Product Validation Report

Evaluation of the fungi detection capability of Microsart® ATMP Fungi and Microsart® ATMP Sterile Release

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1 Introduction

Gene therapy, somatic cell therapy and engineered tissue products are classified as so called Advanced Therapy Medicinal Products (ATMPs). In contrast to parenteral drug products, microbial safety at time of lot release cannot be guaranteed for the majority of ATMP products. Typical shelf-lives of less than 48 hours are in contrast to the current compendial microbiological control test which takes 7 to 14 days before a contamination can be ruled out with certainty. For ATMPs, microbial safety cannot be guaranteed at time of admission. These facts explain the high demand for growth-independent rapid ATMP testing. In order to enhance sensitivity and duration of microbial detection control tests, a more rapid method using real time PCR has been developed by the cooperation partners Minerva Biolabs and Sartorius Stedim Biotech. Following the development of Microsart® ATMP Mycoplasma and Microsart® ATMP Bacteria, this study has been conducted in order to validate the kits Microsart® ATMP Fungi as well as the fungi SR mix from Microsart® Sterile Release used for the detection of fungi contamination in ATMPs. Microsart® ATMP Fungi as well as Fungi SR Mix include a mix of primer, nucleotides and polymerase containing a FAM™ labeled probe specific for a broad range of different fungi genera. False negative results due to PCR inhibitors or improper DNA extraction are detected by the internal amplification control. The internal amplification control can be added to the sample prior to DNA extraction and analysis for verification of the complete process (DNA extraction and PCR reaction). The internal amplification control can also be added directly to the PCR master mix to act as a PCR control only. The amplification of the control reaction is detected at 610 nm (ROX™ channel) and the fungi specific sequence at 520 nm (FAM™ channel).

2 Objective

A study was designed to evaluate the fungal detection capability for the fungal control detection system Microsart® ATMP Fungi and the fungi SR mix of its derivate product Microsart® ATMP Sterile Release, for qPCR. A number of guidance documents are published to demonstrate that rapid and alternative microbiological methods are suitable for their intended use. These include the Parenteral Drug Association (PDA) Technical Report 33, European Pharmacopoeia (Ph. Eur.) chapter 5.1.6 and United States Pharmacopoeia (USP) chapter <1223>. This comprehensive validation plan is intended to show suitability of the new product, Microsart® ATMP Fungi and Microsart® ATMP Sterile Release, for fungi control testing in ATMPs. The validation study was setup to fulfill Ph. Eur. chapter 5.1.6. Part 4-1-1, *Primary validation to be performed by the supplier*. All parameters were validated with characteristic and most challenging test setups. Furthermore, comparability to the compendial method was demonstrated. Selected spiked samples of the sensitivity testing (chapter 7.2) was tested in parallel at an external contract lab according to Ph. Eur. 2.6.1. In this validation at least one lot has been tested. At least two additional lots have been tested for functionality during a separate shelf-life study.

3 Definitions and Abbreviations

ATMP	Advanced Therapy Medicinal Product
CFU/ml	Colony-forming Units per milliliter
DMEM	Dulbecco´s modified Eagles medium
DNA	deoxyribonucleic acid
EP/ Ph. Eur.	European Pharmacopoeia
FBS	Fetal Bovine Serum
GC/ml	genome copies per milliliter
L+S	Labor LS SE & Co. KG Mangelsfeld 4, 5, 6 97 708 Bad Bocklet Deutschland
LOD	limit of detection
MB	Minerva Biolabs GmbH
N/A	not applicable
NEC	negative extraction control
nm	nanometer
NTC	no template control
PC	positive control
PCR	polymerase chain reaction
PDA	Parenteral Drug Association
QC	Quality Control
SSB	Sartorius Stedim Biotech GmbH

4 Responsibilities

Minerva Biolabs GmbH (MB) was responsible for developing the test protocol in agreement with Sartorius Stedim Biotech GmbH (SSB). SSB was responsible for reviewing the test protocol to ensure its accuracy, completeness and validity.

Test initiation was scheduled by MB and SSB after approval of the validation plan by signing and exchanging a copy of the plan cover page and the necessary material for testing have been exchanged and received.

SSB and MB technicians executed the test protocol. MB and SSB were responsible for the execution of dedicated parts of the protocol.

MB drafted the validation report and SSB reviewed and approved the document to ensure its validity. The report was closed by exchanging a signed copy of the report cover page.

Deviations, including test failures and protocol modifications which occurred during the execution of the test protocol had been discussed between MB and SSB.

5 Test Material

The tests were conducted using the following test system, product solutions and material.

5.1 Test System

The test system used for the detection of fungi during this study was as follows:

Table 1. Test System Information

System type	Catalogue No.	Supplied by	Storage Conditions	Lot No.
Microsart® ATMP Fungi (100 tests)	SMB95-1012	SSB/MB	+2 – +8 °C	9519S1049 9519S1069 9519S1099
Fungi SR mix of Microsart® ATMP Sterile Release (10 samples)	SMB95-1007	SSB/MB	+2 – +8 °C	9518S1109
Microsart® ATMP Extraction (50 tests)	SMB95-2001	SSB/MB	Ambient Temperature	95261058 9526L1058 9526U1078 9526S1059 9526S1079

5.2 Sample Matrix

Sample matrix, with defined cell culture medium components (Table 2), were used for specificity testing, LOD (Limit of Detection) determination and robustness testing. For sensitivity testing, a panel of ten different fungal species (Table 3) were diluted in DMEM + 5% FBS to prepare dilution series. Furthermore to demonstrate specificity and robustness, at least four different cell culture samples with different culture media compositions were spiked with 99 CFU/ml of one of the fungal species which was detected with the highest LOD_{95} value during LOD_{95} determination (selected from the two EP 2.6.1/EP 2.6.27 fungi listed Table 12).
All matrices have been heat-treated for 20 min at 95 °C to inactivate all containing DNases. The heat-treated suspensions were stored ≤ -18 °C and intensively vortexed after thawing for immediate use.

Table 2. Matrix Components

Product Ingredient	Manufacturer/Source	Catalogue No.	Lot No.	Storage Conditions
ChondroMAX Differentiation Medium	Sigma Aldrich	SCM123-100ml	SLBT3800	≤ -18 °C
Chrondrocyte Differentiation Medium	Sigma Aldrich	411D-250	745	+2 - +8 °C
DMEM, high glucose, GlutaMAX™ Supplement	Gibco/Thermo Fisher	10566016	2085084	+2 - +8 °C
DMEM liquid medium with stable Gtutamine	Merck/Biochrom AG	FG 0415	0731E 0147G	+2 - +8 °C
DMEM liquid medium w/o Na-Pyruvate, with stable Glutamine	Merck/Biochrom AG	FG0435	1014F	+2 - +8 °C
DMEM/F-12, GlutaMAX™ Supplement	Gibco/Thermo Fisher	10565018	1930078	+2 - +8 °C
FBS	Biochrom AG-	S0615-	0167F	≤ -18 °C

	Gibco/Thermo Fisher	1070106	S0615 0973F- 42Q0685K	
GlutaMAX Supplement	Gibco/Thermo Fisher	35050061	2059587	2°C to 8°C
Human Osteoblast Differentiation Medium	Sigma Aldrich	417D-250	715	+2 - +8 °C
Human Serum	Sigma Aldrich	H6914-100ML	SLBT2873	+2 - +8 °C
KnockOut DMEM	Gibco/Thermo Fisher	10829018	2026761	+2 - +8 °C
Leibovitz´s L-15 Medium	Gibco/Thermo Fisher	11415064	2060481	+2 - +8 °C
LymphoONE™ T-Cell Expansion Xeno-Free Medium	Takara	WK552S	AJ3P037	+2 - +8 °C
McCoys 5A Medium	Gibco/Thermo Fisher	16600082	2085107	+2 - +8 °C
MEM	Sigma Aldrich	M8167-500ml M4655-500ml	RNBG1617 RNBG5441	+2 - +8 °C
Opti-MEM™ I Reduced Serum Medium, GlutaMAX™ Supplement	Gibco/Thermo Fisher	51985026	1929010	+2 - +8 °C
Penicillin-Streptomycin (10,000 U/mL)	Gibco/thermos Fisher	15140122	2041561 MK	-5°C to -20°C
RPMI 1640 Medium (ATCC modification)	Gibco/Thermo Fisher	A1049101	2085378	+2 - +8 °C
RPMI 1640 Medium, no glutamine, no phenol red	Gibco/Thermo Fisher	3240414	2063458	+2 - +8 °C

5.3 Microorganisms and ATMP samples for sensitivity, specificity and robustness testing

For sensitivity, specificity and robustness testing, 12 different microorganisms were used, selected among the most common contaminants (Table 3).
For CFU-based tests, 7 quantified microorganisms with a defined cell count were used. These microorganisms are listed in table 3.

Another 6 microorganisms were tested at DNA-level using lyophilized DNA preparations containing genomic DNA of the particular species (Table 3) provided by MB. Fungi and bacteria were cultivated under ATCC® described conditions followed by DNA extraction, quantification and lyophilisation. Briefly, 5-10 ml are inoculated with the respective species and incubated overnight. A main culture of 50-100 ml is inoculated from the overnight culture and incubated, before harvest at the end of the logarithmic phase. Microorganisms are harvested by centrifugation at 4000 x g at 4-8 °C and genomic DNA is extracted via column purification.

Extracted DNA is analyzed using standardized methods in the following order:

a. partial sequencing and Nanodrop measurement
b. OD ratios between $OD_{260/280}$ = 1.8-2.0 and $OD_{260/230}$ >1.5 are allowed for further analysis
c. DNA concentration of intact DNA is subsequently determined via Qubit
d. Qubit based concentrations are used for genome copy number determination.

Table 3. Microorganisms tested

Species	Strain	Supplier	Lot-No.	Catalogue-No.	Assay
Species used for CFU-based analysis					
Candida albicans[a, b]	ATCC 10231	Microbiologics	443-810	443	Table 12, assay 1 Table 14, assay 9 Table 16, assay 1 Table 17, Assay 2
Candida tropicalis	ATCC 750	Minerva Biolabs	-	-	Table 13, Assay 3
Candida krusei	ATCC 6258	Minerva Biolabs	-	-	Table 13, Assay 5 Table 14, Assay 10
Candida glabrata	ATCC 90030	Minerva Biolabs	-	-	Table 13, Assay 4
Aspergillus brasilliensis[a, b]	ATCC 16404	Microbiologics	392-778-2	392	Table 12, Assay 2 Table 14, Assay 8 Table 16, assay 2 Table 17, Assay 1 Table 17, Assay 2
Aspergillus fumigatus	ATCC 9197	Minerva Biolabs	-	-	Table 13, Assay 6
Penicillium chrysogenum	ATCC 9178	Minerva Biolabs	-	-	Table 13, Assay 7
Species used for DNA-based analysis					
Candida guilliermondii		Minerva Biolabs	2104119471065	210411947	Table 15, Assay 1
Candida albicans		Minerva Biolabs	5113861108	51-1386	Table 15, Assay 1 Table 17, Assay 3 Table 17, Assay 4
Candida haemulonii		Minerva Biolabs	2105706241065	210570624	Table 15, Assay 1
Bacillus subtilis	ATCC 6633	Minerva Biolabs	5100101107	51-0010	Table 15, Assay 2
Pseudomonas aeruginosa	ATCC 10145	Minerva Biolabs	5200711049	52-0071	Table 15, Assay 2
Legionella pneumophila	ATCC 33152	Minerva Biolabs	5201011079	52-0101	Table 15, Assay 2

[a]EP 2.6.1 listed fungal strains
[b]EP 2.6.27 listed fungal strains

Cell samples for specificity and robustness testing consist of four different cell culture samples representing a broad spectrum of host species and applications (Table 4). Cell lines are cultivated according to ATCC® sub culturing guidelines. Cell counts and flask numbers are increased by passaging till reaching the required amount of cells. At the final passage cells are harvested and counted, followed by low speed sedimentation at 950 x g and resuspension in medium.

Table 4. Description of cell samples used as background for spiking experiments

Cell Type	Origin/ATCC	Species	Media Composition
HeLa	Cervix, epithelial, ATCC® CCL-2™	*H. sapiens*	DMEM, 5% FBS
Vero	Kidney, epithelial, ATCC® CCL-81™	*C. aethiops*	DMEM, 5% FBS
CHO-K1	Ovary, epithelial-like, ATCC® CCL-61™	*C. griseus*	DMEM, 5% FBS
Human Peripheral Blood Mononuclear Cells	Primary cells Lonza CC-2702	*H. sapiens*	Tahara lympho one Medium + 5 % HS
Jurkat	T-cell, DSM ACC 282	*H.sapiens*	RPMI, penicillin/Streptomycin, Glutamax, 5% FBS

5.4 Incoming goods inspection and qualification of native material

EZ-CFU from Microbiologics and native material from Minerva Biolabs GmbH of species listed in Table 6 were tested in an incoming good inspection to determine or confirm the cell count.

1. Two pellets of EZ-CFU were rehydrated in 2 ml of rehydration buffer and diluted in a 1:10 dilution step in a standard phosphate buffer pH 7.2. At least three agar plates (Sabouraud agar) were inoculated with 100 µL, incubated for 24-48h and checked for colony formation.

Cell counts of all EZ-CFU were confirmed to be in the range of 10-100 CFU. Consequently all EZ-CFU fulfill the manufacturer's specification and are accepted for validation.

2. 50-100 ml of Sabouraud medium are spiked with few microliter of a concentrated solution of the species of interest. Culture is performed with shaking at the recommended temperature (Table 6). After 24-48h the solution is aliquoted with Glycerol and freeze down. The next day, one aliquots is diluted in a 1:10 dilution step in a standard phosphate buffer pH 7.2. At least three agar plates (Sabouraud agar) were inoculated with 100 µL, incubated for 24-48h and checked for colony formation.

The incubation conditions for each microorganism are listed in Table 6.

Table 5. Fungi Cultivation Media Ingredients

Medium	Manufacturer	Catalog No.	Lot No.
Dextrose	SIGMA	D9434-250G	SLBT1203
Mycological peptone	SIGMA	777199-500G	BCBR2356V
Sabouraud +4% Dextrose agar	Merck Germany	1.05438.0500	VM811438747

Table 6. Incubation Conditions for each fungal species

Species	Strain	Atmosphere	Temperature
*Candida albicans**	ATCC 10231	aerobic	24-26 °C
Candida tropicalis	ATCC 750	aerobic	24-26 °C
Candida krusei	ATCC 6258	aerobic	25-30 °C
Candida glabrata	ATCC 90030	aerobic	20-25 °C
*Aspergillus brasilliensis**	ATCC 16404	aerobic	20-25 °C
Aspergillus fumigatus	ATCC 9197	aerobic	24 °C
Penicillium chrysogenum	ATCC 9178	aerobic	24-26 °C

* EP 2.6.1 listed fungi strains

EZ-CFU standards are cultivated in fungal media and lyophilized in a stabilizing matrix.
Yeast material from Minerva Biolabs are cultivated in liquid fungal media, aliquoted and freeze down with 10-30% Glycerol.
Sporulating fungi are cultivated on fungal media agar plates and spores are collected in PBS/Tween, aliquoted and frozen with 10-30% glycerol.

5.5 Compendial culture method

The two fungi species listed in EP 2.6.1 (*Candida albicans* and *Aspergillus brasilliensis*) were spiked in DMEM + 5 % FBS and cultivated in thioglycolate medium and soya-bean casein medium (TSB) for 14 days according to the recommendation of the European Pharmacopoeia for compendial culture method described in EP 2.6.1.

5.6 Equipment

The following lab equipment was used for the study:

Table 7. Lab Equipment at MB

Equipment	Equipment-ID	Manufacturer	Brand
qPCR cycler	R 04 0843, ES72	Corbett Research	Rotor-Gene 6000
qPCR cycler	275001289, ES12	Applied BioSystems	ABI Prism 7500
qPCR cycler	Model 401513; Serial No DE00700786	Agilent Technologies	Mx3005P
qPCR cycler	CFX96 Optics Ser. No. 785BR11826; C1000 Cycler Ser. No. CT015330	Bio-Rad	CFX Touch
qPCR cycler	272522531	Thermo Fisher	QuantStudio 5
Pipettes for master mix setup 10–300 µl 10–300 µl	 E60, 15011121 E66, 15017290	 Sartorius Sartorius	 Biohit Picus Biohit Picus
Pipettes for Sample Handling 0.2-10 µl 10–300 µl 50-1000 µl	 E69, 15023351 E70, 16010674 E71, 16009571	 Sartorius Sartorius Sartorius	 Biohit Picus Biohit Picus Biohit Picus
Pipettes for DNA Extraction 10-100µl 100-1000µl	 E102, 18020324 E103, 18026710	 Sartorius Sartorius	 Tacta Tacta
Vortex	E29, VB4B016638	VWR	N/A
Glove box	E68, 22160256	GS Glovebox Systemtechnik GmbH	Glovebox P10RT2

Table 8. Lab Equipment at SSB

Equipment	Equipment-ID	Manufacturer	Brand
qPCR cycler	Serial No: CT021642	Bio-Rad	CFX96 touch
Glovebox	12810/12811/12816	GS Glovebox Systemtechnik GmbH	Glovebox P10RT2
Laminar Flow	Heraeus LaminAir HLB 2448	Heraeus	LaminAir
Vortex	No 541-10000-00-0; Ser. No 020314813	Heidolph	REAX top
Micro centrifuge	Model AL220VAC; SN 053164	Roth	Rotilabo-Zentrifuge
Centrifuge	Order No. 75003280; Ser. No. 237292	Heraeus Instruments	Biofuge pico
Pipettes DNA-free 0.5 – 10 µl 2 – 20 µl 20 – 200 µl 100 – 1000 µl	 16007668 16007703 16009618 16008275	Sartorius	Tacta
Electronic Pipettes DNA-free 10 – 300 µl	 16005936	Sartorius	Picus NxT
Pipettes 0.5 – 10 µl 2 – 20 µl 10 – 100 µl 20 – 200 µl 100 – 1000 µl	 16007685 16007711 16006996 16009619 16008272	Sartorius	Tacta

The following consumables were used:

Table 9. DNA-free lab ware used at MB

Article no.	Article name	Manufacturer / Supplier
72.706.400 72.991.103 70.1116.210 70.760.212 70.765.210 70.762.211	Micro tubes, 1.5 ml, DNA-free PCR-tubes, Multiply-µStripPro Biosphere filter tips 0.5 – 20 µl Biosphere filter tips 2 – 100 µl Biosphere filter tips 20 – 300 µl Biosphere filter tips 100 – 1000 µl	Sarstedt
710970X 710975X 711200	PCR tubes, optical, DNA-free, high profile, SingleCap, 8er-SoftStrips 0.2 ml PCR tubes, optical, DNA-free, low profile, SingleCap, 8er-SoftStrips 0.2 ml PCR 0.1 ml 4-tubes & 4-Cap Strips	Biozym

Table 10. DNA-free lab ware used at SSB

Article no.	Article name	Manufacturer / Supplier
72.991.103 70.1116.210 70.760.212 70.765.210 70.762.211	PCR-tubes, Multiply-µStripPro Biosphere filter tips 0.5 – 20 µl Biosphere filter tips 2 – 100 µl Biosphere filter tips 20 – 300 µl Biosphere filter tips 100 – 1000 µl	Sarstedt
710970X 710975X	PCR tubes, optical, DNA-free, high profile, SingleCap, 8er-SoftStrips 0.2 ml PCR tubes, optical, DNA-free, low profile, SingleCap, 8er-SoftStrips 0.2 ml	Biozym

6 Test Procedure

Sample preparation prior testing is strictly required for highest confidence and sensitivity. The design and performance of pre-analytical procedures are part of this study in respect of the intended use but cannot reflect the diversity of the sample material in total. The templates for the PCR analysis are prepared by direct extraction of the sample and subsequent PCR analysis.

6.1 Sample preparation

Microsart® ATMP Extraction and Microsart® ATMP Sterile Release kits are optimized to extract fungal genomic DNA from different sample matrices including cell culture samples, reducing handling steps and thus contamination to a minimum. The Internal Control DNA of Microsart® ATMP Fungi or Microsart® ATMP Sterile Release can be used to monitor the extraction process. Extraction of fungal DNA will be carried out according to the instruction manual.

In detail:
1. Transfer 1 ml sample into a provided DNA-free 1.5 ml processing tube (transparent cap)
2. Centrifuge at 16000 x g for 15 minutes
3. Remove the supernatant carefully and completely

Attention: Samples can only be inactivated or frozen after this sample collection step.

4. Add 500 µl of Lysis Buffer to the sample

Optional: Add 20 µl Internal Control DNA from Microsart® ATMP Fungi Kit or Microsart® ATMP Sterile Release to the sample to monitor the extraction process.

Remark: In this validation study, the Internal Control was always used as a control of the PCR process, except during assay 1 of Table 17 where it was used as a control of the extraction process.

7. Vortex vigorously for 30 seconds until pellet is completely lysed
8. Heat at 80 °C for 10 minutes
9. Spin down at 16200 x g for 10 minutes
10. Remove supernatant carefully and completely, do not withdraw the pellet
11. Add 100 µl Suspension Buffer (violet cap) and suspend pellet by vortexing

6.2 Analytical procedures

The detection of fungal DNA will be carried out according to the instruction manual.

In detail:
Rehydration of the Reagents:
1. Centrifuge tubes with lyophilized components (5 sec at maximum speed)
2. Add 390 µl of Rehydration Buffer to the Fungi Mix (Microsart® ATMP Fungi) or 90 µl of Rehydration Buffer to each Fungi SR Mix (Microsart® ATMP Sterile Release).
3. Add appropriate amount of deionized, DNA-free water

Positive Control DNA 300 µl
Internal Control DNA 800 µl

4. Incubate for 5 minutes at room temperature
5. Vortex and centrifuge again

PCR master mix setup:

Total volume per reaction is 25 µl including 10 µl of sample. When setting up reactions, calculations include positive (PC) and negative controls (NTC). Pipet master mix into a 1.5 ml reaction tube and mix gently.

Pipetting scheme Microsart® ATMP Fungi:

	for 1 reaction	for 26 reactions
Fungi Mix	15 µl	390.0 µl
Internal Control DNA	1.0 µl	26.0 µl

Pipetting scheme Microsart® ATMP Sterile Release:

	for 1 reaction	for 6 reactions
Fungi SR Mix	15 µl	90.0 µl
Internal Control DNA	1.0 µl	6.0 µl

Attention: If the Internal Control DNA was added to the sample during DNA extraction, add 15 µL of the Fungi Mix or the Fungi SR Mix directly to each PCR tube.

Loading the test tubes:

Aliquot 15 µl of master mix into each PCR reaction tube. After pipetting the negative control (10 µl of water or elution buffer of DNA extraction kit), the tube must be sealed before proceeding with the samples. Add 10 µl of sample to each PCR reaction tube. Seal the tubes completely before proceeding with the positive control (10 µl) in order to avoid cross contamination.

Programming the qPCR cycler:

Program Step 1: Pre-incubation

Setting	Hold
Hold Temperature	95°C
Hold Time	3 min 0 sec

Program Step 2: Amplification

Setting	Cycling
Cycles	40
Denaturation	95 °C for 30 sec
Annealing	55 °C for 30 sec
Detection/ Elongation	60 °C for 45 sec

Result Interpretation:

The presence of bacteria in the sample is indicated by an increasing fluorescence signal in the

bacterial FAM™ channel during PCR. In order to interpret results accurately, a baseline has to be set. This threshold was defined as 10% of the maximum fluorescence level of the positive control.

The presence of fungi in the sample is indicated by an increasing fluorescence signal in the fungal FAM™ channel during PCR.

Table 11. Result interpretation Microsart® ATMP Fungi and Microsart® ATMP Sterile Release

Detection of Fungi FAM™ channel	Internal control ROX™ channel	Interpretation
positive (C_q < 40)	irrelevant	Fungi positive
negative (no C_q)	negative (no C_q)	PCR inhibition
negative (no C_q)	positive*	Fungi negative

*see chapter 6.3 for definition of positive

A successfully performed PCR without inhibition is indicated by an increasing fluorescence signal in the internal control channel. The provided Internal Control was added to the PCR master mix or as extraction control. Fungal DNA and Internal Control DNA are competitors in PCR. Because of the very low concentration of Internal Control in the PCR mix, the signal strength in this channel is reduced with increasing fungal DNA loads in the sample.

6.3 System suitability test criteria

Internal control (ROX™) must show C_q-values in the range of +/- 2 cycles of the no-template control (master mix control) if used as PCR control. If the internal control is used as extraction control it must show C_q-values in the range of +/- 3 cycles of the no-template control (master mix control). To exclude contaminations, the extraction control and the master mix control (FAM™) must be negative (no C_q-value or C_q-value > 40). The PCR positive control must show C_q-values of 24 +/- 2 cycles (FAM™).

6.4 Directives for action

During LOD_{95} determination (see Tables 12 and 13) 7 out of 8 NECs have to be negative. If more than one NEC is positive PCR was repeated. If NEC is confirmed positive, extraction was repeated. In case of positive NTC, PCR was repeated.

During specificity testing (see table 15), 6 of 8 NTCs have to be negative. In case more than two NTC were positive, PCR was repeated.

For robustness testing according to Tables 17A, step 1 and 2, two out of two NECs have to be negative. If NECs were positive PCR was repeated. If NEC was confirmed positive, extraction was repeated. In case of positive NTC, PCR was repeated.
For robustness testing as described in Tables 17A, step 3 and 4, seven out of eight NECs have to be negative. If NECs were positive PCR was repeated. If more than one NEC is positive PCR was repeated. If NEC is confirmed positive, extraction was repeated. In case of positive NTC, PCR was repeated.

Due to the environmental abundance of fungal DNA it cannot be guaranteed that all cell culture samples used as spiking matrix are free of fungal DNA. Thus, in case of a positive signal for NEC that was confirmed after re-testing, samples were send for sequencing and parallel sterility testing at an external contract lab in order to distinguish between true- and false-positive signal. This way the NEC of the different sample matrices also provides information regarding specificity. In addition, sequence alignments were done to get an impression of the broad fungi detection capability of Microsart® ATMP Fungi and Microsart® ATMP Sterile Release.

During LOD_{95} determination and robustness for the Fungi SR mix of Microsart® ATMP Sterile Release (see Table 14), 1 out of 2 NEC/NTC have to be negative. If more than one NEC/NTC is positive, PCR was repeated. If NEC/NTC is confirmed positive, extraction was repeated.

6.5 Calculations

N/A

6.6 Reporting requirements

The reports generated by the qPCR machine were printed in color. All run information were printed, including protocol, sample identification, internal amplification control curves (ROX™ filter) and target curves (FAM™ filter) and filed according to the chapter structure of this validation plan. Sample identification should contain information on the species, the contained concentration in CFU/ml or GC/ml or alternatively the type of control (PC for Positive Control, NC for Negative Control, NTC for No Template Control and NEC for Negative Extraction Control).

6.7 Overview of experimental settings

6.7.1 LOD_{95} determination / Fungi Detection Range

The experimental setup of each test is listed in the following tables. All tests of Table 12 were performed six times to have at least 24 results for each CFU concentration of each of the two fungal species listed in EP 2.6.1 and EP 2.6.27 available as EZ-CFU, and 48 results for NEC.

In the event that, after the first assay, two or more samples of a given concentration are negative, the following concentrations of the series were excluded from the study for the next assays.

Please be aware that due to variabilities in GE/CFU ratio of cultures, it is possible not to reproduce exactly the results presented in this validation study if other lot of EZ-CFU are used.

Table 12. Test setup for LOD_{95} determination of fungal species listed in EP 2.6.1 and EP 2.6.27 with Microsart® ATMP Fungi

Detektion Limit								
1	Spike DMEM + 5 % FBS with *Candida albicans* (EZ-CFU)							
	99 CFU/ml → 4 aliquots	50 CFU/ml → 4 aliquots	25 CFU/ml → 4 aliquots	10 CFU/ml → 4 aliquots	5 CFU/ml → 4 aliquots	2.5 CFU/ml → 4 aliquots	1.25 CFU/ml → 4 aliquots	0 CFU/ml → 8 aliquots
	Extract DNA and perform PCR with 10 µl DNA extract in a final volume of 25 µl + IC. Add 2x PCR NTC and 2x PCR PC.							
2	Spike DMEM + 5 % FBS with *Aspergillus brasilliensis* (EZ-CFU)							
	99 CFU/ml → 4 aliquots	50 CFU/ml → 4 aliquots	25 CFU/ml → 4 aliquots	10 CFU/ml → 4 aliquots	5 CFU/ml → 4 aliquots	2.5 CFU/ml → 4 aliquots	1.25 CFU/ml → 4 aliquots	0 CFU/ml → 8 aliquots
	Extract DNA and perform PCR with 10 µl DNA extract in a final volume of 25 µl + IC. Add 2x PCR NTC and 2x PCR PC.							

All tests of Table 13 were performed two times to generate at least eight results for each CFU concentration of each of the fungal species and 16 results for the NEC.

Table 13. Test setup for LOD_{95} determination

Detection Limit							
3	Spike DMEM + 5 % FBS with *Candida tropicalis*						
	99 CFU/ml → 4 aliquots	50 CFU/ml → 4 aliquots	25 CFU/ml → 4 aliquots	10 CFU/ml → 4 aliquots	5 CFU/ml → 4 aliquots	2.5 CFU/ml → 4 aliquots	0 CFU/ml → 8 aliquots
	Extract DNA and perform PCR with 10 µl DNA extract in a final volume of 25 µl + IC. Add 2x PCR NTC and 2x PCR PC.						
4	Spike DMEM + 5 % FBS with *Candida glabrata*						
	99 CFU/ml → 4 aliquots	50 CFU/ml → 4 aliquots	25 CFU/ml → 4 aliquots	10 CFU/ml → 4 aliquots	5 CFU/ml → 4 aliquots	2.5 CFU/ml → 4 aliquots	0 CFU/ml → 8 aliquots
	Extract DNA and perform PCR with 10 µl DNA extract in a final volume of 25 µl + IC. Add 2x PCR NTC and 2x PCR PC.						
5	Spike DMEM + 5 % FBS with *Candida krusei*						
	99 CFU/ml → 4 aliquots	50 CFU/ml → 4 aliquots	25 CFU/ml → 4 aliquots	10 CFU/ml → 4 aliquots	5 CFU/ml → 4 aliquots	2.5 CFU/ml → 4 aliquots	0 CFU/ml → 8 aliquots
	Extract DNA and perform PCR with 10 µl DNA extract in a final volume of 25 µl + IC. Add 2x PCR NTC and 2x PCR PC.						
6	Spike DMEM + 5 % FBS with *Aspergillus fumigatus*						
	99 CFU/ml → 4 aliquots	50 CFU/ml → 4 aliquots	25 CFU/ml → 4 aliquots	10 CFU/ml → 4 aliquots	5 CFU/ml → 4 aliquots	2.5 CFU/ml → 4 aliquots	0 CFU/ml → 8 aliquots
	Extract DNA and perform PCR with 10 µl DNA extract in a final volume of 25 µl + IC. Add 2x PCR NTC and 2x PCR PC.						
7	Spike DMEM + 5 % FBS with *Penicillium crysogenum*						
	99 CFU/ml → 4 aliquots	50 CFU/ml → 4 aliquots	25 CFU/ml → 4 aliquots	10 CFU/ml → 4 aliquots	5 CFU/ml → 4 aliquots	2.5 CFU/ml → 4 aliquots	0 CFU/ml → 8 aliquots
	Extract DNA and perform PCR with 10 µl DNA extract in a final volume of 25 µl + IC. Add 2x PCR NTC and 2x PCR PC.						

The cut-off is defined as the lowest fungi concentration [CFU/ml] which leads in 95 % of the tests to a positive result (23/24 and 8/8 samples have to be positive). For LOD_{95} determination the instrument CFX96 touch was used.

Table 14: Test setup for LOD_{95} determination with Fungi SR mix of Microsart® ATMP Sterile Release.

Detection Limit		
8	Spike DMEM + 5 % FBS with *Aspergillus brasiliensis*	
	2 aliquots 99 CFU/ml + 1 NEC	2 aliquots 50 CFU/ml + 1 NEC
	Extract DNA and perform PCR with 10 µl DNA extract in a final volume of 25 µl + IC. Add 1x PCR NTC and 1x PCR PC.	Extract DNA and perform PCR with 10 µl DNA extract in a final volume of 25 µl + IC. Add 1x PCR NTC and 1x PCR PC.
9	Spike DMEM + 5 % FBS with *Candida albicans*	
	2 aliquots 99 CFU/ml + 1 NEC	2 aliquots 50 CFU/ml + 1 NEC
	Extract DNA and perform PCR with 10 µl DNA extract in a final volume of 25 µl + IC. Add 1x PCR NTC and 1x PCR PC.	Extract DNA and perform PCR with 10 µl DNA extract in a final volume of 25 µl + IC. Add 1x PCR NTC and 1x PCR PC
10	Spike DMEM + 5 % FBS with *Candida krusei*	
	2 aliquots 99 CFU/ml + 1 NEC	2 aliquots 50 CFU/ml + 1 NEC .
	Extract DNA and perform PCR with 10 µl DNA extract in a final volume of 25 µl + IC. Add 1x PCR NTC and 1x PCR PC.	Extract DNA and perform PCR with 10 µl DNA extract in a final volume of 25 µl + IC. Add 1x PCR NTC and 1x PCR PC

All tests of Table 14 were performed four times to generate eight data points for each CFU concentration of each of the fungal species.

6.7.2 PCR specificity

The specificity of the method was tested by spiking the PCR master mix with specific and non-specific DNA as described in Table 15. This assay was performed twice in order to collect at least 16 results for each species.

Table 15. Test setup for specificity testing with genomic DNA

Specificity		
1	Spike PCR master mix with 10 GC/PCR of specific DNA	
	Candida albicans → 8 aliquots *Candida guilliermondii* → 8 aliquots *Candida haemulonii* → 8 aliquots	0 GC/PCR → 8 aliquots
	Perform PCR with 10 µl DNA solution in a final volume of 25 µl + IC. Add 2x PCR PC.	
2	Spike PCR master mix with 10^5 GC/PCR of non-specific DNA	
	Bacillus subtilis → 8 aliquots *Pseudomonas aeruginosa* → 8 aliquots *Legionella pneumophila* → 8 aliquots	0 GC/PCR → 8 aliquots
	Perform PCR with 10 µl DNA solution in a final volume of 25 µl + IC. Add 2x PCR PC.	

6.7.3 Comparison of Microsart® ATMP Fungi with compendial sterility test

The following assays described in Table 16, compares the Microsart® ATMP Fungi system with the compendial sterility test.

Table 16: Test setup for comparison between Microsart® ATMP Fungi and compendial sterility test (Accuracy and precision)

Specificity: Comparison with culture method with defined starting material quantity at external contract lab		
1	Spike DMEM + 5 % FBS with *Candida albicans* at 2x LOD_{95}, LOD_{95} and LOD_{95}/2. One aliquot without spike is processed as NC.	
	1 ml of each sample is used for the sterility test (direct inoculation) at the external contract lab L+S AG	Extract DNA from 1ml starting material with Microsart® ATMP Extraction. Perform the qPCR according to Microsart® ATMP Fungi.
2	Spike DMEM + 5 % FBS with *Aspergillus brasilliensis* at 2x LOD_{95}, LOD_{95} and LOD_{95}/2. One aliquot without spike is processed as NC.	
	1 ml of each sample is used for the sterility test (direct inoculation) at the external contract lab L+S AG	Extract DNA from 1ml starting material with Microsart® ATMP Extraction. Perform the qPCR according to Microsart® ATMP Fungi.

The suspensions of the two listed species were diluted to generate the appropriate concentrations and split into aliquots. A sample of each concentration were used for sterility test (direct inoculation) at L+S. In parallel, aliquots were extracted in duplicates and analyzed on a CFX96 cycler.

6.7.4 Robustness

Robustness testing allowed to test the reproducibility of the assay when the conditions of the experiments were slightly modified. Thus, as detailed in Table 17, we tested the Microsart® ATMP Fungi system with cell suspension, with different PCR cyclers, with free DNA as well as different laboratory environment and operators. Assay 1 to 4 were performed twice.

Table 17. Test setup for robustness testing with Microsart® ATMP Fungi

Robustness					
1	Dilute cell samples listed in table 4 in DMEM with 5 % FBS to generate a concentration of 10^6 and 10^5 cells/ml.				
	Spike with 99 CFU/ml of the fungi with the highest LOD_{95} value selected from the species listed in Table 12 and 13. → 2 aliquots with 10^6 cells/ml → 2 aliquots with 10^5 cells/ml				no spike (NEC) → 2 aliquots
	Add 20 µl Internal Control DNA into lysis buffer to monitor the extraction process. Extract DNA. Perform PCR with 10 µl DNA extract in a final volume of 25 µl. Add 2x PCR NTC and 2x PCR PC (add IC to NTC and PC reactions).				
2	Spike DMEM + 5 % FBS with 99 CFU/ml of the fungi with the highest $LOD_{95.}$ → 8 aliquots				NEC (DMEM + 5% FBS) → 2 aliquots
	Extract DNA and perform PCR with 10 µl DNA extract in a final volume of 25 µl + IC. Add 2x PCR NTC and 2x PCR PC.				
	Rotor Gene 6000	ABI 7500	Mx3005P	CFX96	Quant Studio 5
3	Spike water with *Candida albicans* gDNA.				
	10^4 GC/ml → 8 aliquots	10^3 GC/ml → 8 aliquots	10^2 GC/ml → 8 aliquots	10 GC/ml → 8 aliquots	0 GC/ml (NEC) → 8 aliquots
	Extract DNA				
	Perform PCR with 10 µl of extract in a final volume of 25 µl + IC. In parallel, perform a standard PCR serie with 2x10µl of each of the spiked water (not extracted). Add 2x PCR NTC and 2x PCR PC.				
4	Spike water with cell culture suspension (Vero cells; 10^4 cells/ml) and *Candida albicans* gDNA.				
	10^4 GC/ml → 8 aliquots	10^3 GC/ml → 8 aliquots	10^2 GC/ml → 8 aliquots	10 GC/ml → 8 aliquots	0 GC/ml (NEC) → 8 aliquots
	Extract DNA				
	Perform PCR with 10 µl of extract in a final volume of 25 µl + IC. In parallel, perform a standard PCR serie with 2x10µl of each of the spiked water (not extracted). Add 2x PCR PC.				
5	Extract DNA from culture media listed in Table 2 and from DMEM + 5 % FBS and perform PCR with 10 µl DNA extract in a final volume of 25 µl + IC. Add 2x PCR PC.				
	12 x 8 for DMEM; 1 x 8 for other culture media				

7 Study Results

The study conditions had to provide information on all relevant validation parameters requested by European Pharmacopoeia (Ph. Eur.) chapter 5.1.6 part 4-1-1, Primary validation to be performed by the supplier.

7.1 LOD_{95} Detection Limit

As the method employed is used only to obtain a qualitative result, proof of linearity is not required. If however the concept of linearity is extended to cover the working range, the detection limit becomes extremely important. In practice, the detection limit is determined in the form of the positive threshold (i.e. the cut-off point in the form of the minimum number of amplified target sequences by volume positively detected in 95 % of the sample series).

Link	Procedure	Acceptance Criterion	Results / LOD_{95}
See Table 12; 1 and 2 See Table 13; 3 to 7 See Table 14; 8 and 9	CFU-quantified fungal suspensions (Table 3) were diluted, according to their individual cell count, in DMEM + 5 % FBS to prepare suspensions with concentrations from 99 to 0 CFU/ml. Individual dilution series were prepared for the two fungal species listed in EP 2.6.1 and EP 2.6.27, and for another five fungal species. Dilutions from 99 to 0 CFU/ml were tested according to test procedure described chapter 6.	The cut-off is defined as the lowest fungi concentration [CFU/ml] which leads in 95 % of the tests to a positive result (23/24 and 8/8 samples have to be positive). LOD_{95} must be ⩽ 99 CFU	Passed (LOD_{95} comprised between 10 CFU and 99 CFU)

Candida albicans

CFU/ml						Mean	
99	Run 1	31.32	31.90	32.78	32.80	32.20	24/24
	Run 2	32.96	32.88	33.07	32.25	32.79	
	Run 3	32.29	32.81	36.03	32.09	33.30	
	Run 4	32.46	32.14	32.29	32.33	32.30	
	Run 5	32.32	32.55	32.71	32.36	32.48	
	Run 6	32.58	31.48	31.89	32.43	32.09	
50	Run 1	33.15	33.79	34.11	34.44	33.87	23/24
	Run 2	34.25	33.86	33.04	35.03	34.04	
	Run 3	33.59	33.90	32.63	33.89	33.50	
	Run 4	34.04	32.94	34.27	33.81	33.76	
	Run 5	No Cq	34.77	32.82	34.48	34.02	
	Run 6	32.58	32.57	32.82	34.81	33.19	
25	Run 1	33.42	34.72	34.88	35.32	34.58	21/24
	Run 2	34.67	37.19	33.28	36.08	35.30	
	Run 3	34.96	37.20	38.18	No Cq	36.78	
	Run 4	37.96	34.90	35.11	34.59	35.64	
	Run 5	33.55	No Cq	35.77	No Cq	34.66	
	Run 6	34.51	35.33	36.17	34.90	35.23	

10	Run 1	35.85	36.48	37.27	37.38	36.74	14/24
	Run 2	No Cq	No Cq	37.00	34.65	35.82	
	Run 3	No Cq	No Cq	No Cq	No Cq	No Cq	
	Run 4	No Cq	35.93	No Cq	37.11	36.52	
	Run 5	35.04	No Cq	No Cq	38.91	36.97	
	Run 6	35.54	36.26	35.84	37.52	36.29	

Candida Albicans (Microsart® ATMP Sterile Release)

CFU/ml				Mean	
99	Run 1	31.63	31.96	31.79	8/8
	Run 2	34.21	32.41	33.31	
	Run 3	30.10	30.12	30.11	
	Run 4	30.31	31.12	30.71	
50	Run 1	33.27	32.16	32.71	8/8
	Run 2	33.98	35.53	34.75	
	Run 3	31.99	31.59	31.79	
	Run 4	32.88	32.84	32.86	

Aspergillus brasiliensis

CFU/ml						Mean	
99	Run 1	32.32	35.00	33.22	33.01	33.39	24/24
	Run 2	33.67	33.16	33.58	33.60	33.50	
	Run 3	36.71	33.73	33.64	34.37	34.61	
	Run 4	35.30	36.14	35.24	33.35	35.01	
	Run 5	35.05	33.66	33.10	33.92	33.93	
	Run 6	35.88	34.05	34.86	34.20	34.74	
50	Run 1	35.80	34.68	33.07	33.88	34.36	24/24
	Run 2	34.66	33.43	34.10	34.42	34.15	
	Run 3	35.06	35.01	33.61	34.60	34.57	
	Run 4	33.85	38.19	35.56	34.70	35.57	
	Run 5	35.26	35.39	35.04	35.39	35.27	
	Run 6	35.81	38.35	36.26	34.44	36.21	
25	Run 1	No Cq	39.47	37.41	35.42	37.43	18/24
	Run 2	35.02	No Cq	36.41	35.60	35.67	
	Run 3	No Cq	38.48	36.55	39.44	38.16	
	Run 4	35.45	35.42	No Cq	No Cq	35.44	
	Run 5	34.99	37.80	37.37	39.29	37.36	
	Run 6	38.51	35.66	34.97	No Cq	36.38	
10	Run 1	34.74	36.48	No Cq	35.35	35.52	11/24
	Run 2	37.25	37.67	37.83	No Cq	37.58	
	Run 3	34.69	34.79	No Cq	No Cq	34.74	
	Run 4	37.94	37.83	No Cq	No Cq	37.88	
	Run 5	No Cq	No Cq	No Cq	No Cq	No Cq	
	Run 6	34.79	No Cq	No Cq	No Cq	34.79	

Aspergillus brasiliensis (Microsart® ATMP Sterile Release)

CFU/ml				Mean	
99	Run 1	33.96	33.72	33.84	8/8
	Run 2	32.77	33.83	33.30	
	Run 3	33.34	33.23	33.29	
	Run 4	33.66	34.89	34.28	
50	Run 1	34.67	35.10	34.89	8/8
	Run 2	33.96	33.97	33.97	
	Run 3	33.39	33.69	34.54	
	Run 4	36.14	34.16	35.15	

Candida krusei

CFU/ml						Mean	
99	Run 1	33.69	32.83	33.45	33.39	33.34	8/8
	Run 2	33.25	32.10	31.98	32.37	32.42	
50	Run 1	33.86	35.09	33.84	34.11	34.23	8/8
	Run 2	34.23	34.96	34.20	34.55	34.48	
25	Run 1	34.00	36.83	35.00	34.39	35.06	6/8
	Run 2	No Cq	No Cq	36.73	37.17	36.95	
10	Run 1	No Cq	35.33	36.64	37.25	36.41	4/8
	Run 2	No Cq	37.34	No Cq	No Cq	37.34	

Candida krusei (Microsart® ATMP Sterile Release)

CFU/ml				Mean	
99	Run 1	33.97	34.90	34.44	8/8
	Run 2	32.84	31.81	32.33	
	Run 3	33.70	31.10	32.40	
	Run 4	32.88	33.66	33.27	
50	Run 1	35.03	35.28	35.16	8/8
	Run 2	34.14	33.46	33.80	
	Run 3	34.52	32.54	33.53	
	Run 4	35.15	34.84	35.00	

Candida tropicalis

CFU/ml						Mean	
99	Run 1	31.85	32.10	31.65	31.75	31.84	8/8
	Run 2	30.35	31.85	31.15	31.04	31.10	
50	Run 1	33.10	32.71	34.66	34.00	33.62	8/8
	Run 2	33.06	30.79	32.32	32.03	32.05	
25	Run 1	32.87	38.61	34.39	37.15	35.76	8/8
	Run 2	35.42	31.83	35.96	34.93	34.53	
10	Run 1	38.85	34.83	36.28	35.48	36.36	8/8
	Run 2	34.63	33.96	33.66	36.28	34.63	
5	Run 1	37.32	38.69	39.83	No Cq	38.61	5/8
	Run 2	36.43	No Cq	No Cq	37.29	36.86	

Candida glabrata

CFU/ml						Mean	
99	Run 1	33.54	33.34	37.72	35.42	35.01	8/8
	Run 2	33.12	33.71	33.21	34.21	33.56	
50	Run 1	34.32	35.22	37.22	33.22	35.00	8/8
	Run 2	34.43	35.26	34.35	34.62	34.66	
25	Run 1	37.43	35.74	38.34	39.70	37.80	8/8
	Run 2	36.45	36.03	38.26	34.65	36.35	
10	Run 1	39.12	No Cq	No Cq	No cq	39.12	4/8
	Run 2	38.48	No Cq	36.43	38.12	37.68	

Aspergillus fumigatus

CFU/ml						Mean	
99	Run 1	34.10	36.68	33.19	36.14	35.03	8/8
	Run 2	34.75	34.50	33.77	32.24	33.81	
50	Run 1	No Cq	35.30	No Cq	37.50	36.40	6/8
	Run 2	34.48	36.19	38.75	34.79	36.05	
25	Run 1	38.19	No Cq	No Cq	No Cq	38.19	4/8
	Run 2	36.73	38.07	No Cq	38.45	37.75	

Penicillium chrysogenum

CFU/ml						Mean	
99	Run 1	34.16	34.36	33.05	31.89	33.37	8/8
	Run 2	32.33	34.01	37.24	34.32	34.47	
50	Run 1	33.56	33.55	34.55	38.78	35.11	7/8
	Run 2	34.70	36.23	32.18	No Cq	34.37	
25	Run 1	No Cq	38.59	37.33	37.59	37.84	6/8
	Run 2	36.85	37.57	No Cq	39.27	37.90	
10	Run 1	33.61	No Cq	37.76	No Cq	35.69	2/8
	Run 2	No Cq	No Cq	No Cq	No Cq	No Cq	

LOD_{95} summary

Species	LOD_{95}
Table 13	
Candida albicans	50 CFU/ml
Aspergillus brasilliensis	50 CFU/ml
Table 14	
Candida tropicalis	10 CFU/ml
Candida glabrata	25 CFU/ml
Candida krusei	50 CFU/ml
Aspergillus fumigatus	99 CFU/ml
Penicillium chrysogenum	99 CFU/ml

7.2 Specificity

Specificity testing was comprehensively covered during sensitivity and robustness testing. During evaluation of sensitivity, 7 different fungal species were tested in order to determine the limit of detection when spiked into a complex sample matrix (DMEM + 5 % FBS).To extend our analysis, an *in silico* study was performed in order to predict the detectability of the most common fungi contaminations that could occur during the processing of ATMP products, based on the most recent references about this topic.

7.2.1 Sequence Alignment

Procedure	Acceptance Criterion	Results
Comparison of all primer sequences with a genomic database of fungal species that might contaminate pharmaceutical products. This provides additional *in silico* information about the putative power of detection of the kit.	Fungal species showing ≤ 2 nucleotides mismatch each primer in the alignment with the 18S rRNA genome are considered specifically detectable.	A vast majority of the listed species are detectable based on sequence alignement.

Genus	Reference	Coverage
Alternaria	2,3	97.7 %
Aspergillus	1	95.3 %=
Aureobasidium	6	93.5 %
Bipolaris	2	98 %
Candida	1	86.3 %
Chaetomium	6	3.6%
Cladosporium	2,3	95.5 %
Curvularia	2	100 %
Epidermophyton	4	100 %
Exserohilum	2	97.4 %
Fusarium	2, 3, 6	95.9 %
Memnoniella (Stachybotrys)	6	86,7 %
Microsporum	4	100 %
Myrothecium	6	100 %
Paecilomyces	6	100%
Penicillium	2, 3, 5, 6	98.2 %
*Malassezia**	2	0.1 %
Rhizopus	6	4 %
Scopulariopsis	6	0 %
Trichoderma	6	98 %
Trichophyton	4	100 %

*This genus is normally part of the skin microbiota, but might be involved in skin disorders in patients under parenteral nutrition, immunocompromised patients with increased length of stay in intensive care, very low-birth-weight and premature infants (Source: 7;8)

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7.2.2 Fungi Detection Range

Link	Procedure	Acceptance Criterion	Results
See Table 12; 1 and 2 See Table 13; 3 to 7 See Table 14; 8 to 9	All fungi listed in Table 12, 13 and 14 were tested at concentrations between 0-99 CFU/ml (spiked into DMEM + 5 % FBS) in 3; 4 or 8 replicates	99 CFU/ml must be tested positive for all species.	Passed

	Average C_q value at 99 CFU/ml	% detected at 99 CFU/ml
Tested with ATMP Fungi		
Candida albicans	32.53	100
Aspergillus brasilliensis	34.20	100
Candida krusei	32.88	100
Candida tropicalis	31.47	100
Candida glabrata	34.28	100
Aspergillus fumigatus	34.42	100
Penicillium chrysogenum	33.92	100

Tested with Microsart® ATMP Sterile Release	**Average C_q value at 99 CFU/ml**	**% detected at 99 CFU/ml**
Candida albicans	31.48	100
Aspergillus brasiliensis	33.68	100
Candida krusei	33.11	100

7.2.3 Sample Matrix effects / Cross reactivity

Link	Procedure	Acceptance Criterion	Results
See Table 4 See Table 17, 1 (NEC)	All cell samples listed in Table 4 were tested in duplicates without fungal spike (NEC during robustness testing)	All samples shall show a negative result.	Passed

			Results
Hela	No Cq	No Cq	0/8
	No Cq	No Cq	
	No Cq	No Cq	
	No Cq	No Cq	
Vero	No Cq	No Cq	0/8
	No Cq	No Cq	
	No Cq	No Cq	
	No Cq	No Cq	
CHO-K1	No Cq	No Cq	0/8
	No Cq	No Cq	
	No Cq	No Cq	
	No Cq	No Cq	
HPBMC	No Cq	No Cq	0/8
	No Cq	No Cq	
	No Cq	No Cq	
	No Cq	No Cq	
Jurkat	No Cq	No Cq	0/8
	No Cq	No Cq	
	No Cq	No Cq	
	No Cq	No Cq	

7.2.4 Specificity of PCR with genomic DNA

Link	Procedure	Acceptance Criterion	Results
See Table 15; 1 and 2	All fungi listed in Table 15 were tested with 10 and 0 GC (spiked directly into PCR). All bacteria listed in Table 15 were tested with 10^5 and 0 GC (spiked directly into PCR).	All fungi species must be detected at 10 GC/PCR. No Bacteria Species shall be detected.	Passed (one sample out of 16 was positive for B. subtilis. The very low occurrence indicates that this was due to a contamination and not to a cross reactivity and was consequently not sequenced)

	Run 1	Run 2	
0 GC/PCR (NTC)	No C_q	No C_q	0/16
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	

Fungi Species (Sensitivity)	**Run 1**	**Run 2**	
10 GC/PCR *Candida albicans*	29.44	29.28	16/16
	29.36	29.34	
	29.38	29.31	
	29.24	29.29	
	29.39	29.31	
	29.21	29.39	
	29.64	29.37	
	29.20	29.28	
10 GC/PCR *Candida guilliermondii*	34.42	34.23	16/16
	35.32	34.20	
	34.84	35.11	
	34.37	34.60	
	33.91	34.14	
	34.30	34.60	
	34.69	34.91	
	34.55	34.62	
10 GC/PCR *Candida haemulonii*	29.88	29.95	16/16
	29.60	29.63	
	29.83	29.79	
	30.01	30.20	
	30.05	29.87	
	29.96	30.07	
	29.79	30.00	
	29.56	29.63	

Bacteria species (Cross reactivity)	**Run 1**	**Run 2**	
10^5 GC/PCR *Bacillus subtilis*	No C_q	No C_q	1/16
	38.11	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
10^5 GC/PCR *Pseudomonas aeruginosa*	No C_q	No C_q	0/16
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
10^5 GC/PCR *Legionella pneumophila*	No C_q	No C_q	0/16
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	
	No C_q	No C_q	

7.2.5 Comparison of Microsart® ATMP Fungi with the compendial culture method

Link	Procedure	Acceptance Criterion	Results
See Table 16	For each species listed in Table 16, a dilution series was performed. 1 ml was extracted with Microsart® ATMP Extraction and analyzed with Microsart® ATMP Fungi. In parallel, 1 ml of the species listed in Table 16 were tested with the compendial culture method (direct inoculation) by an external lab.	Microsart® ATMP Fungi shall show equal or higher sensitivity than the culture method. All samples containing spikes at concentrations of 2x LOD_{95} and LOD_{95} shall show a positive result with Microsart® ATMP Fungi.	Passed

	Microsart® ATMP Fungi			**Compendial culture method (External)**		
	2x LOD_{95}	LOD_{95}	$LOD_{95}/2$	2x LOD_{95}	LOD_{95}	$LOD_{95}/2$
Candida albicans	32.25 31.94	32.27 32.12	33.96 32.96	*2/2 Positive* *C. albicans*	*2/2 Positive* *C. albicans*	*0/2 Positive*
Aspergillus brasiliensis	34.38 32.40	37.06 33.17	34.94 34.20	*2/2 Positive* *A. brasisliensis*	*2/2 Positive* *A. brasisliensis*	*2/2 Positive* *A. brasisliensis*

7.3 Robustness

Six samples containing different cell lines in a relevant cell density and in typical cell culture media and buffer were spiked with 99 CFU of one of the fungal species detected with the highest LOD_{95} value during LOD determination (selected from the two fungal species listed in EP 2.6.1 and EP 2.6.27, available as EZ-CFU). Additionally, those two species were tested at three concentrations around their LOD_{95} in comparison with the culture method described in EP 2.6.1.

7.3.1 Cell culture samples spiked with fungi

Link	Procedure	Acceptance Criterion	Results
See Table 17; 1	Five samples containing different cell lines in a relevant cell density and in typical cell culture media and buffer were spiked with 99 CFU/ml of the fungi which was detected with the highest LOD_{95} value during LOD_{95} determination (selected from the two fungi mentioned in the EP 2.6.1 and EP 2.6.27).	All spiked samples shall show a positive result.	Passed

	***Aspergillus brasiliensis* 99 CFU (FAM™)**					
	10^6 cells/ml		***Positive***	***10^5 cells/ml***		***Positive***
Hela	37.54 35.24	31.26 31.84	4/4	33.72 33.34	32.10 32.07	4/4
Vero	33.30 34.45	31.43 32.76	4/4	32.78 33.48	31.39 31.60	4/4
CHO-K1	33.65 34.70	32.40 32.83	4/4	34.49 33.89	33.36 32.40	4/4
HPBMC	32.21 32.39	31.59 31.25	4/4	33.80 33.79	32.12 31.04	4/4
Jurkat	34.99 34.80	34.14 33.50	4/4	31.77 34.30	32.64 33.81	4/4

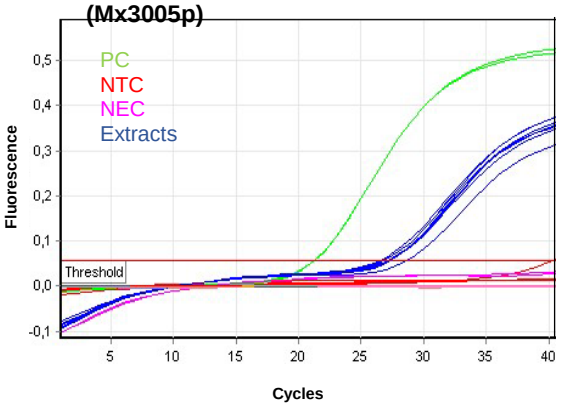
7.3.2 Device compatibility

Link	Procedure	Acceptance Criterion	Results
See Table 17; 2	As the test can basically be performed with any qPCR cycler capable of interpreting FAM™ and ROX™ signals, performance of the test with these machines needs to be validated. As not all qPCR cyclers commercially available are accessible for validation, the following five devices representing block and air heating systems are tested: Rotor-Gene 6000, CFX96 touch, Mx3005P, QuantStudio5 and ABI Prism 7500. The robustness of the method was demonstrated by spiking DMEM + 5 % FBS with 99 CFU/ml of the fungi with the highest LOD_{95} value. At least eight replicates shall be tested on each instrument.	All samples shall show a positive result.	Passed

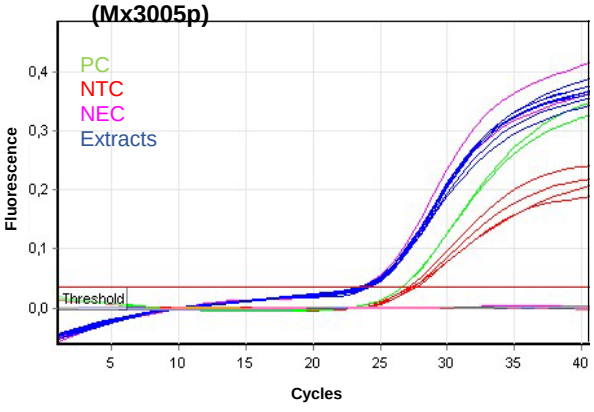
Rotor-Gene 6000 (Microsart® ATMP Fungi)

Extract	C_q (FAM™)		C_q (ROX™)		Result
	Run 1	Run 2	Run 1	Run 2	
Positive Control	21.21	22.20	27.62	27.30	Correct
Positive Control	21.30	22.16	27.14	27.37	Correct
NTC	No Cq	No Cq	27.36	27.47	Correct
NTC	No Cq	No Cq	27.49	27.36	Correct
NEC	No Cq	No Cq	26.61	26.48	Correct
NEC	No Cq	No Cq	26.68	26.19	Correct
Candida albicans	28.70	35.87	23.42	26.16	Positive
	27.14	33.45	23.49	26.29	Positive
	27.16	34.66	24.20	26.01	Positive
	26.84	36.73	23.39	26.00	Positive
	27.27	34.01	23.41	26.26	Positive
	26.54	33.63	23.70	26.40	Positive
	27.39	34.79	24.03	26.10	Positive
	26.90	33.31	23.93	26.10	Positive

***Candida albicans* 99 CFU Extracts (FAM™) (Mx3005p)**



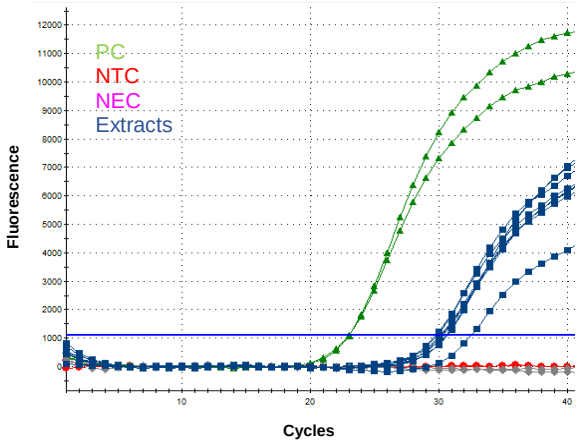
***Candida albicans* 99 CFU Extracts (ROX™) (Mx3005p)**



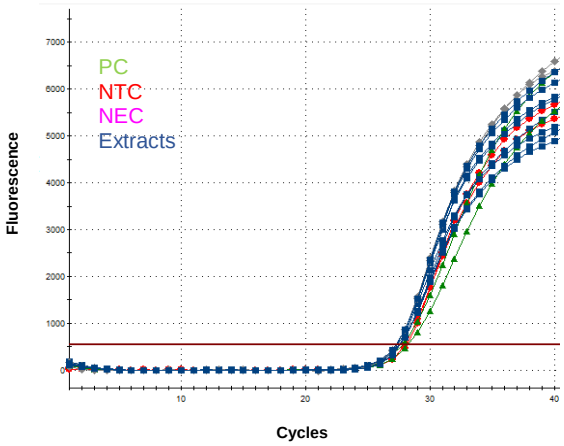
Mx3005p (Microsart® ATMP Fungi)

Extract	C_q (FAM™)		C_q (ROX™)		Result
	Run 1	Run 2	Run 1	Run 2	
Positive Control	23.06	22.93	28.19	27.54	Correct
Positive Control	23.01	23.25	27.47	27.82	Correct
NTC	No Cq	No Cq	28.03	28.56	Correct
NTC	No Cq	No Cq	27.38	28.70	Correct
NEC	No Cq	No Cq	27.84	27.56	Correct
NEC	No Cq	No Cq	27.39	27.57	Correct
Candida albicans	29.90	32.71	28.31	27.48	Positive
	29.80	32.36	27.61	27.43	Positive
	30.24	32.87	27.37	27.89	Positive
	30.15	33.69	27.67	27.66	Positive
	30.40	34.19	27.40	27.53	Positive
	30.72	32.65	27.36	27.77	Positive
	32.62	33.05	27.71	27.52	Positive
	30.62	33.71	27.34	27.53	Positive

***Candida albicans* 99 CFU Extracts (FAM™)**



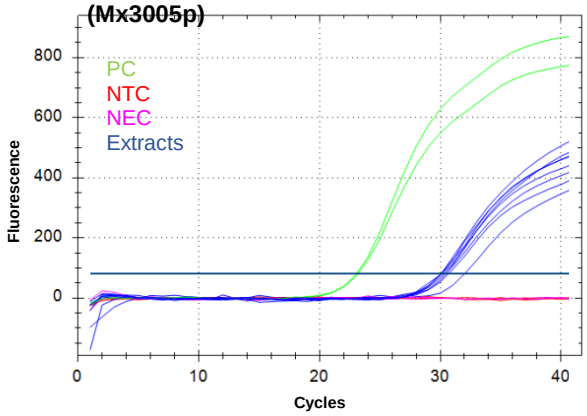
***Candida albicans* 99 CFU Extracts (ROX™)**



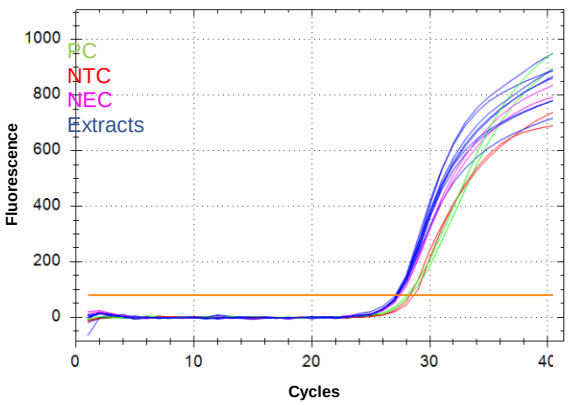
CFX96 (Microsart® ATMP Fungi)

Extract	C_q (FAM™)		C_q (ROX™)		Result
	Run 1	Run 2	Run 1	Run 2	
Positive Control	23.04	23.44	28.09	28.17	Correct
Positive Control	23.15	22.99	28.29	27.45	Correct
NTC	No Cq	No Cq	28.29	28.85	Correct
NTC	No Cq	No Cq	28.64	28.76	Correct
NEC	No Cq	No Cq	27.43	27.53	Correct
NEC	No Cq	No Cq	27.45	27.80	Correct
Candida albicans	30.09	33.78	27.21	27.62	Positive
	30.03	32.30	27.30	27.97	Positive
	30.30	33.36	27.22	28.15	Positive
	30.06	32.38	27.26	27.81	Positive
	30.46	33.16	27.30	27.75	Positive
	30.65	33.06	27.15	27.94	Positive
	31.99	32.39	27.21	28.05	Positive
	30.05	33.38	27.07	27.92	Positive

***Candida albicans* 99 CFU Extracts (FAM™) (Mx3005p)**



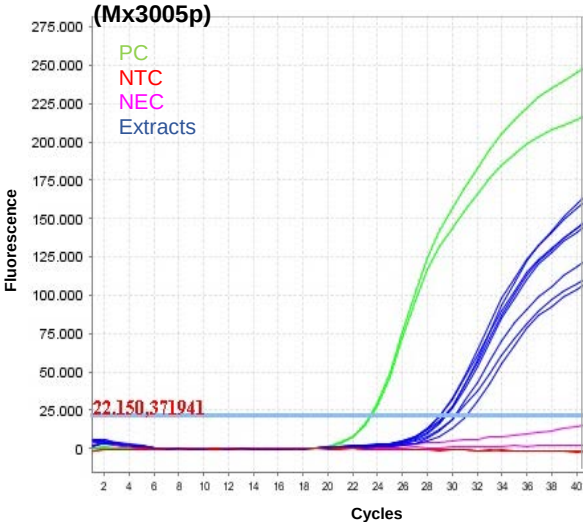
***Candida albicans* 99 CFU Extracts (ROX™)**



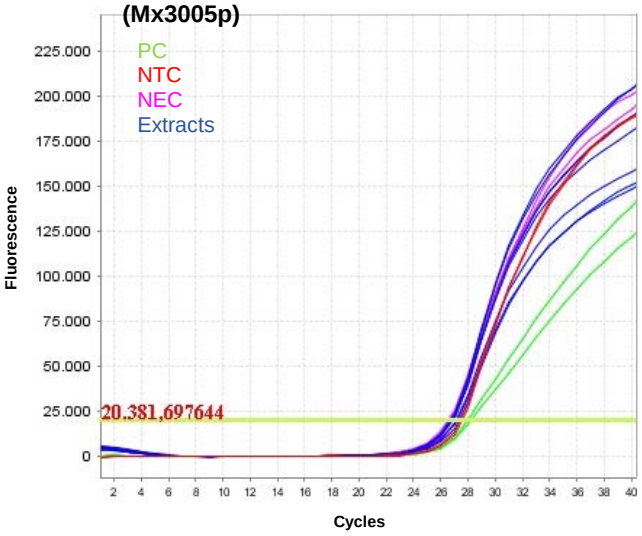
ABI Prism (Microsart® ATMP Fungi)

Extract	C_q (FAM™)		C_q (ROX™)		Result
	Run 1	Run 2	Run 1	Run 2	
Positive Control	23.52	23.39	28.02	27.57	Correct
Positive Control	23.60	23.11	28.33	27.41	Correct
NTC	No Cq	No Cq	27.38	27.37	Correct
NTC	No Cq	No Cq	27.54	27.51	Correct
NEC	No Cq	No Cq	26.86	25.64	Correct
NEC	No Cq	No Cq	26.59	26.24	Correct
Candida albicans	29.51	31.40	26.78	26.14	Positive
	31.17	32.79	27.08	26.20	Positive
	29.61	33.57	26.87	26.41	Positive
	30.25	33.05	27.32	26.07	Positive
	29.11	31.04	26.74	25.76	Positive
	30.37	32.03	27.13	25.64	Positive
	29.11	32.84	26.81	25.99	Positive
	29.44	31.74	26.87	25.36	Positive

***Candida albicans* 99 CFU Extracts (FAM™) (Mx3005p)**



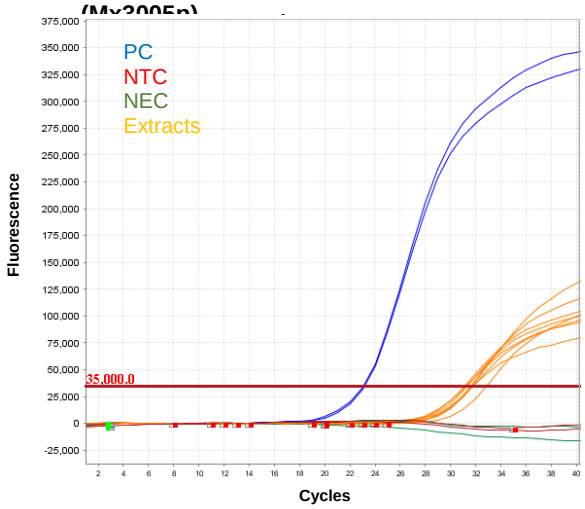
***Candida albicans* 99 CFU Extracts (ROX™) (Mx3005p)**



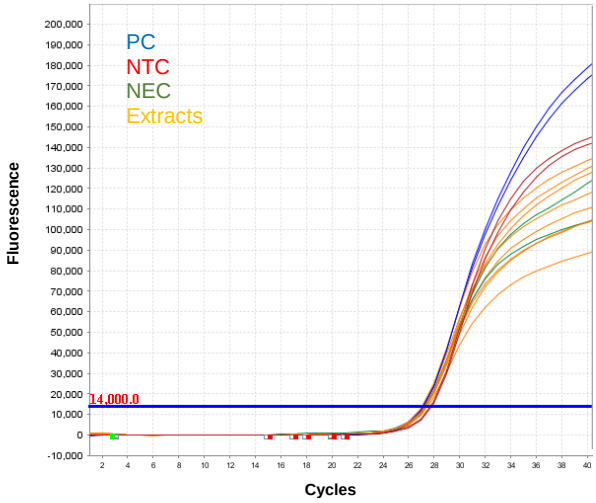
Quant Studio 5 (Microsart® ATMP Fungi)

Extract	C_q (FAM™)		C_q (ROX™)		Result
	Run 1	Run 2	Run 1	Run 2	
Positive Control	23.03	22.98	27.16	27.89	Correct
Positive Control	23.12	23.05	27.14	27.79	Correct
NTC	No Cq	No Cq	27.84	27.47	Correct
NTC	No Cq	No Cq	27.76	27.56	Correct
NEC	No Cq	No Cq	27.20	28.67	Correct
NEC	No Cq	No Cq	27.29	28.64	Correct
Candida albicans	31.73	33.31	27.39	27.54	Positive
	32.93	33.08	27.31	27.53	Positive
	31.64	34.28	27.04	27.90	Positive
	31.82	33.75	27.30	27.69	Positive
	31.35	35.12	27.15	27.78	Positive
	31.85	32.68	27.32	27.50	Positive
	31.16	32.74	27.38	27.50	Positive
	31.26	33.19	27.49	26.93	Positive

***Candida albicans* 99 CFU Extracts (FAM™)**



***Candida albicans* 99 CFU Extracts (ROX™)**



7.3.2 Detection of Free-DNA

Link	Procedure	Acceptance Criterion	Results
See Table 17; 3 and 4	In order to estimate the risk of false positive, water with and without cell suspension was spiked with 10^4; 10^3; 10^2 or 10 GC/ml *Candida albicans*. The samples were divided in eight aliquots, extracted with Microsart® ATMP Extraction and tested with Microsart® ATMP Fungi.	n.a	Without cell lines in the sample, 0% of the free DNA is eliminated during the extraction process. With cell lines in the sample, more than 99% of the free DNA is eliminated during the extraction process.

	10^4 GC/ml		10^3 GC/ml		10^2 GC/ml		10 GC/ml		0 GC/ml	
	Run 1	**Run 2**	**Run 1**	**Run 2**	**Run 1**	**Run 2**	**Run 1**	**Run 2**	**Run 1**	**Run 2**
Extracts without cells	32.40	32.46	34.79	36.21	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq
	31.69	31.89	34.86	39.07	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq
	32.87	32.29	39.69	36.18	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq
	32.05	32.04	34.13	37.44	39.87	No Cq	No Cq	No Cq	No Cq	No Cq
	36.30	34.16	39.69	36.70	36.96	No Cq	No Cq	No Cq	No Cq	No Cq
	34.99	32.10	38.60	36.50	37.57	No Cq	No Cq	No Cq	No Cq	No Cq
	35.17	34.43	No Cq	38.60	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq
	34.68	32.45	37.49	35.77	39.26	No Cq	No Cq	No Cq	No Cq	No Cq
	10^4 GC/ml = 10^2 GC/PCR		**10^3 GC/ml = 10 GC/PCR**		**10^2 GC/ml = 1 GC/PCR**		**10 GC/ml = 0,1 GC/PCR**		**0 GC/PCR**	
	Run 1	**Run 2**	**Run 1**	**Run 2**	**Run 1**	**Run 2**	**Run 1**	**Run 2**	**Run 1**	**Run 2**
Direct PCR without cells	27.76	28.01	32.11	35.36	36.08	No Cq	No Cq	No Cq	No Cq	No Cq
	27.92	27.77	32.43	34.17	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq

After extraction of 10 GC/ml, 10 µl extract contain less than 1GC.
No free DNA was washed away during the extraction process.

	10^4 GC/ml		10^3 GC/ml		10^2 GC/ml		10 GC/ml		0 GC/ml	
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
Extracts with cells	31.06	29.92	35.95	34.98	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq
	31.34	29.51	37.05	35.02	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq
	31.39	30.98	38.32	32.90	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq
	31.30	30.28	36.81	36.44	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq
	31.75	30.15	40.28	35.61	No Cq	37.36	No Cq	No Cq	No Cq	No Cq
	31.01	30.88	36.57	37.49	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq
	31.03	30.42	36.73	35.85	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq
	31.21	30.58	36.42	34.09	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq
	10^4 GC/ml = 10^2 GC/PCR		10^3 GC/ml = 10 GC/PCR		10^2 GC/ml = 1 GC/PCR		10 GC/ml = 0,1 GC/PCR		0 GC/PCR	
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
Direct PCR without cells	26.42	26.01	32.27	30.38	37.28	33.94	No Cq	No Cq	No Cq	No Cq
	26.22	25.94	32.49	30.94	38.14	35.45	No Cq	No Cq	No Cq	No Cq

After extraction 100 GC/ml, 10 µl extract contain less than 1GC.
Thus, more than 90% free DNA was washed away during the extraction process.

7.3.3 False positive rate

Link	Procedure	Acceptance Criterion	Results
See Table 2 See Table 17; 5	Cell culture Media without spike were extracted with Microsart® ATMP Extraction and tested with Microsart® ATMP Fungi. 12 independent experiments were performed for DMEM + 5%FBS, each with eight replicates. For other culture media, one experiment was performed, with eight replicates.	n.a	All the run conducted with Microsart® ATMP Extraction and Microsart® ATMP Fungi were valid indicating that none of those media affect the performance of the kit.

	Negative Results	% negative results
DMEM + 5% FBS	8/8	100%
	8/8	
	8/8	
	8/8	
	8/8	
	8/8	
	8/8	
	8/8	
	8/8	
	8/8	
	8/8	
	8/8	

	Negative Results	ROX™	% negative results
DMEM	8/8	8/8 Correct	100%
Fetal bovine serum (FBS)	8/8	8/8 Correct	100%
DMEM high Glucose, GlutaMAX	8/8	8/8 Correct	100%
DMEM w/o Na-Pyruvat mit stable Glutamin	8/8	8/8 Correct	100%
RPMI 1640	8/8	8/8 Correct	100%
MEM (1)	8/8	8/8 Correct	100%
MEM (2)	8/8	8/8 Correct	100%
KnockOut DMEM	8/8	8/8 Correct	100%
DMEM/F-12 GlutaMAX	8/8	8/8 Correct	100%
Opti-MEM Reduced Serum GlutaMAX	8/8	8/8 Correct	100%
McCoys 5A Medium	8/8	8/8 Correct	100%
Leibovitz L-15 Medium	8/8	8/8 Correct	100%
Chrondrocyte Differentiation Medium	8/8	8/8 Correct	100%
Human Osteoblast Differentiation Medium	8/8	8/8 Correct	100%
ChondroMAX Differentiation Medium	8/8	8/8 Correct	100%
Tahara Lympho One + HS	8/8	8/8 Correct	100%
RPMI, penicillin/Streptomycin, Glutamax, 5% FBS	8/8	8/8 Correct	100%

8 Conclusion

Microsart® ATMP Fungi and the Fungi SR mix from Microsart® ATMP Sterile Release were intensively validated in compliance with the designed validation protocol. The validation protocol reflects the method itself and variations expected by the diversity of samples from different customers during QC testing in the manufacturing process of ATMP samples. Microsart® ATMP Fungi should be applied for fungi detection for any kind of sample material occurring in the manufacturing process of ATMP.

Microsart® ATMP Fungi and its derivate product Microsart® ATMP Sterile Release were designed as state-of-the-art new product for the detection of fungi contaminations in ATMP products. The product validation has been conducted according to the European Pharmacopoeia 2.6.1 and 2.6.27 regulations for sterility testing and microbiological control of cellular products such as ATMP.

Specificity of the tested fungi revealed a detection limit (LOD_{95}) comprised between 10 and 99 CFU/ml and a great sensitivity of the PCR system, able to detect as little as 10 GC/PCR for various species.
In silico sequence alignments analysis demonstrated that Microsart® ATMP Fungi and Microsart® ATMP Sterile Release can detect > 37 % of fungi. This feature greatly increase the chances to detect any kind of fungal contamination, including some species difficult to detect with the classical culture method. A direct comparison with the culture method recommended by EP 2.6.1 was performed and showed 100% of similarities between the results obtained through both methods.

As robustness is a key issue in evaluating the characteristics of a product, the kit has been validated with various relevant samples, with different cyclers and by different experimenter in different laboratory environments. All collected results are consistent and confirm the accuracy and reproducibility of Microsart® ATMP Fungi and of Microsart® ATMP Sterile Release in those diverse surroundings for the detection of fungal DNA.

It was shown that the product Microsart® ATMP Fungi and Microsart® ATMP Sterile Release offer an appropriate replacement solution to the culture method described in EP 2.6.1 and EP 2.6.27 and can be safely used to detect the presence of fungal contamination in cell culture derived samples including ATMPs.

9 Reference Documents

1. European Pharmacopoeia 8th edition, Strasbourg, FR; European Directorate for the Quality of Medicines; 2014, 5.1.6
2. European Pharmacopoeia 8th edition, Strasbourg, FR; European Directorate for the Quality of Medicines; 2014; 2.6.21 Nucleic Acid Amplification Techniques
3. US Pharmacopoeial Convention (USP). USP 38/NF 33 <1223>, 2015

10 Appendix

Labor LS

Bericht über Resultate der Prüfung auf Sterilität			Version: 01
Titel:	**Bericht zur Prüfung auf Sterilität an "Cell culture medium samples"**		
LS-Nr.:	191017-0115-001 bis 016		
Probe/Produkt:	Cell culture medium sample 16 Gebinde mit jeweils 1 ml		
Standort:	Labor LS 97708 Bad Bocklet – Großenbrach Deutschland		
Kunde/Sponsor:	Sartorius Stedim Biotech GmbH August-Spindler-Straße 11 37079 Göttingen Deutschland		
Beginn der Untersuchungen:	17.10.19	Ende der Untersuchung:	04.11.19
Auftrag vom:	17.10.19	Probeneingang am:	17.10.19

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Die Prüfergebnisse beziehen sich ausschließlich auf die angegebene Probe.
Vervielfältigungen sind auszugsweise nur mit schriftlicher Genehmigung durch die Labor LS gestattet.

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1. Prüfungsdurchführung

1.1 Prüfung auf Sterilität

Die Prüfungen wurden am 17. Oktober 2019 mittels Direktbeschickung unter einer qualifizierten Sicherheitswerkbank (LS SWB 024) durchgeführt.

Hierbei wurde jeweils der Gesamtinhalt pro Gebinde aseptisch in einen 400 ml Kolben Nährmedium überführt. Gemäß Kundenvorgaben wurden die Proben mit der Bezeichnung „a" in 400 ml Thioglykolat-Bouillon mit Enthemmerzusatz (Charge 66-0909190952) überführt. Die Proben mit der Bezeichnung „b" wurden in 400 ml Caso-Bouillon (Charge 19-0909190952) mit Enthemmerzusatz überführt.

1.2 Inkubation

Nach der Prüfungsdurchführung wurden die Testansätze bei 22,5 ± 2,5 °C (Caso-Bouillon, KL 018) bzw. 32,5 ± 2,5 °C (Thioglykolat-Bouillon, KL 017) in qualifizierten Klimakammer mit kontinuierlicher Temperaturaufzeichnung für mind. 14 Tage inkubiert.

1.3 Auswertung

Alle Testansätze wurden arbeitstäglich auf makroskopisch sichtbares Wachstum überprüft. Sobald Trübung bzw. makroskopisch sichtbares Wachstum detektiert wurde, wurde die Inkubation unterbrochen. Je ein Aliquot des betroffenen Flüssignährmediums wurde auf 2 Platten Columbia-Blut-Agar und einer SDA-(Sabouraud-Dextrose-Agar)-Platte ausplattiert. Je eine Blut-Agar-Platte wurde aerob, die zweite anaerob bei 30 - 35°C inkubiert. Die SDA-Platte bei 28,5 – 31,5°C. Nachdem Keimwachstum auf den Platten nachweisbar war, wurde mittels VITEK®MS bzw. mikroskopisch (bei Schimmelpilzen) identifiziert.

Die Nährmedienansätze, bei welchen kein makroskopisch sichtbares Wachstum erkennbar war, wurden bis zum Ende der 14-tägigen Inkubationszeit inkubiert. Die Endauswertung erfolgte im 4-Augen-Prinzip.

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2. Ergebnisse

Alle Ergebnisse der visuellen Auswertung sowie der Identifizierung sind der folgenden Tabelle zu entnehmen:

Tabelle 1: Übersicht Ergebnisse

LS-Nr.:	Bezeichnung	Makroskopisch sichtbares Wachstum am Tag	Ergebnis [1]	Nährmedien	Ergebnis Identifizierung
191017-0115-001	1 a	-	negativ	Thio +	-
191017-0115-002	2 a	7	negativ [2]	Thio +	-
191017-0115-002	3 a	7	positiv	Thio +	*Candida albicans*
191017-0115-004	4 a	7	positiv	Thio +	*Candida albicans*
191017-0115-005	5 a	7	negativ [2]	Thio +	-
191017-0115-006	6 a	8	positiv	Thio +	*Aspergillus brasiliensis*
191017-0115-007	7 a	7	positiv	Thio +	*Aspergillus brasiliensis*
191017-0115-008	8 a	7	positiv	Thio +	*Aspergillus brasiliensis*
191017-0115-009	1 b	-	negativ	Caso +	-
191017-0115-010	2 b	-	negativ	Caso +	-
191017-0115-011	3 b	7	positiv	Caso +	*Candida albicans*
191017-0115-012	4 b	7	positiv	Caso +	*Candida albicans*
191017-0115-013	5 b	-	negativ	Caso +	-
191017-0115-014	6 b	11	positiv	Caso +	*Aspergillus brasiliensis*
191017-0115-015	7 b	7	positiv	Caso +	*Aspergillus brasiliensis*
191017-0115-016	8 b	7	positiv	Caso +	*Aspergillus brasiliensis*

[1] negativ: kein Keimwachstum, positiv: Keimwachstum
[2] Testabbruch nach 7 Tagen. Anschließend kein Keimwachstum nachweisbar (siehe Abweichung A6886)

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Die Zuordnung der jeweiligen LS-Nummer der Sterilitätsprüfung zur entsprechenden LS-Nummer der Keimidentifizierung ist in folgender Tabelle zusammengefasst:

Tabelle 2: Zuordnung LS-Nummer Sterilitätsprüfung und Keimidentifizierung

LS-Nr. Prüfung auf Sterilität	LS-Nr. Identifizierung
191017-0115-002	191017-0115-017
191017-0115-003	191017-0115-018
191017-0115-004	191017-0115-019
191017-0115-005	191017-0115-020
191017-0115-006	191017-0115-027
191017-0115-007	191017-0115-021
191017-0115-008	191017-0115-022
191017-0115-011	191017-0115-023
191017-0115-012	191017-0115-024
191017-0115-014	191017-0115-028
191017-0115-015	191017-0115-025
191017-0115-016	191017-0115-026

3. Zusammenfassung und Bewertung

In 10 der 16 Prüfmuster wurde mikrobiologisches Wachstum detektiert. 4 Prüfansätze zeigten kein makroskopisch sichtbares Wachstum und entsprechen nach 14 Tagen Inkubation den Akzeptanzkriterien des Steriltests gemäß Ph. Eur., Kapitel 2.6.1.

Bei 2 Prüfansätzen wurde nach 7 Tagen makroskopisch sichtbare Veränderung des Mediums detektiert. Nach Anlegen von Subkulturen auf festem Nährmedium war kein Keimwachstum detektierbar. Eine Abweichungsmeldung wurde gestartet (Vorgangsnummer A6888); im Zuge der Ursachenanalyse wurden Sequenzierungen zum Nachweis bakterieller DNA bzw. der DNA von Hefen oder Schimmelpilzen eingeleitet. In keinem Versuch war der Nachweis von DNA möglich. Die beiden Testansätze (LS-Nr. 191017-0115-002 und 191017-0115-005) zeigten bei wiederholter visueller Prüfung kein makroskopisch sichtbares Wachstum.

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Unterschriften zur Autorisierung			
erstellt:	Labor LS	Katharina Schlereth (Abteilungsleitung)	02. DEZ. 2019 (Datum/Unterschrift)
geprüft/ genehmigt:	Labor LS	Stefan Gärtner (Abteilungsleitung)	03. DEZ. 2019 (Datum/Unterschrift)

Ende des Dokuments