycoplasma DNA and a recovery control plasmid, respectively: fluorescein amidite (FAM), and Yellow 555. All controls are included in the MycoTOOL RT Kit.

Each biological sample to be tested for mycoplasma contamination requires the following separate qPCR reactions on a 96-well PCR plate:

1. Cell sample spiked with recovery control plasmid prior to DNA preparation
2. Plasmid containing mycoplasma DNA (positive PCR control)
3. Water (negative PCR control)
4. Mycoplasma-free cell sample spiked with recovery control plasmid prior to DNA preparation (negative process control)

Materials and methods

Materials – Mammalian and Mycoplasma cells

For each sample, we used 5×10^6 cells/ml of a standard CHO cell culture that had been confirmed to be mycoplasma-free in fourfold determination. We obtained stocks of ten different mollicute reference strains from three different sources and determined their genomic copy per colony forming units (GC/CFU) ratio using an in-house method (Table 1). The mollicute strains chosen represent all strains required by the EP2.6.7² and additionally include *M. orale*, *M. salivarium* and *M. hominis*.

Validation design

The validation of MycoTOOL RT followed the criteria as mentioned in the EP2.6.7² and ICH-Q2 R1⁷ guidelines:

1. Limit of detection (LOD)

We spiked CHO cell culture samples with each mollicute reference strain (Table 2) in a dilution series ranging from 10 - 0.1 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 23 out of 24 samples.

Acceptance criteria by EP2.6.7 were met if:

- the LOD was ≤ 10 CFU/ml.

2. Specificity

We spiked CHO cell culture samples with three gram-positive bacterial species (*Streptococcus bovis*, *Lactobacillus acidophilus*, *Clostridium sporogenes*) in a tenfold dilution series ranging from 10^8 - 10^2 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 EP2.6.7 were met if:

- the spiked samples showed a qPCR quantification cycle (C_q) 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 EP2.6.7 were met if:

- all spiked samples gave positive results.
- the mean of all samples was within a range of 4 C_q values.

4. Precision

3 different analysts spiked 8 CHO cell culture samples with *M. orale* at a concentration three times higher than the calculated LOD and performed the MycoTOOL RT assay on different days.

Acceptance criteria by EP2.6.7 were met if:

- all spiked samples gave a positive result for each analyst.
- the mean of all samples was within a range of 4 C_q values.

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 10 unspiked CHO cell samples. We performed the MycoTOOL RT assay with all samples. The spiked and unspiked samples were placed alternately on the microtiter plate of the MagNA Pure 96 for DNA isolation as well as on the multiwell plate of the LightCycler 480 II system for qPCR.

Acceptance criteria by EP2.6.7 were met if:

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

6. Comparability

We performed comparability studies to show equivalency between the two compendial methods and the MycoTOOL RT assay. We spiked a 15 ml pre-harvest CHO cell culture sample with mollicute reference strains (Table 4). The spike was performed in tenfold dilution series ranging from 100 - 0.001 CFU/ml. MycoTOOL RT and the two compendial methods were performed in triplicates for all samples.

Acceptance criteria by EP2.6.7 were met if:

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

Results

Determination of GC/CFU Ratio

To ensure that the viability of the mollicute reference strains is sufficiently high, a GC/CFU ratio of ≤ 100 is recommended.⁸ All tested mollicute reference strains showed GC/CFU ratios < 100 (Table 1).

Mollicute species	Source	Total mean GC/CFU Ratio (n = 3)
<i>M. hominis</i>	ATCC 23114	59.1
<i>M. orale</i>	ATCC 23714	74.2
<i>M. aginini</i>	ATCC 23838	25.4
<i>A. laidlawii</i>	EDQM	30.3
<i>M. hyorhinis</i>	EDQM	8.1
<i>M. hyorhinis*</i>	ATCC 29052	0.7
<i>M. pneumoniae</i>	NCTC 10119	11.6
<i>M. salivarium</i>	ATCC 23064	7.3
<i>M. fermentans</i>	ATCC 19989	36.2
<i>Sp. citri</i>	ATCC 27556	51.8

* Fastidious strain

Table 1: GC/CFU ratios for mollicute reference strains.

Validation results

General acceptance criteria for MycoTOOL RT results were that all positive controls gave positive signals and all negative controls gave negative signals. Thus a sample was regarded as truly positive or negative only if all controls yielded the expected signals.

1. LOD

The LOD was determined to be ≤ 10 CFU/ml for each mycoplasma reference strain (Table 2). The data confirm that MycoTOOL RT is a highly sensitive detection method.

Mollicute species	Source	LOD [CFU/ml]
<i>M. hominis</i>	ATCC 23114	0.2
<i>M. orale</i>	ATCC 23714	0.5
<i>M. aginini</i>	ATCC 23838	0.3
<i>A. laidlawii</i>	EDQM	0.1
<i>M. hyorhinis</i>	EDQM	2.0
<i>M. pneumoniae</i>	NCTC 10119	7.0
<i>M. salivarium</i>	ATCC 23064	3.0
<i>M. fermentans</i>	ATCC 19989	0.2
<i>Sp. citri</i>	ATCC 27556	0.1
<i>M. hyorhinis*</i>	ATCC 29052	10.0

* Fastidious strain

Table 2: Limits of detection for mollicute reference strains.

2. Specificity

Cross-detection was observed for *L. acidophilus* above a spike concentration of 10^4 CFU/ml, for *S. bovis* above 10^6 CFU/ml and *C. sporogenes* above 102 CFU/ml.

3. Robustness

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

4. Precision

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

		Analyst 1	Analyst 2	Analyst 3
PCR result	mycoplasma: <i>M. orale</i>	8/8	8/8	8/8
	recovery control	8/8	8/8	8/8
	ΔC_q (mycoplasma: <i>M. orale</i>)	38.89	37.00	38.93
	ΔC_q (mycoplasma: <i>M. orale</i>)		1.93	

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

5. Cross-contamination

All *M. orale* spiked samples yielded positive results (10 out of 10) and all unspiked samples yielded negative results (10 out 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 contaminations with a sensitivity ≤ 10 CFU/ml. In addition, MycoTOOL RT is able to detect strains that are non-cultivable.

Mollicute species	Source	Detection limit		
		Culture method [CFU/ml]	Indicator cell culture method [CFU/ml]	MycoTOOL RT [CFU/ml]
<i>M. hominis</i>	ATCC 23114	1	0.1	0.1
<i>M. orale</i>	ATCC 23714	0.1	ND	1
<i>M. aginini</i>	ATCC 23838	0.01	1	1
<i>A. laidlawii</i>	EDQM	0.01	0.1	0.1
<i>M. hyorhinis</i>	EDQM	1	1	10
<i>M. pneumoniae</i>	NCTC 10119	1	ND	1
<i>M. salivarium</i>	ATCC 23064	0.1	ND	0.1
<i>M. fermentans</i>	ATCC 19989	10	1	0.1
<i>Sp. citri</i>	ATCC 27556	ND	ND	0.1
<i>M. hyorhinis*</i>	ATCC 29052	ND	1	10

* Fastidious strain ND = not detectable

Table 4: Comparison between the different mycoplasma detection methods.

Summary and discussion

This validation study demonstrates the compliance of MycoTOOL RT with the EP2.6.7² 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 EP2.6.7² 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

- 1) Volokhov DV et al. Mycoplasma testing of cell substrates and biologics: Review of alternative non-microbiological techniques. Mol Cell Probes 2011; 25: 69-77
- 2) Council of Europe. European Pharmacopoeia, 9th ed. Chapter 2.6.7: Mycoplasmas. Strasbourg, France; 2017
- 3) United States Pharmacopoeial Convention. United States Pharmacopoeia – National Formulary (USP-NF), 39th ed. Chapter 63: Mycoplasma Tests; Rockville, MD, USA; 2016
- 4) Ministry of Health, Labour and Welfare. Japanese Pharmacopoeia, 17th ed. Mycoplasma testing for cell substrates used for the production of biotechnological/biological products. Tokyo, Japan; 2016
- 5) International Committee on Harmonization. Q5D: Derivation and characterization of cell substrates used for production of biotechnological/biological products. Geneva, Switzerland; 1997
- 6) Food and Drug Administration. Points to consider in the characterization of cell lines used to produce biologicals. Rockville, MD, USA; 1993
- 7) ICH Expert Working Group. ICH Q2(R1) Validation of Analytical Procedures: Text and Methodology. Geneva, Switzerland; 2005
- 8) Dabrazhynetskaya A et al. Preparation of reference strains for validation and comparison of mycoplasma testing methods. J Appl Microbiol 2011; 111: 904-914

Regulatory disclaimer

QC Sample Preparation Kit: For use in quality control/manufacturing process only. MycoTOOL Mycoplasma Real-Time PCR Kit: For customers in the European Economic Area: Contains SVHC: octyl/ nonylphenol ethoxylated. For use in analytical activities such as monitoring and quality control and under controlled conditions only – acc. to Art. 56.3 and 3.23 REACH Regulation. LightCycler® 480 Instrument and MagNa Pure Systems: For life science research only. Not for use in diagnostic procedures.