10CFU Sensitivity Standards

INTRODUCTION

Mycoplasma are known as important contaminants of biological products derived from cell lines in the biopharmaceutical industry affecting every parameter of a cell culture system. Contaminated cultures can result in production loss and unsafe products. Mycoplasma are the smallest of self-propagating organisms. Unlike viruses, mycoplasmas can reproduce outside of living cells. Many species within the genera *Mycoplasma*, *Acholeplasma* and *Spiroplasma* thrive as parasites in humans, birds, plants and animal hosts. Some species can cause disease in humans. Such contaminations can arise from the contamination of the source cell lines themselves (cell substrates) or from adventitious introduction of mycoplasmas during production. Based on this, contamination risk guidelines and technical papers are published to give guidance on mycoplasmas safety for the manufacture of biological products as for instance the European Pharmacopoeia, chapter 2.6.7., *"Mycoplasmas"* (EP 2.6.7) or the Japanese Pharmacopoeia, chapter G3 (JP G3).

PRODUCT DESCRIPTION

The intended use of mycoplasma qPCR detection kits is the detection of *Mollicutes (Mycoplasma, Acholeplasma, Spiroplasma)* contamination in cell cultures and other cell culture derived biologicals in respect of EP 2.6.7.
European Pharmacopoeia 2.6.7 "Mycoplasmas" requires a sensitivity of 10 Colony Forming Units (CFU)/ml sample volume for NAT-based methods like PCR to replace the traditional culture method. This feature of the test method must be shown by the performing lab as part of robustness testing in presence of the sample matrix. 10CFU™ Sensitivity Standards are designed for verifying the sensitivity of a mycoplasma detection kit according to the requirements of the EP 2.6.7 "Mycoplasmas" and have to be validated in combination with a dedicated kit in respect to sample matrix and lab specific conditions.
As most cell culture labs and production facilities cannot accept vital mycoplasma in their facility due to safety regulations, or do not have access to a microbiology lab able to cultivate mycoplasma, these preparations allow safe and reliable validation of the procedure.

PURPOSE AND PRINCIPLE OF OPERATION

Each vial contains 10 CFU of irreversibly inactivated mycoplasma. By adding the sample matrix of interest, a sample according to EP 2.6.7 is prepared which has to be tested positive by the method applied. Obviously, the inactivated sample material is not suitable for the culture method anymore. As a result of proficiency tests on NAT-methods for mycoplasma detection it became obvious that in means of highest sensitivity DNA extraction is indispensable. The DNA extract can directly be used for PCR. The particular PCR test must show positive amplification for EP- compliance.

PREPARATION PROCEDURE

All mycoplasma listed below were cultivated in 50 ml culture broth as described in EP 2.6.7 in Hayflick/Frey medium to early logarithmic phase to avoid an atypical high ratio of non-vital to vital mycoplasma and correspondingly a high GU*: CFU ratio. The growth kinetic of the organisms under specific culture conditions were determined in former experiments to identify the best time point for harvesting. The culture broth was divided into two portions:

1. One portion was used for quantification of the mycoplasma. The broth was vortexed intensively and subjected to ultrasonic treatment for 5 minutes prior titration to break up mycoplasma clusters. An aliquot remained untreated for vitality control. Two tenfold dilution series were prepared in culture broth. Each dilution series has been performed in multiples by different operators for highest precision. Of each dilution step two Hayflick/Frey agar plates were inoculated with 20 µl each, incubated at 37 °C (30 °C for *Spiroplasma citri*) and checked frequently for colony formation by microscope. Frequent counting was stopped at constant colony numbers and titer calculated as CFU/ml culture broth.
2. The second portion of the culture broth was transferred into 1.5 ml reaction tubes at a volume of 500 µl/tube and heat inactivated (10 min, 95 °C), then adjusted to 200 CFU/ml and filled in 50 µl aliquots before lyophilization. All tubes were stored at 4-8 °C until use.

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3. Although mycoplasma were inactivated prior to lyophilization, the lack of vitality was proven by media fills with Hayflick and Frey medium and cultivation at 37 °C (30 °C for *Spiroplasma citri*) for 23 days. Growth was monitored by qPCR.

All strains have been obtained from the National Collection of Type Cultures (NCTC), UK, and cultivated in low passages. The corresponding NCTC codes are listed in component-table below.

Please note: This standard material was not titrated for genome copies (GU) as EP 2.6.7 does not provide sensitivity limits on DNA level. No guarantee for a particular GU to CFU ratio is provided with this product and the ratio may vary from lot to lot.

MYCOPLASMA CULTIVATION MEDIA

For the titration of mycoplasma spike, the quality of the culture medium is of severe relevance for the subsequent spiking experiments. Mycoplasma were cultivated and titrated in an in-house preparation of Hayflick/Frey culture medium. Each lot of the mycoplasma culture material was intensively validated and quality controlled according to EDQM standards.

QUALITY CONTROL

For quality control, 10CFU™ Sensitivity Standards undergo intensive functionality and vitality testing and validation.

- Functionality Testing

1. From each lot 2 vials of 10CFU™ Sensitivity Standards were rehydrated with 1 ml of cell culture supernatant (100 % confluent cells in DMEM and 10 % FCS), 2 vials were rehydrated with 1 ml of 10 mM Tris/HCl buffer (pH 8.0), and 2 vials were rehydrated in 1 ml DMEM and 10 % FCS (negative controls).
2. For each sample 2 separate DNA extraction assays are performed independently using Venor®GeM Sample Preparation Kit (Minerva Biolabs GmbH). DNA extracts are used as template and analyzed in qPCR using Microsart® ATMP Mycoplasma Kit (Sartorius Stedim Biotech GmbH).
3. All preparations must show detectable amplification within the defined sensitivity limits.
4. Procedure and analysis are performed by highly qualified and trained operators according to up-to-date instructions of use.
5. Procedure is carried out under sterile conditions at all times. In order to avoid DNA cross-contaminations, spatial segregation of sequential steps (in 4 separate modules) is ensured.

- Vitality Testing

Mycoplasma vitality tests are performed with each lot to exclude the transport of vital microorganisms.

1. From each lot, 2 vials of 10CFU™ Sensitivity Standards were rehydrated with 1 ml of culture medium (DMEM and 5 % FCS), and incubated at 37 °C for 7 days.
2. After 7 days another vial was rehydrated with culture medium.
3. DNA extraction (using Venor®GeM Sample Preparation Kit) and qPCR (using Microsart® ATMP Mycoplasma Kit) were carried out in duplicates.
4. To prove the lack of vitality, the C_t-value is shown to not exceed the measurement tolerance of the system (ΔC_t > 2) in reference to the negative control.

- Stability

All 10CFU™ Sensitivity Standards were lyophilized for sustained product stability.

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- Detection Limits and Lot Release Criteria

Mollicute suspensions with 3 dilution series were prepared in 1:10 dilution steps in DMEM supplemented with 5 % (v/v) FCS and tested in 3 separate qPCR amplification runs. Each dilution series was performed in 8 repeats per sample so that 24 PCR results per dilution were obtained (n= 3 x 8 =24). A range of average C_t-value for each species was determined as a release criterion.

Species	Detection Range (10 CFU/ml)		
	higher than	aim C_t-Value	lower than
Acholeplasma laidlawii	30.82	32.32	40
Mycoplasma fermentans	30.16	31.66	40
Mycoplasma hyorhinis	32.03	33.53	40
Mycoplasma orale	32.35	33.85	40
Mycoplasma pneumoniae	30.78	32.28	40
Mycoplasma gallisepticum	31.88	33.38	40
Mycoplasma synoviae	32.14	33.64	40
Mycoplasma arginini	32.43	33.93	40
Spiroplasma citri	33.91	35.41	40

CENTIFICATE OF ANALYSIS

A lot-specific Certificate of Analysis with average C_t-value is available for download as pdf from https://www.minerva-biolabs.com/.

PRODUCT FEATURES AND PACKAGING

The product is available in 2 different package sizes: one set includes all Mycoplasma species listed in EP 2.6.7, each of the subsets contain one specific species of the mycoplasmas list in EP 2.6.7:

- Set of all EP 2.6.7 listed species

102-0002 Mycoplasma Set

- Subsets of each Mycoplasma species listed in EP 2.6.7 and JP G3

102-1003 *Mycoplasma arginini*
102-2003 *Mycoplasma orale*
102-3003 *Mycoplasma gallisepticum*
102-4003 *Mycoplasma pneumoniae*
102-1103 *Mycoplasma salivarium*
102-5003 *Mycoplasma synoviae*
102-6003 *Mycoplasma fermentans*
102-7003 *Mycoplasma hyorhinis*
102-8003 *Acholeplasma laidlawii*
102-9003 *Spiroplasma citri*

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Cat. No. Species	Components	Amount	NCTC code	Volume
102-0002 *Mycoplasma Set*	*Mycoplasma arginini*	2 vials	10129	lyophilized
	Mycoplasma orale	2 vials	10112	lyophilized
	Mycoplasma gallisepticum	2 vials	10115	lyophilized
	Mycoplasma pneumoniae	2 vials	10119	lyophilized
	Mycoplasma synoviae	2 vials	10124	lyophilized
	Mycoplasma fermentans	2 vials	10117	lyophilized
	Mycoplasma hyorhinis	2 vials	10130	lyophilized
	Acholeplasma laidlawii	2 vials	10116	lyophilized
	Spiroplasma citri	2 vials	10164	lyophilized
	Negative Control	2 vials	N/A	lyophilized
102-1003 *M. arginini*	*Mycoplasma arginini*	3 vials	10129	lyophilized
	Negative Control	2 vials	N/A	lyophilized
102-2003 *M. orale*	*Mycoplasma orale*	3 vials	10112	lyophilized
	Negative Control	2 vials	N/A	lyophilized
102-3003 *M. gallisepticum*	*Mycoplasma gallisepticum*	3 vials	10115	lyophilized
	Negative Control	2 vials	N/A	lyophilized
102-4003 *M. pneumoniae*	*Mycoplasma pneumoniae*	3 vials	10119	lyophilized
	Negative Control	2 vials	N/A	lyophilized
102-1103 *M. salivarium*	*Mycoplasma salivarium*	3 vials	10113	lyophilized
	Negative Control	2 vials	N/A	lyophilized
102-5003 *M. synoviae*	*Mycoplasma synoviae*	3 vials	10124	lyophilized
	Negative Control	2 vials	N/A	lyophilized
102-6003 *M. fermentans*	*Mycoplasma fermentans*	3 vials	10117	lyophilized
	Negative Control	2 vials	N/A	lyophilized
102-7003 *M. hyorhinis*	*Mycoplasma hyorhinis*	3 vials	10130	lyophilized
	Negative Control	2 vials	N/A	lyophilized
102-8003 *A. laidlawii*	*Acholeplasma laidlawii*	3 vials	10116	lyophilized
	Negative Control	2 vials	N/A	lyophilized
102-9003 *S. citri*	*Spiroplasma citri*	3 vials	10164	lyophilized
	Negative Control	2 vials	N/A	lyophilized

The expiry date of the unopened package is specified on the package label. The kit components are stored until use at +2 to +8 °Cmust be stored after opening at <-18 °C.

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Part	Component	Concentration	Manufacturer	Cat. No.	Change Control
Buffer	Tris	10 mM	Merck Millipore	1.08382.1000	yes ☐ / no ☒
	EDTA	0.1 mM	Roth	8043.1	yes ☐ / no ☒
	Sodium azide	0.02 %	Merck Millipore	1.099.880.100	yes ☐ / no ☒
	pH	8.0	N/A	N/A	yes ☐ / no ☒
Mycoplasma	Mycoplasma, titrated in culture broth (Hayflick/ Frey)		Minerva Biolabs	N/A	yes ☒ / no ☐

FIGURES OF SELECTED PRODUCTS

TRADEMARKS

10CFU is a trademarks of Minerva Biolabs. Venor is a registered trademark of Minerva Biolabs. Microsart is a registered trademark of Sartorius Stedim.

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