Validation of the MycoTOOL Mycoplasma Real-Time PCR Kit

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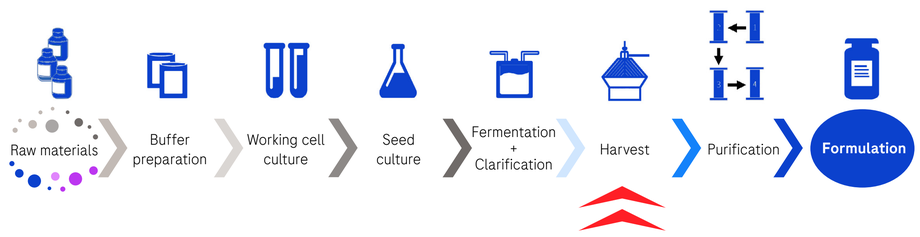
Abstract

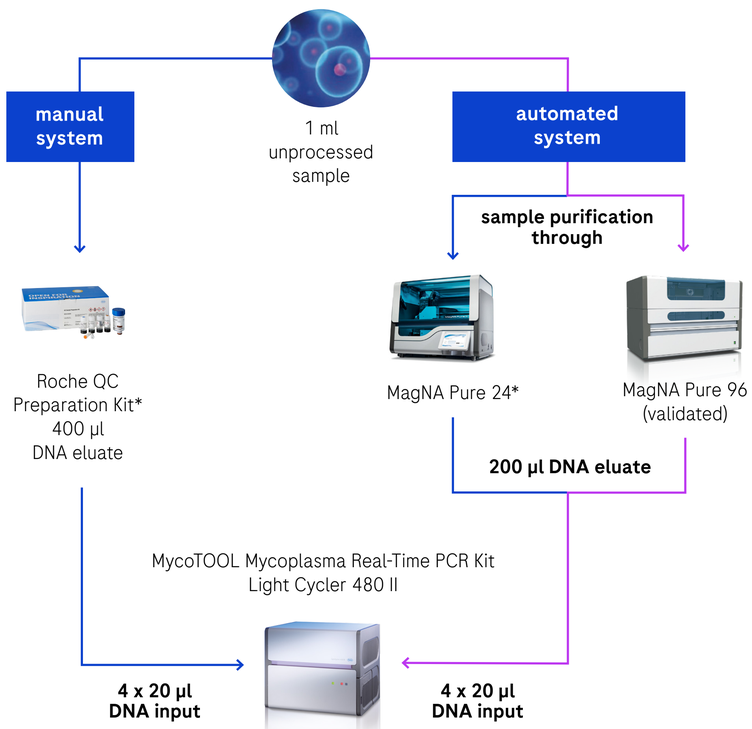
Mycoplasma contamination of biopharmaceuticals – as a consequence of cell culture contamination in the manufacturing process – poses a potential health risk to patients.[1] To minimize this risk, regulatory agencies require manufacturers of biopharmaceuticals to ensure the absence of mycoplasmas in the manufacturing process and end product.[2-6] To test for mycoplasmas, key regulatory guidelines such as EP2.6.7[2], USP<63>[3] and JP[4], 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.



01
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



02
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 x 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 EP2.6.7[2] and additionally include *M. orale, M. salivarium and M. hominis.*

Validation design

The validation of MycoTOOL RT followed the criteria as mentioned in the EP2.6.7[2] and ICH-Q2 R[17] 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 MycTOOL 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 10^6 - 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 01.

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 04). 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 recommend.[8] All tested mollicute reference strains showed GC/CFU ratios < 100 (Table 01).

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

* Fastidious strain

T01
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 02). The data confirm that MycoTOOL RT is a highly sensitive detection method.

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

* Fastidious strain

T02
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 03).

		Analyst 1	Analyst 2	Analyst 3
PCR result	mycoplasma: *M. orale*	8/8	8/8	8/8
	recovery control	8/8	8/8	8/8
ØCq (mycoplasma: *M. orale*)		38.89	37.00	38.93
ΔCq (mycoplasma: *M. orale*)			1.93	

T03
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	Detection limit: Indicator cell culture method [CFU/ml]	Detection limit: Myco TOOL RT [CFU/ml]
M. hominis	ATCC 23114	1	0.1	0.1
M. orale	ATCC 23714	0.1	ND	1
M. aginini	ATCC 23838	0.01	1	1
A. laidlawii	EDQM	0.01	0.1	0.1
M. hyorhinis	EDQM	1	1	10
M. pneumoniae	NCTC 10119	1	ND	1
M. salivarium	ATCC 23064	0.1	ND	0.1
M. fermentans	ATCC 19989	10	1	0.1
Sp. citri	ATCC 27556	ND	ND	0.1
*M. hyorhinis**	ATCC 29052	ND	1	10

* Fastidious strain ND = not detectable

T04
Comparison between the different mycoplasma detection methods.

Summary and discussion

This validation study demonstrates the compliance of MycoTOOL RT with the EP2.6.7[2] 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 EP2.6.7[2] 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 nonmicrobiological techniques. Mol Cell Probes 2011; 25: 69-77
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Regulatory Disclaimer

QC Sample Preparation Kit and MycoTOOL Mycoplasma Real-Time PCR Kit: For use in quality control/ manufacturing process only. LightCycler® 480 Instrument and MagNa Pure Systems: For life science research only. Not for use in diagnostic procedures.