Product Validation Report

Evaluation of the bacteria detection capability of Microsart® ATMP Bacteria and Microsart® ATMP Sterile Release

Product Identifier	
Version number	1
Release date	25.09.2018 (ATMP Bacteria) 06.12.2019 (ATMP Sterile Release)
Document Identifier	
Version number	2
ID#	VA06.01EN
Release date	06.12.2019
Changes from last version	• ATMP Bacteria Extraction renamed to ATMP Extraction • ATMP Sterile Release includes the former ATMP Bacteria Patient with the addition of a PCR Kit the detection of fungal DNA • Minor revisions of text and data
Distribution	QM, R&D, Marketing

Approval			
Test Facility 1	Minerva Biolabs GmbH, Schkopauer Ring 12681 Berlin, Germany		
Written by:	Dr. Denis Ciato Head of Product Development	Date:	07.02.2020
Reviewed by:	Matthias Hornschuh Scientist	Date:	07.02.2020
Reviewed by:	Dr. Dirk Vollenbroich Managing Director	Date:	07.02.2020
Test Facility 2	Sartorius Stedim Biotech GmbH, August-Spindler-Straße 11, 37079 Goettingen, Germany		
Reviewed by:	Dr. Diana Patzelt/Dr. Alexandra Mueller-Scholz Scientist Applications for Molecular Biology	Date:	24.02.2020
Reviewed by:	Kai Nesemann Product Manger	Date:	24.02.2020
Reviewed by:	Gerhard Haake/Anna Vreemann Quality Assurance Microbiology	Date:	2020-02-24

Table of Contents

1 Introduction

Gene therapy, somatic cell therapy and tissue engineered 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. Furthermore some cases of microbial contamination have been reported for “aseptic” procurement, representing a relevant contamination burden for the process [1]. These facts explain the high demand for growth-independent rapid ATMP testing. In order to enhance sensitivity and duration of bacterial detection control tests, a more rapid method using real time PCR has been developed by the cooperation partners Minerva Biolabs and Sartorius Stedim Biotech. In this study, the kits Microsart® ATMP Bacteria and the Bacteria SR Mix from Microsart® ATMP Sterile Release were validated for the detection of bacterial contamination in ATMPs. The detection efficacy and efficiency of fungal DNA of Microsart® ATMP Sterile Release is not included in this report as being part of the Validation Report of Microsart® ATMP Fungi. Microsart® ATMP Bacteria as well as Bacteria SR Mix PCR include a mix of primer, nucleotides and polymerase containing a FAM™ labeled probe specific for a broad range of different bacterial 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 bacteria specific sequence at 520 nm (FAM™ channel).

2 Objective

A study was designed to evaluate the bacterial detection capability for the bacterial control detection Kits Microsart® ATMP Bacteria and the Bacteria 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 [2] and United States Pharmacopoeia (USP) chapter <1223> [3]. This comprehensive validation plan is intended to show suitability of the new products, Microsart® ATMP Bacteria and Microsart® ATMP Sterile Release, for bacterial 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. Furthermore, recommendations of the Paul Ehrlich Institute have been implemented in the validation plan to fulfill the expectations of the German federal institute for vaccines and biomedicines [4] as well as the European Pharmacopoeia chapter 2.6.27 regarding the *Microbiological examination of cell-based preparations* [5]. 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.

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

Sartorius Stedim Biotech GmbH (SSB) was responsible for developing the test protocol in agreement with Minerva Biolabs GmbH (MB). MB 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 bacteria during this study was as follows:

Table 1. Test System Information

System type	Catalogue No.	Supplied by	Storage Conditions	Lot No.
Microsart® ATMP Bacteria (100 reactions)	SMB95-1008	SSB/MB	+2 – +8 °C	9516S1097 9516S2097 9516S1028 9516S2028 9516S2048
Bacteria SR Mix of Microsart® ATMP Sterile Release (Former Microsart® ATMP Bacteria Patient) (10 patients)	SMB95-1007	SSB/MB	+2 – +8 °C	9516S1078
Microsart® ATMP Extraction (Former Microsart® Bacteria Extraction) (50 extractions)	SMB95-2001	SSB/MB	Ambient Temperature	95261077 95261107 95261028

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 12 different bacterial species 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 bacterial species which was detected with the highest LOD_{95} value during LOD_{95} determination (selected from the five EP 2.6.1/EP 2.6.27 bacteria listed Table 13A).
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 Formulation

Product Ingredient	Manufacturer/Source	Catalogue No.	Lot No.	Storage Conditions
DMEM medium	Merck/Biochrom AG	FG 0415	0374E	+2 - +8 °C
DMEM w/o Na-Pyruvat mit stable Glutamin	Merck/Biochrom AG	FG0435	1014F	+2 - +8 °C
DMEM high Glucose, GlutaMAX	Gibco/Thermo Fisher	10566016	1930187	+2 - +8 °C
RPMI 1640	Gibco/Thermo Fisher	A1049101	1941065	+2 - +8 °C
RPMI 1640, GlutaMAX	Gibco/Thermo Fisher	72400013	1912692	+2 - +8 °C
MEM	Sigma Aldrich	M8167-500ml	RNBG1617	+2 - +8 °C
MEM	Sigma Aldrich	M4655-500ml	RNBG5441	+2 - +8 °C
KnockOut DMEM	Gibco/Thermo Fisher	10829018	1904921	+2 - +8 °C
DMEM/F-12 GlutaMAX	Gibco/Thermo Fisher	10565018	1930078	+2 - +8 °C

Product Ingredient	Manufacturer/Source	Catalogue No.	Lot No.	Storage Conditions
Opti-MEM Reduced Serum GlutaMAX	Gibco/Thermo Fisher	51985026	1929010	+2 - +8 °C
McCoys 5A Medium	Gibco/Thermo Fisher	16600082	1897165	+2 - +8 °C
Leibovitz L-15 Medium	Gibco/Thermo Fisher	11415064	1922999	+2 - +8 °C
Chrondrocyte Differentiation Medium	Sigma Aldrich	411D-250	745	+2 - +8 °C
Human Osteoblast Differentiation Medium	Sigma Aldrich	417D-250	715	+2 - +8 °C
Mesencult ACF Basal Medium	Sigma Aldrich	05456	17H83230	+2 - +8 °C
Mesencult ACF Basal Medium + Mesencult ACF Supplement	Sigma Aldrich	05457	16K74902-4	≤ -18 °C
ChondroMAX Differentiation Medium	Sigma Aldrich	SCM123-100ml	SLBT3800	≤ -18 °C
StemPro Osteo/Chond Dif Basal + StemPro Chondrogenesis Supplement	Thermo Fisher	A1006901 A1006401	1941369 1933294	2-8°C ≤ -18 °C
StemPro Osteo/Chond Dif Basal + StemPro Osteogenesis Supplement	Thermo Fisher	A1006601 A1006901	1950435 1941369	2-8°C ≤ -18 °C
Fetal bovine serum (FBS)	Biochrom AG	S0615	0167F	≤ -18 °C

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

For sensitivity, specificity and robustness testing, 18 different microorganisms were used, selected according to recommendations found in literature [Montag et. al, 2009; Paul Ehrlich Institute – Statement, 2 June 2015]. For CFU-based tests, 12 quantified microorganisms with a defined cell count (EZ-CFU), originated from the company “microbiologics” were used. These microorganisms are listed in table 3.

Table 3. Bacterial species tested

Species	Classification	Strain	Supplier	LOT-No.	Catalogue-No.
Species used for CFU-based analysis					
Bacteroides vulgatus	Gram neg.	ATCC 8482	Microbiologics	445-56-2 445-76	0445C
Escherichia coli	Gram neg.	ATCC 8739	Microbiologics	483-555-1	0483C
Pseudomonas aeruginosa,[a,b]	Gram neg.	ATCC 9027	Microbiologics	484-824 484-890-1	0484C
Pseudomonas protegens	Gram neg.	ATCC 17386	Microbiologics	524-40-2	0524C
Streptococcus pyogenes [b]	Gram neg.	ATCC 19615	Microbiologics	385-127-2 385-156-1	0385C
Bacillus cereus	Gram pos.	ATCC 10876	Microbiologics	998-141-1 998-161	0998C
Bacillus subtilis subsp. spizizenii[a,b]	Gram pos.	ATCC 6633	Microbiologics	486-402-1 486-448-2 486-550	0486C
Clostridium sporogenes[a,b]	Gram pos.	ATCC 19404	Microbiologics	317-138-1 317-162-1 317-172-1	0317C
Enterococcus faecalis	Gram pos.	ATCC 29212	Microbiologics	366-249-1	0366C
Kocuria rhizophila	Gram pos.	ATCC 9341	Microbiologics	688-143-1	0688C
Staphylococcus aureus subsp. aureus[a,b]	Gram pos.	ATCC 6538	Microbiologics	485-374-1 485-374-2 485-413	0485C
Staphylococcus epidermidis	Gram pos.	ATCC 12228	Microbiologics	371-154-1 371-234	0371C
Species used for DNA-based analysis					
Bacteroides fragilis	Gram neg.	ATCC 25285	Minerva Biolabs	-	-
Enterobacter cloacae supsp. cloacae	Gram neg.	ATCC 13047	Minerva Biolabs	2110300541065	2110-30054
Klebsiella pneumoniae	Gram neg.	ATCC 13883	Minerva Biolabs	2132301041075	2132-30104
Serratia marcescens	Gram neg.	ATCC 13880	Minerva Biolabs	2117301211065	2117-30121
Clostridium perfringens	Gram pos.	ATCC 13124	Minerva Biolabs	2108007561065	2108-00756
Yersinia enterocolitica	Gram neg.	ATCC 9610	Minerva Biolabs	2140047801075	2140-04780

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

Another 6 microorganisms were tested at DNA-level using lyophilized DNA preparations containing genomic DNA of the particular species (Table 3) provided by MB. 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 late logarithmic phase. Bacteria are harvested by centrifugation at 4000 x g at 4-8°C and genomic DNA is extracted via column purification.

Bacteroides fragilis was ordered as "KWIK Stik" from Doenitz Prolab. 10 ml of TSB were inoculated and incubated under ATCC® described conditions for two days. Genomic DNA was extracted via column purification with subsequent alcohol precipitation.

Extracted DNA is analyzed using standardized methods in the following order: a. initial QC consists of partial sequencing and is accompanied by Nanodrop measurement, b. OD ratios between $OD_{260/280}$ = 1.8-2.0 and $OD_{260/230}$ >1.5 are allowed for further analysis and c. DNA concentration of intact DNA is subsequently determined via Qubit and d. Qubit based concentrations are used for genome copy number determination.

Cells 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 adjustment to 10^6 and 10^5 cells/ml in DMEM containing 5% FCS.

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™	*Homo sapiens*	DMEM, 5% FBS
Vero	Kidney, epithelial, ATCC® CCL-81™	*Cercopithecus aethiops*	DMEM, 5% FBS
CHO-K1	Ovary, epithelial-like, ATCC® CCL-61™	*Cricetulus griseus*	DMEM, 5% FBS
RK13	Kidney, epithelial, ATCC® CCL-37™	*Oryctolagus cuniculus*	DMEM, 5% FBS

5.4 Incoming goods inspection and qualification of EZ-CFU

All EZ-CFU of species listed in Table 6 were tested in an incoming good inspection to confirm the stated cell count. 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 (TSA) were inoculated with 100 µl, incubated for 24 to 48 h and checked for colony formation. The incubation conditions for each microorganism are listed in Table 6.
C. sporogenes and *B. vulgatus* are fastidious anaerobes. Without the use of an anaerobic gas chamber these microorganisms do not grow on agar plates. For this reason a most probable number test have been implemented for those species. Two pellets of EZ-CFU were rehydrated in 2 ml of rehydration buffer and diluted according to their individual cell count in a standard phosphate

buffer, pH 7.2., 25 ml fluid thioglycolate medium were inoculated with different concentrations in the range of 100 to 0.125 CFU/ml and incubated at 37 °C under anaerobic conditions. After 14 days, the turbidity of the medium was examined.

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

Table 5. Bacteria Cultivation Media and Phosphate Buffer Ingredients

Medium	Manufacturer	Catalog No.	Lot No.
Tryptic soy agar (TSA)	Merck Germany	1.05458.0500	VM676858505 VM717058547
Tryptic soy broth (TSB)	Merck Germany	1.05459.0500	-
Soybean casein digest broth media	Merck Germany	STBMTSB12DP	F7PA54851
Fluid thioglycolate medium	VWR Germany	301051ZA	88458
Clear thioglycolate medium	Merck germany	STBMCTM12DP	F7SA54849
NaH_2PO_4 x 2 H_2O	Merck Germany	1.06345.5000	K93435445341
Na_2HPO_4 x 7 H_2O	Merck Germany	1.06574.1000	AM0732274437

Table 6. Incubation Conditions for each bacterial species (EZ-CFU)

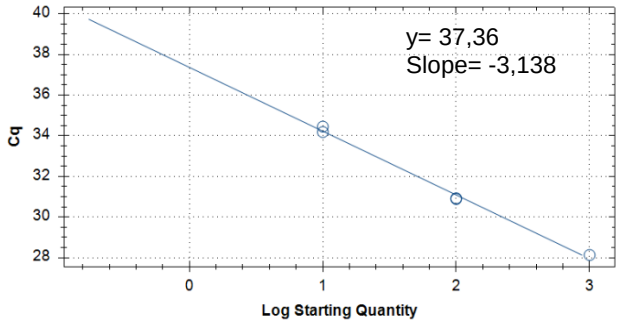
Species	Strain	Atmosphere	Temperature
*Bacillus subtilis subsp. spizizenii**	ATCC 6633	aerobic	30 °C
*Clostridium sporogenes**	ATCC 19404	anaerobic	37 °C
*Pseudomonas aeruginosa**	ATCC 9027	aerobic	37 °C
*Staphylococcus aureus subsp. aureus**	ATCC 6538	aerobic	37 °C
Bacteroides vulgatus	ATCC 8482	anaerobic	37 °C
Escherichia coli	ATCC 8739	aerobic	37 °C
Pseudomonas protegens	ATCC 17386	aerobic	26 °C
Streptococcus pyogenes	ATCC 19615	aerobic	37 °C
Bacillus cereus	ATCC 10876	aerobic	30 °C
Enterococcus faecalis	ATCC 29212	aerobic	37 °C
Kocuria rhizophila	ATCC 9341	aerobic	30 °C
Staphylococcus epidermidis	ATCC 12228	aerobic	37 °C

* EP 2.6.1 listed bacteria strains

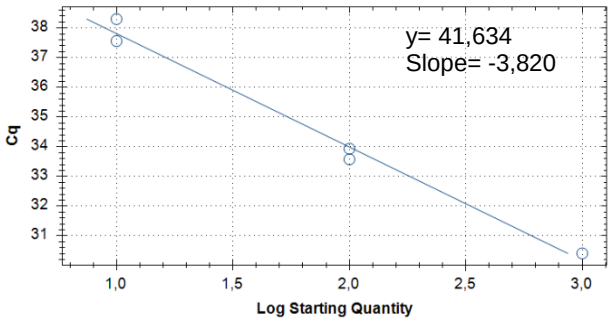
EZ-CFU standards are cultivated in bacterial media and lyophilized in a stabilizing matrix. Both, cultivation media as well as lyophilization matrix are potential sources of free environmental or process derived bacterial DNA. In order to assess the ratio of specific vs. unspecific DNA in EZ-CFU standards, the following procedure was exemplarily performed for *B. subtilis*, *P. aeruginosa*, *S. aureus and E. coli*:

EZ-CFU were rehydrated in 2 ml of rehydration buffer, diluted 1:10 and 100 µl aliquots were separated into pellet and supernatant via centrifugation at 18.500 g for 5 minutes. Supernatant (containing free DNA) and pellet (containing intact bacteria) were processed in parallel using Microsart® ATMP Extraction (the product previously named Microsart® Bacteria Extraction corresponds to the exact same product). DNA extracts were quantified via Microsart® ATMP Bacteria or a specific qPCR detection system for the appropriate species, respectively. Microsart® ATMP Bacteria shows total intact bacteria as well as total free-DNA in supernatant, while specific qPCR performed with AquaScreen® shows specific bacterial DNA in pellet and supernatant. Finally, a ratio of specific vs. unspecific DNA is calculated.

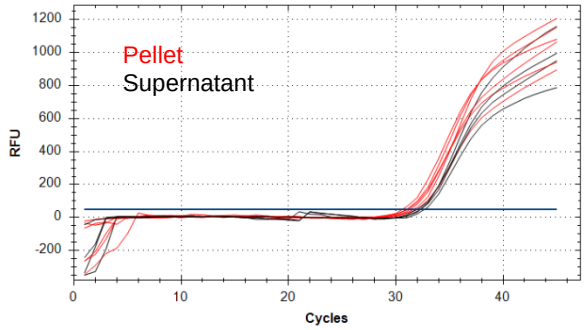
Standard Curve Microsart® ATMP Bacteria



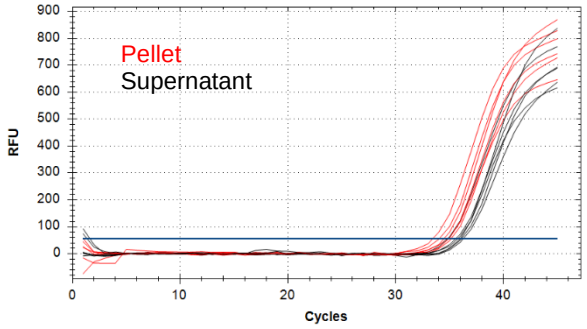
Standard Curve AquaScreen® E.coli



Extracts Microsart® ATMP Bacteria (E. coli)



Extracts AquaScreen® (E. coli)



	Microsart® ATMP Bacteria		AquaScreen® E.coli		Ratio specific/unspecific
	GC/PCR	GC/ml	GC/PCR	GC/ml	
Pellet	80,97	1619,30	79,49	1589,70	1,01
Supernatant	37,19	3718,75	35,94	3594,25	1,03

5.5 Compendial culture method

Bacteria listed in Table 7 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.

Table 7. Incubation Conditions for each Bacterial Species

Species	Strain	Atmosphere
Bacillus subtilis	ATCC 6633	aerobic
Staphylococcus aureus	ATCC 6538	aerobic
Clostridium sporogenes	ATCC 19404	anaerobic
Pseudomonas aeruginosa	ATCC 9027	aerobic
Streptococcus pyogenes	ATCC 19615	aerobic
Pseudomonas protegens	ATCC 17386	aerobic

5.6 Equipment

The following lab equipment was used for the study:

Table 8. Lab Equipment at MB

Equipment	Equipment-ID	Manufacturer	Brand
qPCR cycler	R 04 0843, ES72	Corbett Research	RotorGene 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
Pipettes for master mix setup 0.5-10 µl 10–100 µl 100–1000 µl 10–300 µl 10–300 µl	 E03, M25061D E04, L10238D E05, O10885D E60, 15011121 E66, 15017290	 Eppendorf Sartorius	 Reference 2 Biohit Picus
Pipettes for Sample Handling 0.2-10 µl 10–300 µl 50-1000 µl	 E69, 15023351 E70, 16010674 E71, 16009571	Sartorius	Biohit Picus
Pipettes for DNA Extraction 10-100µl 10-100µl 100-1000µl 100-1000µl	 E88, L56940G E13, L10224D E89, M45254G E14, O10970D	Eppendorf	Reference 2
Vortex	E29, VB4B016638	VWR	N/A
Glove box	E68, 22160256	GS Glovebox Systemtechnik GmbH	Glovebox P10RT2

Table 9. 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	 16007685 16007711 16006996 16009619	Sartorius	Tacta

Equipment	Equipment-ID	Manufacturer	Brand
100 – 1000 µl	16008272		

The following consumables were used:

Table 10. 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 11. 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 Kit are optimized to extract genomic bacterial 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 Bacteria and Microsart® ATMP Sterile Release can be used to monitor the extraction process. Extraction of bacterial DNA was carried out according to the suitable instruction manual.

In detail:

1. Transfer 1 ml sample into a provided DNA-free 1.5 ml processing tube (transparent cap)
2. Centrifuge at 16200 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 Bacteria kit or from Microsart® ATMP Sterile Release kit to the sample to monitor 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 bacterial 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 (Microsart® ATMP Bacteria) of Rehydration Buffer to each Bacteria Mixes or 90 µl (Microsart® ATMP Sterile Release) of Rehydration Buffer to each Bacteria SR Mixes
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 Bacteria:

	for 1 reaction	for 26 reactions
Bacteria 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
Bacteria 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 Bacteria Mix or Bacteria 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 Rotorgene 6000 (5-plex):

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
Gain setting	automatic (auto gain)
Slope Correct	activated
Ignore First	deactivated

Programming the qPCR cycler ABI Prism® 7500:

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

Programming the qPCR cycler Mx3005p®:

Segment 1 (Pre-Melt)	95°C, 3 min 0 sec
Segment 2	95 °C for 30 sec
	55 °C for 30 sec
	60 °C for 45 sec, data collection
Cycles	40
Analysis mode:	adaptive baseline (baseline correction)

Programming the qPCR cycler CFX96 touch:

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, all channels

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 bacteria in the sample is indicated by an increasing fluorescence signal in the bacterial FAM™ channel during PCR.

Table 12. Result interpretation Microsart® ATMP Bacteria and Microsart® ATMP Sterile Release

Detection of Bacteria FAM™ channel	Internal Control ROX™ channel	Interpretation
positive (C_q < 40)	irrelevant	Bacteria positive
negative (no C_q)	negative (no C_q)	PCR inhibition
negative (no C_q)	positive*	Bacteria 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. Bacterial 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 bacterial 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 specificity testing and LOD_{95} determination (see Tables 13A and 14) 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.

For robustness testing (see Tables 17) 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. Due to the environmental abundance of bacterial DNA it cannot be guaranteed that all cell culture samples used as spiking matrix are free of bacterial 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 bacteria detection capability of Microsart® ATMP Bacteria and Microsart® ATMP Sterile Release.

During LOD_{95} determination for the Bacteria SR Mix of Microsart® ATMP Sterile Release (see Table 13B), 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 / Bacteria Detection Range

The experimental setup of each test is listed in the following tables. All tests of Table 13A were performed six times to have at least 24 results for each CFU concentration of each of the five bacterial species listed in EP 2.6.1 and EP 2.6.27 available as EZ-CFU, and 48 results for NEC.

Table 13A. Test setup for LOD_{95} determination of bacterial species listed in EP 2.6.1 and EP 2.6.27

Detection Limit								
1	Spike DMEM + 5 % FBS with *Bacillus subtilis*							
	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 *Clostridium sporogenes*							
	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.							
3	Spike DMEM + 5 % FBS with *Pseudomonas aeruginosa*							
	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.							
4	Spike DMEM + 5 % FBS with *Staphylococcus aureus*							
	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.							
5	Spike DMEM + 5 % FBS with *Streptococcus pyogenes*							
	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.							

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.

Tests of Table 13B were performed three times to generate at least six results for each CFU concentration and three NECs.

Table 13B: Test setup for LOD_{95} determination with Bacteria SR Mix of Microsart® ATMP Sterile Release.

Detection Limit		
6	Spike DMEM + 5 % FBS with *Bacillus subtilis*	
	2 aliquots 99 CFU/ml + 1 NEC	2 aliquots 25 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.
7	Spike DMEM + 5 % FBS with *Clostridium sporogenes*	
	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 two times to generate at least eight results for each CFU concentration of each of the bacterial species and 16 results for the NEC.

Table 14. Test setup for LOD_{95} determination

Detection Limit						
8	Spike DMEM + 5 % FBS with *Bacteroides vulgatus*					
	99 CFU/ml → 4 aliquots	50 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.					
9	Spike DMEM + 5 % FBS with *Escherichia coli*					
	99 CFU/ml → 4 aliquots	50 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.					
10	Spike DMEM + 5 % FBS with *Pseudomonas protegens*					
	99 CFU/ml → 4 aliquots	50 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.					
11	Spike DMEM + 5 % FBS with *Bacillus cereus*					
	99 CFU/ml → 4 aliquots	50 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.					
12	Spike DMEM + 5 % FBS with *Enterococcus fecalis*					
	99 CFU/ml → 4 aliquots	50 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.					
13	Spike DMEM + 5 % FBS with *Kocuria rhizophila*					
	99 CFU/ml → 4 aliquots	50 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.					
14	Spike DMEM + 5 % FBS with *Staphylococcus epidermidis*					
	99 CFU/ml → 4 aliquots	50 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 bacteria 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 were used.

6.7.2 Specificity of PCR and bacteria detection range

In addition to the species tested for the LOD_{95} determination, the species listed in table 15 were tested using genomic DNA standards.
The specificity of the method was tested by spiking the PCR master mix with bacterial genomic 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 PCR quantification standards

Specificity		
1	Spike PCR master mix with 10 GC/PCR	
	Bacteroides fragilis → 8 aliquots *Enterobacter cloacae* → 8 aliquots *Klebsiella pneumoniae* → 8 aliquots *Serratia marcescens* → 8 aliquots *Clostridium perfringens* → 8 aliquots *Yersinia enterocolitica* → 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 Bacteria with compendial sterility test

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

Table 16: Test setup for comparison between Microsart® ATMP Bacteria and compendial sterility test

Specificity: Comparison with culture method with defined starting material quantity at external contract lab		
1	Spike DMEM + 5 % FBS with *Bacillus subtilis* 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 labor L+S	Extract DNA from 1ml starting material with Microsart® ATMP Extraction. Perform the qPCR according to Microsart® ATMP Bacteria.
2	Spike DMEM + 5 % FBS with *Clostridium sporogenes* 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 labor L+S	Extract DNA from 1ml starting material with Microsart® ATMP Extraction. Perform the qPCR according to Microsart® ATMP Bacteria.
3	Spike DMEM + 5 % FBS with *Pseudomonas aeruginosa* 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 labor L+S	Extract DNA from 1ml starting material with Microsart® ATMP Extraction. Perform the qPCR according to Microsart® ATMP Bacteria.
5	Spike DMEM + 5 % FBS with *Staphylococcus aureus* 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 labor L+S	Extract DNA from 1ml starting material with Microsart® ATMP Extraction. Perform the qPCR according to Microsart® ATMP Bacteria.
5	Spike DMEM + 5 % FBS with *Streptococcus pyogenes* 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 labor L+S	Extract DNA from 1ml starting material with Microsart® ATMP Extraction. Perform the qPCR according to Microsart® ATMP Bacteria.
6	Spike DMEM + 5 % FBS with *Pseudomonas protegens* 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 labor L+S	Extract DNA from 1ml starting material with Microsart® ATMP Extraction. Perform the qPCR according to Microsart® ATMP Bacteria.

EZ-CFU of the six species listed in Table 16 were rehydrated in 2 ml of rehydration buffer according to the user manual. The suspensions 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 17A, we tested the Microsart® ATMP Bacteria system with cell suspension, with free DNA as well as with different PCR cyclers. Assay 1 to 4 were performed twice.
The Bacteria SR Mix of Microsart® ATMP Sterile Release was also, as detailed in Table 17B, tested with different PCR cyclers. The assay was performed three times.

Table 17A. Test setup for robustness testing for Microsart® ATMP Bacteria

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 *Bacillus subtilis*. → 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 *Clostridium sporogenes*. → 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	
3	Spike water with *Escherichia coli* 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 2x10 µl 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 *Escherichia coli* 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 2x10 µl 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 % FCS 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 13A; 1 to 5 See Table 13B: 6 to 7 See Table 14; 8 to 14	The EZ-CFU of each bacterial species (Table 13, 14 and 15) will be 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 will be prepared for the five bacterial species listed in EP 2.6.1 and EP 2.6.27, available as EZ-CFU, and for another seven bacterial species. Dilutions from 99 to 0 CFU/ml will be tested according to test procedure described chapter 6.	The cut-off is defined as the lowest bacteria 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 ⩽ 99CFU	Passed (LOD_{95} comprised between 2.5 CFU and 99 CFU)

Bacillus subtilis (Microsart® ATMP Bacteria)

CFU/ml						Mean	
99	Run 1	31.04	31.31	31.17	31.35	31.22	24/24
	Run 2	31.79	31.52	31.11	30.89	31.33	
	Run 3	31.10	31.43	31.16	30.51	31.05	
	Run 4	31.40	30.96	31.15	31.07	31.14	
	Run 5	31.39	31.29	32.01	31.40	31.52	
	Run 6	31.46	30.76	31.31	32.99	31.63	
50	Run 1	33.05	32.15	32.93	32.96	32.77	24/24
	Run 2	33.25	32.65	39.39	32.21	34.37	
	Run 3	32.13	32.29	32.37	32.79	32.39	
	Run 4	33.31	30.65	32.15	33.21	32.33	
	Run 5	31.50	32.35	32.37	34.01	32.56	
	Run 6	31.78	32.16	32.19	32.17	32.07	
25	Run 1	34.36	34.10	34.45	33.46	34.09	23/24
	Run 2	33.33	33.65	31.31	33.34	32.91	
	Run 3	33.17	32.18	31.10	32.74	32.29	
	Run 4	32.54	35.36	34.07	33.91	33.97	
	Run 5	35.07	33.26	35.06	34.11	34.37	
	Run 6	No C_q	32.90	34.97	34.16	34.01	
10	Run 1	37.09	36.67	35.57	No C_q	36.44	22/24
	Run 2	34.38	34.86	34.59	34.70	34.63	
	Run 3	33.91	34.25	33.67	34.05	33.97	
	Run 4	34.02	34.66	35.70	34.34	34.68	
	Run 5	35.59	33.79	35.12	No C_q	34.83	
	Run 6	34.42	33.41	34.99	34.65	34.37	
5	Run 1	38.55	36.84	39.42	34.99	37.45	18/24
	Run 2	38.63	No C_q	No C_q	No C_q	38.63	
	Run 3	No C_q	No C_q	No C_q	36.89	36.89	
	Run 4	35.33	39.67	37.04	33.20	36.31	
	Run 5	35.64	37.28	36.25	35.36	36.13	
	Run 6	36.28	36.84	37.76	37.00	36.97	

Bacillus subtilis (Microsart® ATMP Sterile Release)

CFU/ml					Mean	
99	Run 1	32.41	31.07	31.74		
	Run 2	32.35	34.42	33.38	6/6	
	Run 3	33.05	31.53	32.29		
25	Run 1	35.38	33.39	34.38		
	Run 2	35.42	35.23	35.32	6/6	
	Run 3	34.34	33.53	33.93		

Clostridium sporogenes (Microsart® ATMP Bacteria)

CFU/ml						Mean	
99	Run 1	33.04	33.42	33.13	34.74	33.58	
	Run 2	33.48	33.33	33.97	34.05	33.70	
	Run 3	34.01	33.53	34.03	33.90	33.87	
	Run 4	32.85	32.30	33.24	33.69	33.02	24/24
	Run 5	33.31	33.88	34.00	33.29	33.62	
	Run 6	33.72	33.79	34.44	33.94	33.98	
50	Run 1	34.07	35.01	34.21	35.42	34.68	
	Run 2	35.60	36.56	35.40	35.50	35.76	
	Run 3	34.57	34.34	34.50	35.02	34.61	
	Run 4	34.50	34.89	34.76	No C_q	34.72	23/24
	Run 5	35.39	35.95	38.41	35.53	36.32	
	Run 6	35.85	35.97	35.81	37.24	36.22	
25	Run 1	39.12	36.14	35.71	36.83	36.95	
	Run 2	No C_q	37.22	37.12	38.07	37.47	
	Run 3	39.24	36.85	No C_q	36.52	37.54	
	Run 4	37.04	36.76	35.77	38.09	36.91	19/24
	Run 5	No C_q	No C_q	No C_q	38.38	38.38	
	Run 6	38.54	36.47	38.87	39.27	38.29	

Clostridium sporogenes (Microsart® ATMP Sterile Release)

CFU/ml					Mean	
99	Run 1	35.41	33.40	34.40		
	Run 2	35.14	34.30	34.72	6/6	
	Run 3	34.57	34.71	34.64		
50	Run 1	35.46	34.46	34.96		
	Run 2	34.86	34.42	34.64	6/6	
	Run 3	35.39	34.70	35.04		

Pseudomonas aeruginosa

CFU/ml						Mean	
99	Run 1	29.02	29.07	29.21	29.16	29.11	
	Run 2	30.67	31.01	31.35	31.35	31.09	
	Run 3	30.07	30.53	30.22	39.94	32.69	
	Run 4	30.50	31.14	31.23	30.53	30.85	24/24
	Run 5	31.52	30.74	30.76	31.76	31.19	
	Run 6	30.36	30.92	31.35	30.75	30.84	
50	Run 1	31.27	31.42	31.13	31.10	31.23	
	Run 2	32.17	31.98	31.85	31.45	31.86	
	Run 3	31.82	31.58	31.61	32.12	31.78	
	Run 4	32.31	32.14	33.08	32.01	32.38	24/24
	Run 5	33.13	32.24	32.30	32.37	32.51	
	Run 6	32.05	32.43	32.65	32.14	32.32	
25	Run 1	32.93	31.48	31.92	32.13	32.11	
	Run 2	32.65	32.28	32.51	33.27	32.68	
	Run 3	32.24	32.10	31.92	32.00	32.06	24/24
	Run 4	33.06	33.72	33.07	33.42	33.32	
	Run 5	33.50	33.46	33.33	33.50	33.45	

CFU/ml						Mean	
	Run 6	33.44	33.52	33.21	32.63	33.20	
10	Run 1	32.88	32.87	32.63	33.29	32.92	24/24
	Run 2	33.70	33.26	33.46	33.66	33.52	
	Run 3	32.38	33.18	32.91	33.34	32.95	
	Run 4	34.34	34.22	34.60	34.87	34.51	
	Run 5	34.39	33.93	36.64	34.19	34.79	
	Run 6	34.02	33.95	34.47	34.94	34.34	
5	Run 1	34.23	35.43	34.45	34.48	34.65	24/24
	Run 2	36.10	36.52	37.06	35.92	36.40	
	Run 3	34.05	34.43	34.07	34.65	34.30	
	Run 4	35.44	34.66	34.98	35.59	35.17	
	Run 5	35.21	35.51	34.41	35.14	35.07	
	Run 6	34.66	35.48	36.33	35.05	35.38	
2.5	Run 1	36.16	37.32	No C_q	36.21	36.56	20/24
	Run 2	37.16	38.46	37.37	No C_q	37.66	
	Run 3	36.19	36.62	37.25	39.12	37.29	
	Run 4	34.22	37.10	No C_q	36.64	35.99	
	Run 5	36.22	35.96	35.14	36.58	35.97	
	Run 6	35.22	38.45	35.31	No C_q	36.33	

Staphylococcus aureus

CFU/ml						Mean	
99	Run 1	33.24	33.28	33.11	32.29	32.98	24/24
	Run 2	33.43	32.51	32.11	32.79	32.71	
	Run 3	31.17	31.27	31.41	31.43	31.32	
	Run 4	33.15	33.16	33.35	32.44	33.02	
	Run 5	32.56	33.05	32.15	33.45	32.80	
	Run 6	32.00	33.12	32.06	32.55	32.43	
50	Run 1	35.51	34.84	33.53	34.07	34.49	24/24
	Run 2	33.65	34.48	34.33	33.89	34.09	
	Run 3	32.62	32.39	32.09	32.13	32.31	
	Run 4	34.42	34.90	34.00	34.25	34.39	
	Run 5	33.82	34.51	34.93	33.70	34.24	
	Run 6	33.82	33.48	34.29	33.10	33.67	
25	Run 1	36.04	36.94	36.14	37.80	36.73	23/24
	Run 2	35.02	35.72	34.98	34.53	35.06	
	Run 3	32.94	34.12	32.95	32.09	33.02	
	Run 4	35.46	No C_q	37.08	35.02	35.85	
	Run 5	35.31	34.17	34.70	35.48	34.91	
	Run 6	34.32	34.18	35.47	36.96	35.23	
10	Run 1	No C_q	38.02	37.05	No C_q	37.54	22/24
	Run 2	36.11	39.54	35.62	31.45	35.68	
	Run 3	34.36	34.50	34.59	34.22	34.42	
	Run 4	38.68	37.72	36.77	37.17	37.58	
	Run 5	37.26	35.73	37.02	38.83	37.21	
	Run 6	38.00	35.76	37.01	35.38	36.54	

Streptococcus pyogenes

CFU/ml						Mean	
99	Run 1	34.42	34.06	34.44	34.75	34.42	24/24
	Run 2	33.61	35.89	36.89	33.98	35.09	
	Run 3	35.41	34.92	36.88	35.01	35.55	
	Run 4	34.87	37.03	35.58	35.95	35.86	
	Run 5	34.22	34.48	34.25	34.11	34.26	
	Run 6	34.44	34.87	34.81	34.69	34.70	
50	Run 1	37.01	37.52	No C_q	36.76	37.10	18/24
	Run 2	35.61	35.37	35.17	35.29	35.36	
	Run 3	No C_q	37.70	No C_q	No C_q	37.70	
	Run 4	37.83	38.31	No C_q	No C_q	38.07	

CFU/ml						Mean	
	Run 5	34.50	35.25	35.29	35.34	35.10	
	Run 6	35.84	35.95	35.03	35.25	35.52	
25	Run 1	38.28	No C_q	36.47	No C_q	37.37	13/24
	Run 2	38.09	38.78	No C_q	No C_q	38.43	
	Run 3	No C_q	No C_q	39.17	No C_q	39.17	
	Run 4	No C_q	No C_q	No C_q	No C_q	No C_q	
	Run 5	37.56	36.29	36.86	37.41	37.03	
	Run 6	36.69	37.52	37.15	36.69	37.01	

Bacteroides vulgatus

CFU/ml						Mean	
99	Run 1	19.71	19.92	19.95	20.74	20.08	8/8
	Run 2	20.09	19.90	20.06	19.86	19.98	
50	Run 1	20.66	20.74	20.59	21.26	20.81	8/8
	Run 2	20.45	21.03	20.21	21.47	20.79	
10	Run 1	23.33	22.88	22.77	23.35	23.08	8/8
	Run 2	22.99	22.97	23.18	22.96	23.02	
5	Run 1	23.66	24.21	23.73	24.06	23.91	8/8
	Run 2	23.97	24.57	25.02	25.15	24.68	
2.5	Run 1	26.25	25.73	24.85	24.65	25.37	8/8
	Run 2	25.38	25.72	25.09	25.10	25.32	

Escherichia coli

CFU/ml						Mean	
99	Run 1	32.44	33.17	32.79	32.18	32.64	8/8
	Run 2	31.40	31.56	31.16	31.63	31.44	
50	Run 1	33.70	33.45	35.40	33.06	33.90	8/8
	Run 2	32.07	33.17	33.07	32.63	32.73	
10	Run 1	35.96	34.53	34.48	35.04	35.00	8/8
	Run 2	34.49	33.97	34.46	34.32	34.31	
5	Run 1	35.25	34.71	35.11	No C_q	35.02	6/8
	Run 2	35.41	36.09	36.03	No C_q	35.84	
2.5	Run 1	35.59	34.68	37.94	35.62	35.96	8/8
	Run 2	36.66	34.76	36.57	36.70	36.17	

Pseudomonas protegens

CFU/ml						Mean	
99	Run 1	32.17	31.88	32.02	33.56	32.40	8/8
	Run 2	32.79	33.14	32.42	33.01	32.84	
50	Run 1	32.97	32.98	33.73	33.22	33.22	8/8
	Run 2	33.77	34.15	34.06	34.01	34.00	
10	Run 1	37.16	37.32	36.00	35.63	36.53	8/8
	Run 2	37.74	36.28	36.67	36.72	36.85	
5	Run 1	37.05	36.63	No C_q	37.27	36.98	7/8
	Run 2	37.35	36.48	38.05	37.94	37.45	
2.5	Run 1	36.47	36.71	37.81	35.86	36.71	7/8
	Run 2	37.90	No C_q	38.05	37.37	37.77	

Bacillus cereus

CFU/ml						Mean	
99	Run 1	32.51	32.11	31.73	32.27	32.15	8/8
	Run 2	32.16	32.40	32.17	32.03	32.19	
50	Run 1	32.93	34.20	33.76	34.00	33.72	8/8

	Run 2	34.62	33.36	33.36	33.44	33.69	
10	Run 1	36.11	35.97	36.00	35.62	35.92	8/8
	Run 2	35.02	36.28	34.64	34.47	35.10	
5	Run 1	35.03	37.25	36.62	37.05	36.49	8/8
	Run 2	36.17	37.39	36.30	36.27	36.53	
2.5	Run 1	36.78	No C_q	37.05	No C_q	36.91	6/8
	Run 2	39.73	36.88	35.54	36.85	37.25	

Enterococcus fecalis

CFU/ml						Mean	
99	Run 1	34.52	34.72	34.19	34.84	34.57	8/8
	Run 2	33.15	35.66	36.48	36.03	35.33	
50	Run 1	No C_q	36.12	36.78	35.63	36.18	7/8
	Run 2	36.54	36.80	37.03	36.73	36.77	
10	Run 1	36.44	36.33	38.51	37.95	37.31	8/8
	Run 2	36.25	37.25	37.51	38.62	37.41	
5	Run 1	No C_q	37.75	36.48	36.84	37.02	6/8
	Run 2	35.64	No C_q	37.61	38.44	37.23	
2.5	Run 1	38.24	37.37	No C_q	39.58	38.40	6/8
	Run 2	36.86	38.11	No C_q	39.03	38.00	

Kocuria rhizophila

CFU/ml						Mean	
99	Run 1	31.79	32.29	32.04	32.12	32.06	8/8
	Run 2	35.34	32.62	33.35	32.54	33.46	
50	Run 1	33.07	33.08	33.04	33.08	33.07	8/8
	Run 2	33.51	34.76	33.86	33.40	33.88	
10	Run 1	35.48	35.12	35.07	35.32	35.25	8/8
	Run 2	37.51	39.23	36.33	37.59	37.66	
5	Run 1	35.76	35.76	37.58	35.64	36.18	7/8
	Run 2	No C_q	36.65	35.52	36.20	36.12	
2.5	Run 1	37.89	35.83	38.31	37.91	37.48	8/8
	Run 2	38.06	37.52	37.39	37.87	37.71	

Staphylococcus epidermis

CFU/ml						Mean	
99	Run 1	35.84	38.19	34.63	35.09	35.94	8/8
	Run 2	37.95	38.65	37.14	35.88	37.40	
50	Run 1	36.10	No C_q	36.66	39.99	37.58	5/8
	Run 2	32.66	No C_q	No C_q	37.62	35.14	
10	Run 1	35.87	No C_q	39.77	38.41	38.02	3/8
	Run 2	No C_q	No C_q	No C_q	No C_q	No C_q	
5	Run 1	No C_q	No C_q	No C_q	No C_q	No C_q	0/8
	Run 2	No C_q	No C_q	No C_q	No C_q	No C_q	
2.5	Run 1	No C_q	No C_q	No C_q	No C_q	No C_q	0/8
	Run 2	No C_q	No C_q	No C_q	No C_q	No C_q	

LOD_{95} summary

Species	LOD_{95}
Table 13	
Bacillus subtilis	25 CFU/ml

Clostridium sporogenes	50 CFU/ml
Pseudomonas aeruginosa	5 CFU/ml
Staphylococcus aureus	25 CFU/ml
Streptococcus pyogenes	99 CFU/ml
Table 14	
Bacteroides vulgatus	2.5 CFU/ml
Escherichia coli	10 CFU/ml
Pseudomonas protegens	10 CFU/ml
Bacillus cereus	5 CFU/ml
Enterococcus fecalis	99 CFU/ml
Kocuria rhizophila	10 CFU/ml
Staphylococcus epidermis	99 CFU/ml

7.2 Specificity

Specificity testing was comprehensively covered during sensitivity and robustness testing. During sensitivity, 12 different bacterial species were tested in order to determine the limit of detection when spiked into a complex sample matrix (DMEM + 5 % FBS). Another six species were tested as PCR quantification standards by spiking directly into PCR master mix and an *in silico* study was performed in order to predict the specificity of the primers. In terms of robustness testing, four 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 bacterial species detected with the highest LOD_{95} value during LOD determination (selected from the five bacterial species listed in EP 2.6.1 and EP 2.6.27, available as EZ-CFU). Additionally, six species were tested at three concentrations around their LOD_{95} in comparison with the culture method described in EP 2.6.1.

7.2.1 Sequence Alignment

Procedure	Acceptance Criterion	Results
Comparison of all primer sequences with the genomic database of relevant bacterial species. This provides additional information as only a limited number of bacteria can be part of this study.	Bacterial species showing ≤ 3 nucleotides mismatch in the alignment of the primer and probe sequence with the 16S rRNA genome are considered specifically detectable.	94.7 % of bacteria sequences are putatively detectable based on sequence alignments.

Strains of all bacterial species which were part of this study	**Primer Mismatches**		
	Forward Primer	**Probe**	**Reverse Primer**
Bacteroides fragilis ATCC® 25285	0	0	1
Bacteroides vulgatus ATCC® 8482	0	0	1
Enterobacter cloacae subsp. cloacae ATCC® 13047	0	0	0
Escherichia coli ATCC® 8739	0	0	0
Klebsiella pneumoniae ATCC® 13883	0	0	0
Pseudomonas aeruginosa ATCC® 9027	0	0	0
Pseudomonas protegens ATCC® 17386	0	0	0
Serratia marcescens ATCC® 13880	0	0	0
Streptococcus pyogenes ATCC® 19615	0	0	0
Bacillus cereus ATCC® 10876	0	0	0
Bacillus subtilis ATCC® 6633	0	0	0
Clostridium perfringens ATCC® 13124	0	0	0
Clostridium sporogenes ATCC® 3584	0	0	0
Enterococcus faecalis ATCC® 29212	0	0	0
Kocuria rhizophila ATCC® 9341	0	0	2
Propionibacterium acnes ATCC® 6919	1	0	2
Staphylococcus aureus ATCC® 6538	0	0	0

Strains of all bacterial species which were part of this study	Primer Mismatches		
	Forward Primer	Probe	Reverse Primer
Staphylococcus epidermidis ATCC® 12228	0	0	0

	Primer/Probe Mismatches			
	0	1	2	3
Bacteria	48.8%	69.4%	85.7%	94.7%
Archea	n.a	n.a	0.1%	40.4%
Eukaryotes	0%	0%	0.1%	0.3%

7.2.2 Bacteria Detection Range

Link	Procedure	Acceptance Criterion	Results
See Table 13A; 1 to 5 See Table 13B; 6 to 7 See Table 14; 8 to 14	All bacteria listed in Table 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
Bacillus subtilis (Microsart® ATMP Bacteria)	31.31	100
Bacillus subtilis (Microsart® ATMP Sterile Release)	32.47	100
Clostridium sporogenes (Microsart® ATMP Bacteria)	33.63	100
Clostridium sporogenes (Microsart® ATMP Sterile Release)	34.59	100
Pseudomonas aeruginosa	30,97	100
Staphylococcus aureus	32.55	100
Streptococcus pyogenes	34.98	100
Bacteroides vulgatus	20.05	100
Escherichia coli	32.04	100
Pseudomonas protegens	32.62	100
Bacillus cereus	32.17	100
Enterococcus fecalis	34.95	100
Kocuria rhizophila	32.76	100
Staphylococcus epidermidis	36.67	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 bacterial spike (NEC during robustness testing)	All samples shall show a negative result.	Passed

7.2.4 Specificity of PCR

		Results
Hela	No C_q No C_q	0/2
Vero	No C_q No C_q	0/2
CHO-K1	No C_q No C_q	0/2
RK13	No C_q No C_q	0/2

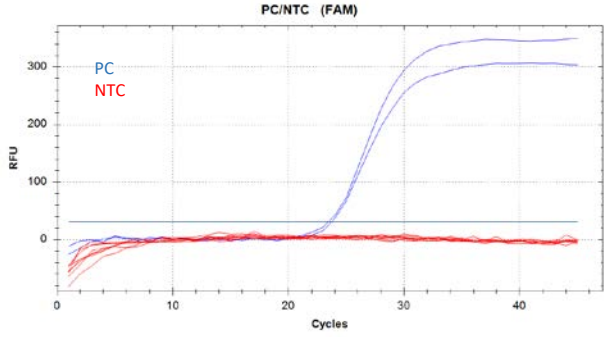
Link	Procedure	Acceptance Criterion	Results

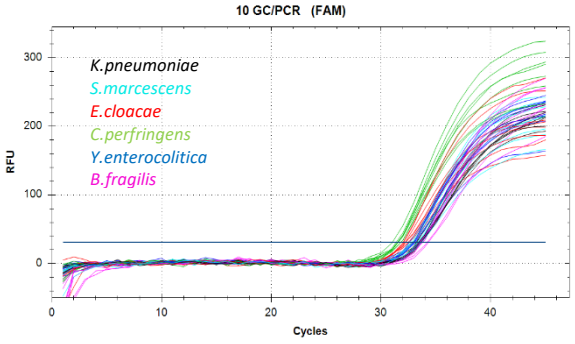
See Table 15; 1	All bacteria listed in Table 16 were tested with 10 and 0 GC (spiked directly into PCR).	All species must be detected at 10 GC/PCR.	Passed

	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	

	Run 1	Run 2	
10GC/PCR *Bacteroides fragilis*	33.60	34.13	16/16
	33.55	33.44	
	33.78	33.61	
	34.21	33.76	
	33.47	33.70	
	33.79	33.95	
	33.72	34.03	
	34.32	34.24	
10GC/PCR *Enterobacter cloacae*	32.07	31.93	16/16
	32.02	32.26	
	32.55	32.08	
	32.23	32.13	
	32.89	32.03	
	32.25	32.13	
	32.20	32.18	
	32.46	32.11	
10GC/PCR *Klebsiella pneumoniae*	33.43	32.76	16/16
	33.55	33.32	
	33.26	32.83	
	33.51	33.22	
	33.22	33.41	
	33.65	32.86	
	33.42	33.00	
	33.66	32.96	

	Run 1	Run 2	
10GC/PCR *Serratia marcescens*	32.74	32.70	16/16
	32.60	33.22	
	32.97	32.70	
	33.39	32.90	
	33.31	32.72	
	33.04	32.95	
	32.85	32.95	
	33.67	33.04	
10GC/PCR *Clostridium perfringens*	31.04	31.46	16/16
	31.50	31.33	
	31.38	31.51	
	31.81	31.34	
	31.52	31.52	
	31.55	31.26	
	31.57	31.23	
	31.81	31.37	
10GC/PCR *Yersinia enterocolitica*	32.91	32.76	16/16
	33.01	33.05	
	33.04	33.22	
	33.12	33.18	
	32.99	33.40	
	32.57	32.75	
	33.06	32.84	
	33.51	33.45	





7.2.5 Comparison of Microsart® ATMP Bacteria 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 Bacteria shall show equal or higher	Passed (100% similarity with culture

	Microsart® ATMP Bacteria. In parallel, 1 ml of the species listed in Table 16 were tested with the compendial culture method (direct inoculation) by an external lab.	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 Bacteria.	method; see Appendix)

	Microsart® ATMP Bacteria			Compendial culture method (External)		
	2x LOD_{95}	LOD_{95}	$LOD_{95}/2$	2x LOD_{95}	LOD_{95}	$LOD_{95}/2$
Bacillus subtilis	33.16 33.23	34.23 34.32	35.47 34.38	*B. subtilis*	*B. subtilis*	*B. subtilis*
Staphylococcus aureus	35.42 34.13	35.77 35.67	36.56 39.90	*S. aureus*	*S. aureus*	*S. aureus*
Clostridium sporogenes	34.20 34.10	34.87 33.43	35.45 35.61	*C. sporogenes*	*C. sporogenes*	*C. sporogenes*
Pseudomonas aeruginosa	36.40 36.22	36.74 37.96	37.22 No Cq	*P. aeruginosa*	*P. aeruginosa*	Negative
Streptococcus pyogenes	34.89 35.09	35.53 35.93	36.55 35.88	*S. pyogenes*	*S. pyogenes*	*S. pyogenes*
Pseudomonas protegens	34.14 33.28	34.38 34.51	36.52 35.61	Gram – Oxidase +	Gram – Oxidase +	Gram – Oxidase +

7.3 Robustness

7.3.1 Cell culture samples spiked with bacteria

Link	Procedure	Acceptance Criterion	Results
See Table 17A; 1	Four samples containing different cell lines in a relevant cell density and in typical cell culture media were spiked with 99 CFU/ml of *Bacillus subtilis*.	All spiked samples shall show a positive result.	Passed

7.3.2 Device compatibility

	***Bacillus subtilis* 99 CFU (FAM™)**			
	10^6 cells/ml		***10^5 cells/ml***	
Hela	30.73	29.67	30.64	28.80
	30.49	28.46	29.16	29.50
	4/4		**4/4**	
Vero	30.46	29.78	30.95	29.07
	30.96	29.71	30.45	29.53
	4/4		**4/4**	
CHO-K1	30.29	29.31	30.03	29.30
	29.81	29.42	30.40	28.73
	4/4		**4/4**	
RK13	30.75	29.82	31.04	29.25
	31.76	29.94	30.38	29.69
	4/4		**4/4**	

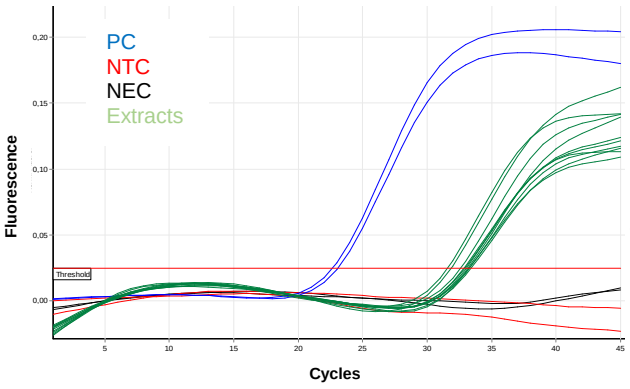
Link	Procedure	Acceptance Criterion	Results
See Table 17A; 2 See Table 17B; 6	As the test can basically be performed with any qPCR cycler capable of interpreting FAM™ and ROX™ signals, performance of the test with these machines needs to be validated. As not all qPCR cyclers commercially available are	All samples shall show a positive result.	Passed

	accessible for validation, the following four devices representing block and air heating systems are tested: Rotor-Gene 6000, Mx3005p, CFX96 touch and ABI Prism 7500. The robustness of the method was demonstrated by spiking DMEM + 5 % FBS with 99 CFU/ml of *Clostridium sporogenes*. At least eight replicates shall be tested on each instrument.		

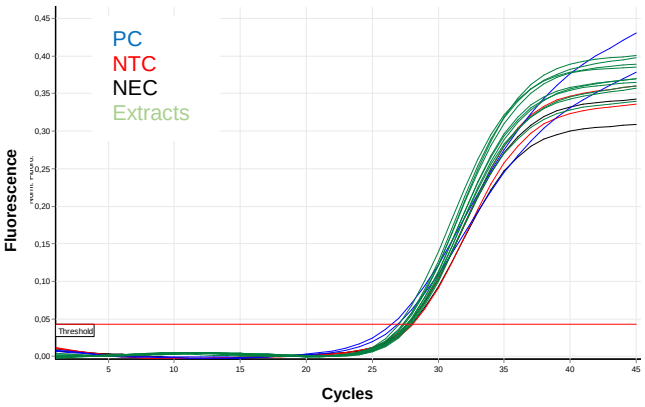
Rotor-Gene 6000 (Microsart® ATMP Bacteria)

Extract	C_q (FAM™)		C_q (ROX™)		Result
	Run 1	Run 2	Run 1	Run 2	
Positive Control	23.06	28.07	26.96	27.43	Correct
Positive Control	22.65	28.22	26.50	27.28	Correct
NTC	No C_q	No C_q	27.72	27.90	Correct
NTC	No C_q	No C_q	28.10	27.83	Correct
NEC	No C_q	No C_q	27.97	28.04	Correct
NEC	No C_q	No C_q	27.73	27.68	Correct
Clostridium sporogenes	31.86	30.81	27.05	27.54	Positive
	33.28	30.49	27.90	27.16	Positive
	33.41	31.83	27.76	27.92	Positive
	32.87	31.56	27.65	27.91	Positive
	31.59	31.17	27.37	27.40	Positive
	33.18	30.63	27.81	27.27	Positive
	33.06	31.60	27.37	27.81	Positive
	33.27	32.01	27.65	27.80	Positive

***C. sporogenes* 99 CFU Extracts (FAM™) (Rotor-Gene 6000)**



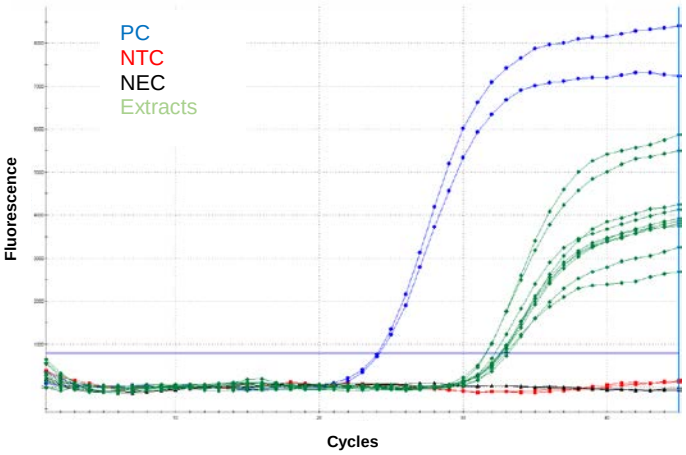
***C. sporogenes* 99 CFU Extracts (ROX™M) (Rotor-Gene 6000)**



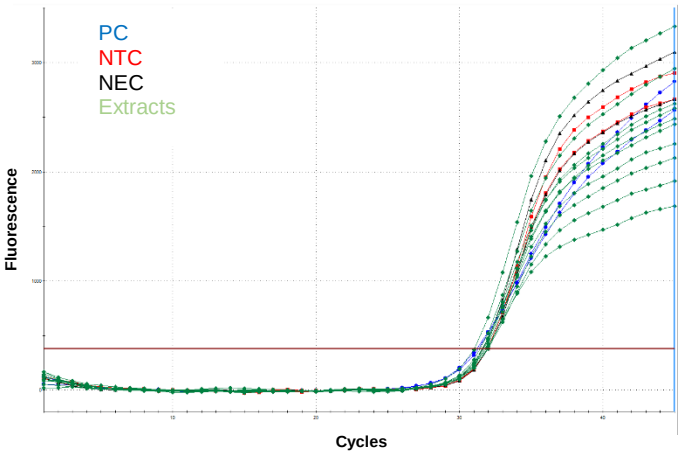
Mx3005p (Microsart® ATMP Bacteria)

Extract	C_q (FAM™)		C_q (ROX™)		Result
	Run 1	Run 2	Run 1	Run 2	
Positive Control	22.63	24.15	33.55	31.64	Correct
Positive Control	24.96	24.79	32.93	31.68	Correct
NTC	No C_q	No C_q	33.16	32.18	Correct
NTC	No C_q	No C_q	32.58	32.19	Correct
NEC	No C_q	No C_q	33.26	31.71	Correct
NEC	No C_q	No C_q	33.06	32.04	Correct
Clostridium sporogenes	32.02	32.64	33.32	32.34	Positive
	32.20	32.27	32.76	31.94	Positive
	31.70	31.64	33.97	31.79	Positive
	32.39	31.70	33.58	31.37	Positive
	31.81	32.62	33.26	32.14	Positive
	32.52	32.97	33.78	32.28	Positive
	32.13	32.70	32.89	31.98	Positive
	31.87	33.17	33.08	32.38	Positive
	32.07	32.73	33.73	32.03	Positive
	32.04	32.81	32.67	32.14	Positive

***C. sporogenes* 99 CFU Extracts (FAM™) (Mx3005p)**



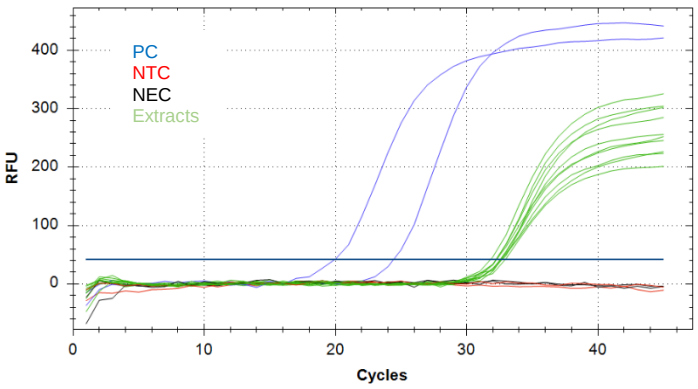
***C. sporogenes* 99 CFU Extracts (ROX™) (Mx3005p)**



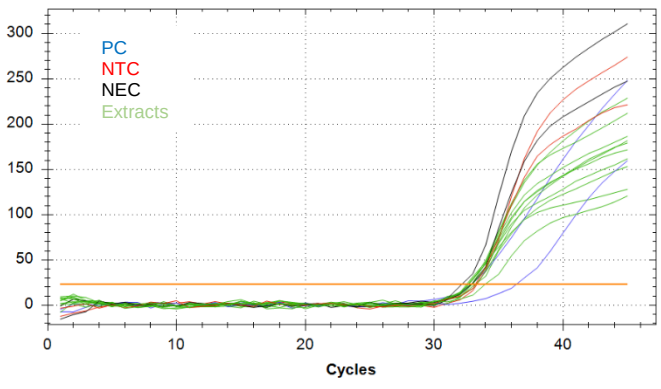
CFX96 (Microsart® ATMP Bacteria)

Extract	C_q (FAM™)		C_q (ROX™)		Result
	Run 1	Run 2	Run 1	Run 2	
Positive Control	24.01	23.86	30.36	28.53	Correct
Positive Control	24.19	24.00	30.30	28.33	Correct
NTC	No C_q	No C_q	33.19	29.37	Correct
NTC	No C_q	No C_q	33.31	29.58	Correct
NEC	No C_q	No C_q	32.13	29.3	Correct
NEC	No C_q	No C_q	32.86	29.85	Correct
Clostridium sporogenes	33.78	33.09	30.91	29.16	Positive
	33.61	35.03	30.81	28.84	Positive
	33.80	34.38	30.92	28.78	Positive
	34.07	33.94	31.26	28.85	Positive
	34.08	34.08	31.08	29.21	Positive
	34.48	34.47	31.09	29.65	Positive
	34.71	38.50	30.86	28.92	Positive
	34.12	34.09	31.25	29.14	Positive
	34.45	33.60	30.8	28.86	Positive
	34.27	33.70	31.21	28.75	Positive

***C. sporogenes* 99 CFU Extracts (FAM™) (CFX96)**



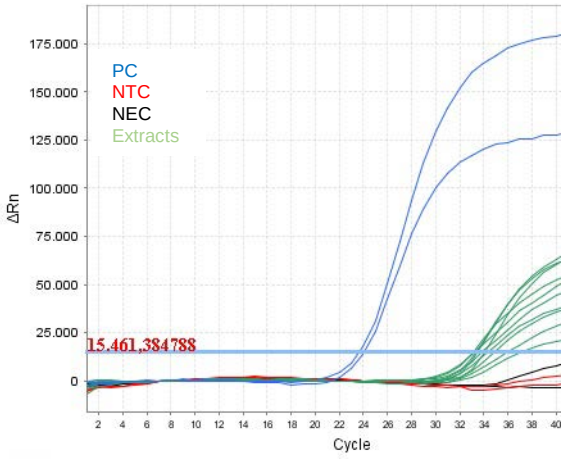
***C. sporogenes* 99 CFU Extracts (ROX™) (CFX96)**



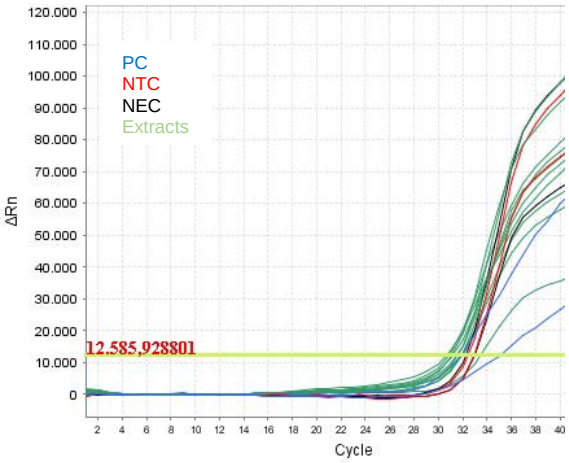
ABI Prism (Microsart® ATMP Bacteria)

Extract	C_q (FAM™)		C_q (ROX™)		Result
	Run 1	Run 2	Run 1	Run 2	
Positive Control	25.14	24.66	33.63	35.06	Correct
Positive Control	25.96	25.88	34.20	31.89	Correct
NTC	No C_q	No C_q	34.44	32.96	Correct
NTC	No C_q	No C_q	34.37	32.48	Correct
NEC	No C_q	No C_q	33.91	32.23	Correct
NEC	No C_q	No C_q	33.62	32.94	Correct
Clostridium sporogenes	31.40	34.38	30.64	31.64	Positive
	32.40	33.92	33.19	31.24	Positive
	33.48	33.37	34.45	31.12	Positive
	32.77	33.49	32.56	31.90	Positive
	32.88	36.06	34.02	31.54	Positive
	32.37	34.42	33.10	31.91	Positive
	33.42	33.11	33.09	30.93	Positive
	33.91	33.26	33.44	30.76	Positive
	34.07	37.22	32.59	33.52	Positive
	35.02	34.94	36.19	32.00	Positive

***C. sporogenes* 99 CFU Extracts (FAM™) (ABI)**



***C. sporogenes* 99 CFU Extracts (ROX™) (ABI)**



7.3.2 Detection of Free-DNA

Link	Procedure	Acceptance Criterion	Results
See Table 17A; 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 *Escherichia coli*. The samples were divided in eight aliquots, extracted with Microsart® ATMP Extraction and tested with Microsart® ATMP Bacteria.	n.a	Without cell lines in the sample, more than 99% of the free DNA is eliminated during the extraction process. With cell lines in the sample, more than 90% 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	39.00	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	No C_q	No C_q	No C_q	No C_q	No C_q
	39.93	39.89	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q
	39.38	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	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	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	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q
	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	31.37	31.97	35.57	35.01	No C_q	38.11	No C_q	No C_q	No C_q	No C_q
	31.29	31.28	35.14	35.02	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q

After extraction 10^3 GC/ml, 10 µl extract contain less than 1GC.
Thus, more than 99% 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	No C_q	38.14	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q
	36.92	35.43	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q
	38.08	35.21	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q
	39.68	37.18	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q
	35.84	35.62	No C_q	35.49	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q
	35.44	35.84	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q
	36.54	No C_q	34.56	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q
	36.00	36.79	36.20	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q
	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	32.78	34.80	34.93	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q
	32.49	34.81	36.31	No C_q	38.76	No C_q	No C_q	No C_q	No C_q	No C_q

After extraction 10^2 GC/ml with cell lines, 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 17A; 5	Cell culture Media without spike were extracted with Microsart® ATMP Extraction and tested with Microsart® ATMP Bacteria. 12 independent experiments were performed for DMEM + 5%FCS, each with eight replicates. For other culture media, one experiment was performed, with eight replicates.	n.a	All the run conducted with Microsart® Bacteria Extraction and Microsart® ATMP Bacteria were valid indicating that none of those media affect the performance of the kit.

	Negative Results	% negative results
DMEM + 5% FCS	8/8	98,95%
	8/8	
	8/8	
	8/8	
	8/8	
	7/8	
	8/8	
	8/8	
	8/8	
	8/8	
	8/8	
	8/8	

	Negative Results	ROX™	% negative results
DMEM	8/8	8/8 Correct	100%
DMEM high Glucose, GlutaMAX	8/8	8/8 Correct	100%
RPMI 1640	8/8	8/8 Correct	100%
RPMI 1640, GlutaMAX	8/8	8/8 Correct	100%
MEM (1)	8/8	8/8 Correct	100%
MEM (2)	8/8	8/8 Correct	100%
KnockOut DMEM	7/8	8/8 Correct	87.5%
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	7/8	8/8 Correct	87.5%
Mesencult ACF Basal Medium	8/8	8/8 Correct	100%
Mesencult ACF Basal Medium + Mesencult ACF Supplement	8/8	8/8 Correct	100%
ChondroMAX Differentiation Medium	8/8	8/8 Correct	100%
StemPro Osteo/Chond Dif Basal + StemPro Chondrogenesis Supplement	8/8	8/8 Correct	100%
StemPro Osteo/Chond Dif Basal + StemPro Osteogenesis Supplement	8/8	8/8 Correct	100%

8 Conclusion

Microsart® ATMP Bacteria and the Bacteria 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 Bacteria should be applied for Bacteria detection for any kind of sample material occurring in the manufacturing process of ATMP.

Microsart® ATMP Bacteria and its derivate product Microsart® ATMP Sterile Release were designed as state-of-the-art new product for the detection of bacterial contaminations in ATMP products. The products 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. The study provided detailed information about the performance of the kit regarding:

- all bacteria species that are requested by the EP 2.6.1 and 2.6.27
- the influence of samples matrices frequently used for manufacturing ATMPs and cell culture in general
- the influence of common lab to lab alternative sample preparation techniques

Specificity of the tested bacteria revealed a detection limit (LOD_{95}) comprised between 2.5 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 Bacteria and Microsart® ATMP Sterile Release can detect > 94 % of Gram positive and Gram negative bacteria. This feature greatly increase the chances to detect any kind of bacterial 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 standard cell culture material and with different PCR cyclers using the kits as directed. All collected results are consistent and confirm the accuracy and reproducibility of Microsart® ATMP Bacteria and Microsart® ATMP Sterile Release in those diverse surroundings for the detection of bacterial DNA.

It was shown that the products Microsart® ATMP Bacteria and Microsart® ATMP Sterile Release offers an alternative to the requirements of EP 2.6.1 and EP 2.6.27 and can be safely used to detect the presence of bacterial contamination in cell culture derived samples including ATMPs.

9 Reference Documents

1. T. Montag et al., Bundesgesundheitsbl 2010, 53:45–51: Probleme der mikrobiellen Sicherheit bei neuartigen Therapien
2. European Pharmacopoeia 8th edition, Strasbourg, FR; European Directorate for the Quality of Medicines; 2014, 5.1.6
3. US Pharmacopoeial Convention (USP). USP 38/NF 33 <1223>, 2015
4. Paul Ehrlich Institut: Stellungnahme zur mikrobiologischen Kontrolle von hämatopoietischen Stammzellzubereitungen, URL: http://www.pei.de/SharedDocs/Downloads/blut/stammzellen-genehmigung-21a-amg/pei-stellungnahme-mikrobiologische-kontrolle-stammzellen.pdf?__blob=publicationFile&v=3, 02.06.2015
5. European Pharmacopoeia 8th edition, Strasbourg, FR; European Directorate for the Quality of Medicines; 2015; 2.6.27 Microbiological examination of cell based preparations

10 Appendix

Labor LS

1. Prüfungsdurchführung

1.1 Prüfung auf Sterilität

Die Prüfungen wurden am 23.05.2018 mittels Direktbeschickung unter einer qualifizierten Sicherheitswerkbank (LS SWB 024) durchgeführt.

Hierbei wurde jeweils der Gesamtinhalt pro Gebinde in einen 400 ml Kolben Nährmedium überführt. Gemäß Kundenvorgaben wurden die Proben mit der Bezeichnung „a" in 400 ml Thio-Bouillon übeführt. Die Proben mit der Bezeichnung „b" wurden in 400 ml Caso-Bouillon überführt.

1.2 Inkubation

Nach der Prüfungsdurchführung wurden die Testansätze bei 22,5 ± 2,5 °C (Caso-Bouillon, KL 023) bzw. 32,5 ± 2,5 °C (Thio-Bouillon, KL 022) in einer qualifizierten Klimakammer mit kontinuierlicher Temperaturaufzeichnung inkubiert.

1.3 Auswertung und Resultate

Alle Testansätze wurden arbeitstäglich auf makroskopisch sichtbares Wachstum überprüft. Nach 6 Tagen (am 29.05.2018) Inkubationszeit, wiesen die ersten Ansätze eine visuelle Trübung auf. Die geforderte Inkubationszeit von 14 Tagen wurde somit vorzeitig aufgrund der Ergebnisse der visuellen Auswertung beendet. Bei diesen Testansätzen erfolgte unmittelbar die Anlage einer Subkultur mit nachfolgender Identifizierung.

Alle weiteren Testansätze wurden weiterhin bis zum Ende der 14-tägigen Inkubationszeit inkubiert und arbeitstäglich auf makroskopisch sichtbares Wachstum überprüft. Die Endauswertung erfolgte im 4-Augen-Prinzip.

Alle Resultate der visuellen Auswertung sowie der Identifizierung sind der *Tabelle 1: Übersichtstabelle aller Testansätze* zu entnehmen.

Die Zuordnung der LS-Nummern der Sterilitätsprüfung zu den entsprechenden LS-Nummern der Identifizierung wird in *Tabelle 2: Zuordnung LS-Nummern Sterilität und Identifizierung* dargestellt.

Labor LS

Tabelle 1: Übersichtstabelle aller Testansätze

LS-Nr.:	Produkt-name	Bezeich-nung	Sterilität*	betroffene Nährmedien	Ergebnis Identifizierung
180523-0197-001	DMEM + 5% FCS	1 a	E	Thio	-
		1 b	E	Caso	-
180523-0197-002	DMEM + 5% FCS	2 a	EN	Thio	Bacillus subtilis group
		2 b	EN	Caso	Bacillus subtilis group
180523-0197-002	DMEM + 5% FCS	3 a	EN	Thio	Bacillus subtilis group
		3 b	EN	Caso	Bacillus subtilis group
180523-0197-004	DMEM + 5% FCS	4 a	EN	Thio	Bacillus subtilis group
		4 b	EN	Caso	Bacillus subtilis group
180523-0197-005	DMEM + 5% FCS	5 a	E	Thio	-
		5 b	E	Caso	-
180523-0197-006	DMEM + 5% FCS	6 a	EN	Thio	Staphylococcus aureus
		6 b	EN	Caso	Staphylococcus aureus
180523-0197-007	DMEM + 5% FCS	7 a	EN	Thio	Staphylococcus aureus
		7 b	EN	Caso	Staphylococcus aureus
180523-0197-008	DMEM + 5% FCS	8 a	EN	Thio	Staphylococcus aureus
		8 b	EN	Caso	Staphylococcus aureus
180523-0197-009	DMEM + 5% FCS	9 a	E	Thio	-
		9 b	E	Caso	-
180523-0197-010	DMEM + 5% FCS	10 a	EN	Thio	Clostridium sporogenes
		10 b	EN	Caso	Clostridium sporogenes
180523-0197-011	DMEM + 5% FCS	11 a	EN	Thio	Clostridium sporogenes
		11 b	E	Caso	-
180523-0197-012	DMEM + 5% FCS	12 a	EN	Thio	Clostridium sporogenes
		12 b	E	Caso	-
180523-0197-013	DMEM + 5% FCS	13 a	E	Thio	-
		13 b	E	Caso	-
180523-0197-014	DMEM + 5% FCS	14 a	E	Thio	-
		14 b	E	Caso	-
180523-0197-015	DMEM + 5% FCS	15 a	EN	Thio	Pseudomonas aeruginosa
		15 b	EN	Caso	Pseudomonas aeruginosa
180523-0197-016	DMEM + 5% FCS	16 a	EN	Thio	Pseudomonas aeruginosa
		16 b	EN	Caso	Pseudomonas aeruginosa
180523-0197-017	DMEM + 5% FCS	17 a	E	Thio	-
		17 b	E	Caso	-
180523-0197-018	DMEM + 5% FCS	18 a	EN	Thio	Streptococcus pyogenes
		18 b	EN	Caso	Streptococcus pyogenes
180523-0197-019	DMEM + 5% FCS	19 a	EN	Thio	Streptococcus pyogenes
		19 b	EN	Caso	Streptococcus pyogenes
180523-0197-020	DMEM + 5% FCS	20 a	EN	Thio	Streptococcus pyogenes
		20 b	EN	Caso	Streptococcus pyogenes

Bericht über Resultate der Prüfung auf Sterilität
Produktbezeichnung: DMEM + 5% FCS
LS-Nr.: 180523-0197-001 bis 024

Version: 01
Seite: 3 von 5

Labor LS

LS-Nr.:	Produkt-name	Bezeich-nung	Sterilität*	betroffene Nährmedien	Ergebnis Identifizierung
180523-0197-021	DMEM + 5% FCS	21 a	E	Thio	-
		21 b	E	Caso	-
180523-0197-022	DMEM + 5% FCS	22 a	E	Thio	-
		22 b	EN	Caso	gramnegative Stäbchen, Oxidase positiv
180523-0197-023	DMEM + 5% FCS	23 a	E	Thio	gramnegative Stäbchen, Oxidase positiv
		23 b	EN	Caso	-
180523-0197-024	DMEM + 5% FCS	24 a	E	Thio	-
		24 b	EN	Caso	gramnegative Stäbchen, Oxidase positiv

* E: Entspricht, EN: Entspricht nicht

Tabelle 2: Zuordnung LS-Nummern Sterilität und Identifizierung

LS-Nummern der Prüfung auf Sterilität	Zugehörige LS-Nummern der Identifizierung
180523-0197-002	180523-0197-025
180523-0197-003	180523-0197-026
180523-0197-004	180523-0197-027
180523-0197-006	180523-0197-028
180523-0197-007	180523-0197-029
180523-0197-008	180523-0197-030
180523-0197-010	180523-0197-031
180523-0197-011	180523-0197-039
180523-0197-012	180523-0197-040
180523-0197-015	180523-0197-032
180523-0197-016	180523-0197-033
180523-0197-018	180523-0197-034
180523-0197-019	180523-0197-035
180523-0197-020	180523-0197-036
180523-0197-022	180523-0197-037
180523-0197-023	180523-0197-041
180523-0197-024	180523-0197-038

Labor LS

2. Wachstumskontrollen

2.1 Eingesetzte Testkeime

Bei LS Nr.: 180523-0197-001 wurden Wachstumskontrollen im Anschluss an die Sterilprüfung durchgeführt. Die Ergebnisse entsprechen den Anforderungen und sind dem Analysenzertifikat zu entnehmen.

Unterschriften zur Autorisierung			
erstellt:	Labor LS	Sandra Räder (Facheitung)	25. JUNI 2018 (Datum/Unterschrift)
geprüft/ genehmigt:	Labor LS	Stefan Gärtner (Abteilungsleitung)	25. JUNI 2018 (Datum/Unterschrift)

Ende des Dokuments

Appendix of the Product Validation Report for Microsart® ATMP Bacteria

This technical note summarizes additional LOD_{95} data (limit of detection) for bacterial species which have not been covered during product validation but are of relevance for kit users, which includes *Cutibacterium acnes* (ATCC 11827), *Serratia marcescens* (ATCC 14756) and *Peptostreptococcus anaerobius* (ATCC 27337).

Link	Procedure	Acceptance Criterion	Results / LOD_{95}
See chapter 6.7.1 (LOD_{95} determination) of the Product Validation Report Microsart® ATMP Bacteria	CFU-quantified bacterial suspensions were diluted, according to their individual cell count, in DMEM to prepare suspensions with concentrations from 99 to 0 CFU/ml. Individual dilution series were prepared. Dilutions from 99 to 0 CFU/ml were tested according to the test procedure described in chapter 6.7.1 of the validation report. Only data of relevant concentrations for LOD_{95} determination are shown below.	The cut-off is defined as the lowest bacteria concentration [CFU/ml] which leads in 95 % of the tests to a positive result (here 16/16 and 8/8 samples have to be positive). LOD_{95} must be ≤ 99 CFU	Passed (LOD_{95} is between 25 CFU and 50 CFU) (And all control reactions fulfilled acceptance criteria)

Serratia marcesens

CFU/ml						Mean	
99	Run 1	35,81	35,41	36,81	34,63	35,67	15/16*
	Run 2	35,30	34,12	34,27	35,47	34,79	
	Run 3	33,98	34,20	34,75	34,06	34,25	
	Run 4	32,05	32,94	N/A*	33,13	32,71	
50	Run 1	37,08	35,00	35,39	34,98	35,61	16/16
	Run 2	34,06	35,12	36,66	35,48	35,33	
	Run 3	36,00	37,60	36,06	36,59	36,56	
	Run 4	32,40	34,04	34,35	33,20	33,50	
10	Run 1	No Cq	No Cq	37,92	No Cq	37,92	10/16
	Run 2	37,05	38,57	38,96	36,38	37,74	
	Run 3	No Cq	36,80	No Cq	No Cq	36,80	
	Run 4	35,48	35,17	35,97	36,99	35,90	

Cutibacterium acnes

CFU/ml						Mean	
25	Run 1	38,35	37,41	35,65	38,49	37,48	7/8*
	Run 2	N/A*	39,02	39,22	39,20	39,15	
10	Run 1	No Cq	38,48	39,67	No Cq	39,08	6/8
	Run 2	38,09	37,77	38,80	38,81	38,37	
5	Run 1	39,10	No Cq	No Cq	No Cq	39,10	4/8
	Run 2	39,02	37,26	No Cq	38,76	38,35	

*Two samples in total showed PCR inhibition most likely due to residual lysis buffer in the final DNA extract

2

Peptostreptococcus anaerobius

CFU/ml						Mean	
50	Run 1	34,99	36,08	34,31	38,39	35,94	8/8
	Run 2	32,57	32,14	31,88	33,19	32,45	
25	Run 1	36,93	36,05	36,02	36,61	36,40	8/8
	Run 2	33,15	33,92	32,30	33,16	33,13	
10	Run 1	38,67	37,93	36,42	38,34	37,84	8/8
	Run 2	35,64	34,63	34,40	34,96	34,91	
5	Run 1	38,76	38,72	38,23	37,75	38,37	8/8
	Run 2	36,19	35,36	35,26	36,18	35,75	

Species	**LOD_{95} Summary**
Serratia marcesens (ATCC 14756)	50 CFU/ml
Cutibacterium acnes (ATCC 11827)	25 CFU/ml
Peptostreptococcus anaerobius (ATCC 27337)	5 CFU/ml