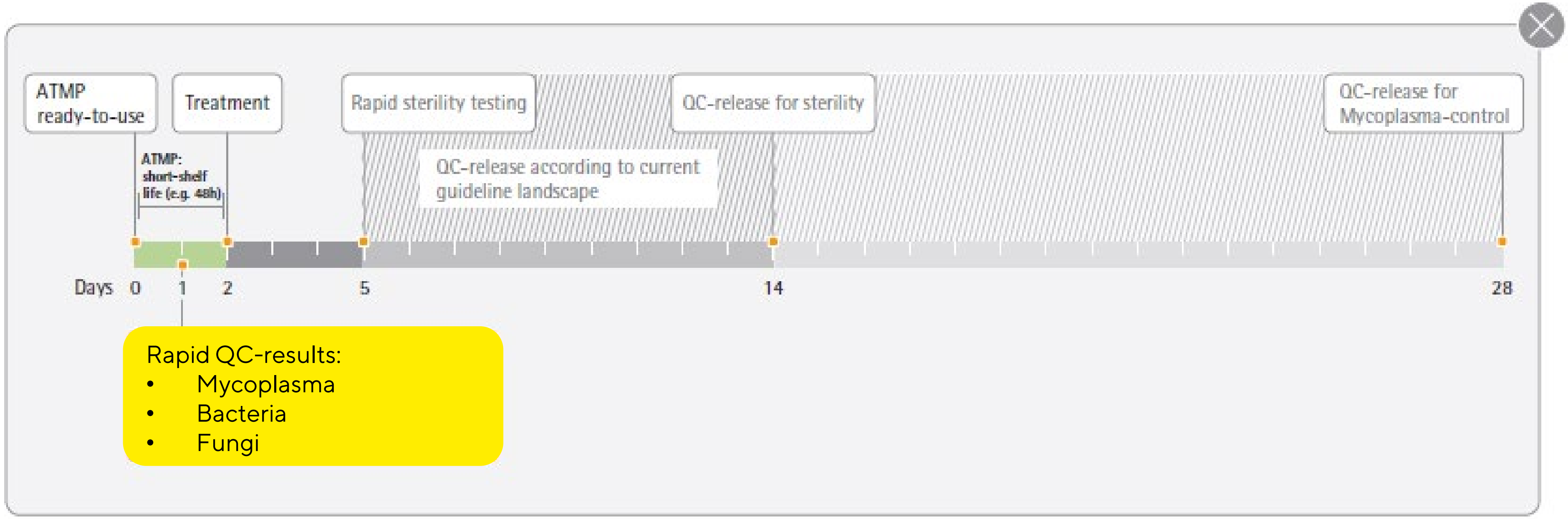
Simplifying Progress

Rapid sterility of ATMPs prior treatment - Validation of a qPCR-based test

Alexandra Mueller-Scholz, Sartorius Lab Instruments

NIST RMTM Workshop - Apr 19 , 2022

Safety of the Patient is a Risk



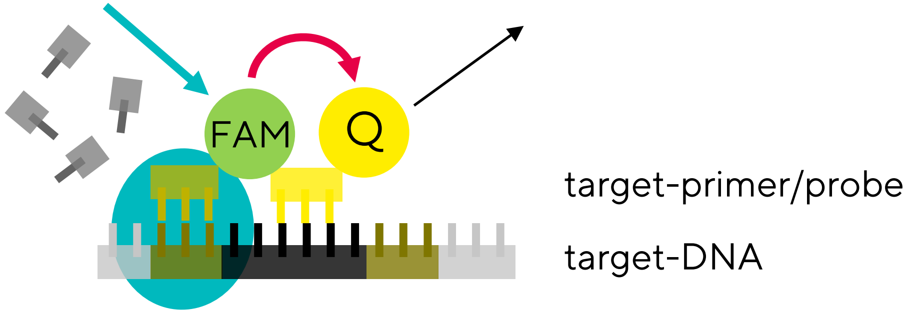
Rapid QC-results:
- Mycoplasma
- Bacteria
- Fungi

The Challenge:
- Growth-based QC-release takes too long.
- How do you ensure non-contaminated ATMPs?

Strategies to Reduce False Results

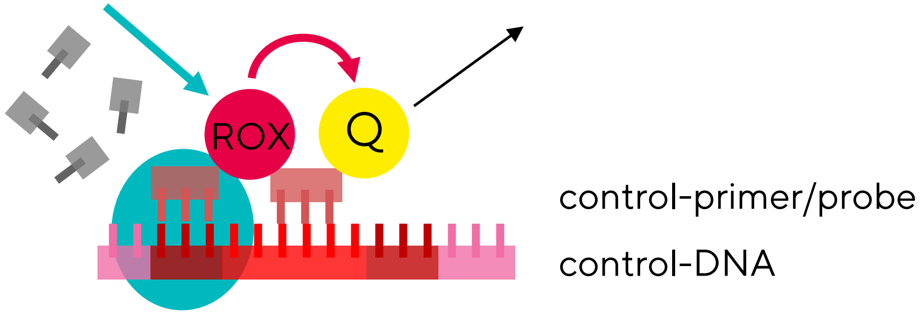
Reduce false-POSITIVES

- ✓ *specificity of TaqMan ® system*



Reduce false-NEGATIVES

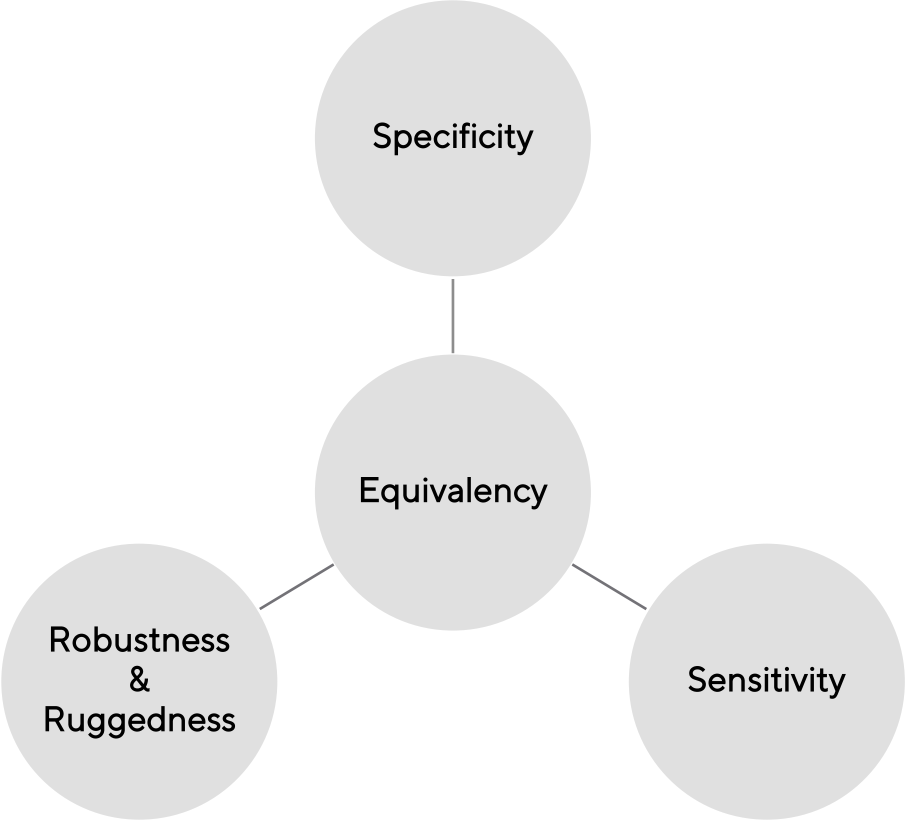
- ✓ *control of PCR-inhibition by integrated internal control*



Reliable Results for Bacterial and Fungal Detection in ATMPs PRIOR Treatment

Guidance for validation of rapid / alternativ methods:

- PDA, TR 33
- USP <1223>
- USP <1071>
- EP 5.1.6 part 4-1-1 primary validation by supplier
- EP 2.6.27



Specificity

Equivalency

Robustness & Ruggedness

Sensitivity

Including Guidance of the German Governmental Regulatory Agency (part of EMEA)

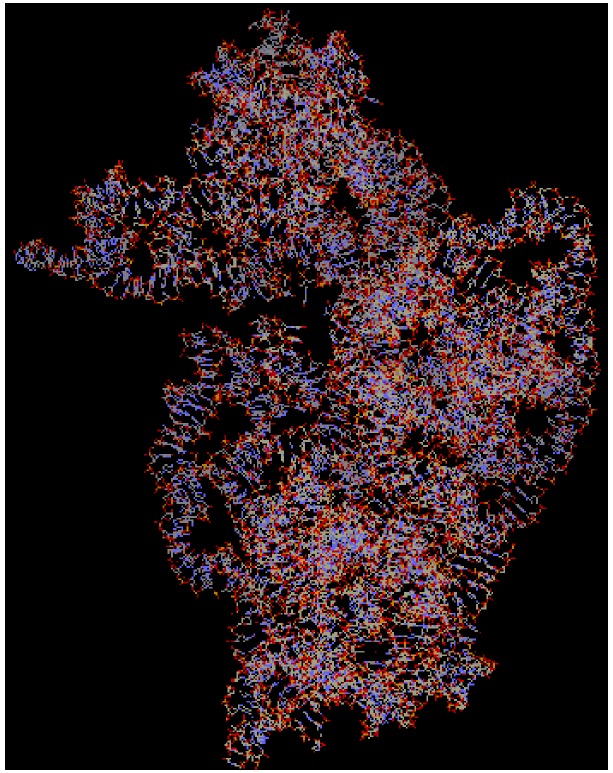
Paul-Ehrlich-Institut

Challenge
To be accepted by regulatory bodies

In-silico prediction by sequence alignment and BLAST

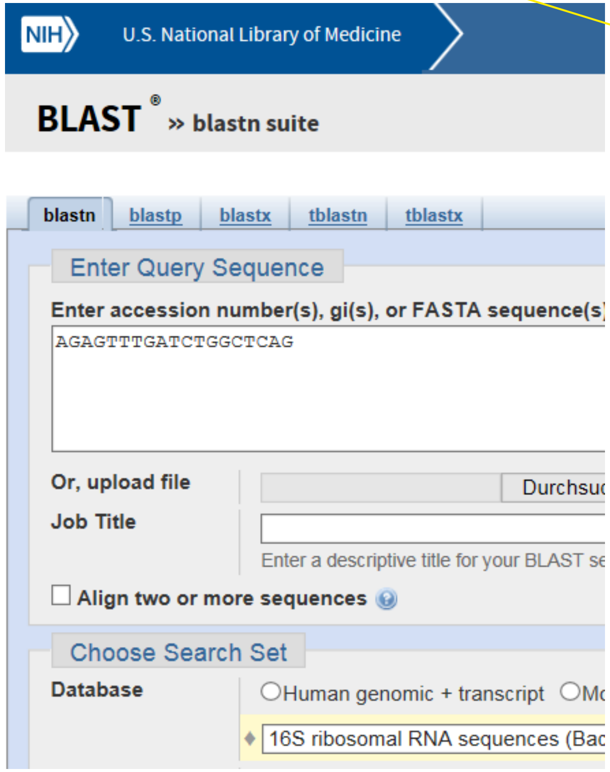
Acceptance criterion: ≤ 3 nucleotides mismatch of primers and probe

16S / 18S rDNA



http://www.biochem.umd.edu/biochem/kahn/bchm465-01/ribosome/16SrRNA.html

example



Enter accession number(s), gi(s), or FASTA sequence(s

AGAGTTTGATCTGGCTCAG

Or, upload file

Job Title

☐ Align two or more sequences

Database ○Human genomic + transcript ○Mo

16S ribosomal RNA sequences (Bac

https://www.ncbi.nlm.nih.gov/

Detection Range - Bacteria

Acceptance criterion: ≤ 3 nucleotides mismatch of primers and probe

	Primer 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 %**

Specificity

Detection Range - Fungi

Acceptance criterion: ≤ 3 nucleotides mismatch of primers and probe

Strains of all Fungi species which were part of this study	Primer Mismatches		
	Forward Primer	**Probe**	**Reverse Primer**
Candida albicans (HQ876034.1)	0	0	0
Candida tropicalis (EU348785.1)	0	0	0
Candida glabrata (AB094140.2)	0	0	0
Candida guiliermondii (AJ508274.1)	0	0	0
Candida haemulonii (AB013572.1)	0	0	0
Candida parapsilosis (AY497758.1)	0	0	0
Aspergillus brasilliensis (D63697.1)	0	0	0
Aspergillus fumigatus (AF548061)	0	0	0
Penicillium crysogenum (M55628.1)	0	0	0

37 % of the whole kingdom fungi are putatively detected based on sequence alignments.

Challenge
Do we detect enough species?

Cross Reactivity to Cell Lines / Sample Matrix Effects

Bacteria

		Results
Hela	No Cq	0/2
	No Cq	
Vero	No Cq	0/2
	No Cq	
CHO-K1	No Cq	0/2
	No Cq	
RK13	No Cq	0/2
	No Cq	
CHO-DG44	No Cq	0/2
	No Cq	
CHO XM111-10	No Cq	0/2
	No Cq	
L-9296 (NCTC)	No Cq	0/2
	No Cq	

Fungi

			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	

26 species included in Validation

EP 2.6.1
USP<71>

Currently tested = 6

Bacillus subtilis
Clostridium sporogenes
Pseudomonas aeruginosa
Staphylococcus aureus

Candida albicans
Aspergillus brasiliensis

23/24 positive

+

regulatory advice;
EP 2.6.27; user feedback

Recommended Extension = 20

Colony Forming Units (CFU)

Streptococcus pyogenes
Bacteroides vulgatus
Escherichia coli
Pseudomonas protegens
Bacillus cereus
Enterococcus faecalis
Kocuria rhizophila
Staphylococcus epidermidis
Serratia marcescens
Propionibacterium acnes

Candida tropicalis
Candida glabrata
Candida krusei
Aspergillus fumigatus
Penicillium chrysogenum

Genome Copies (GC)

Bacteroides fragilis
Enterobacter cloacae
Klebsiella pneumoniae
Clostridium perfringens
Yersinia enterocolitica

8/8 positive

Limit of Detection (LOD_{95}): 99, 50, 25, 10, 5, 2.5, 1.25 CFU/ml

Limit of Detection (LOD_{95}) – Fungi

currently tested

recommended extension

Species (CFU-based)	Acceptance criterium	LOD_{95} (CFU/ml)	Acceptance passed?
Candida albicans	23/24	50	✓
Aspergillus brasiliensis	23/24	50	✓
Candida tropicalis	8/8	10	✓
Candida glabrata	8/8	25	✓
Candida krusei	8/8	50	✓
Aspergillus fumigatus	8/8	99	✓
Penicillium chrysogenum	8/8	99	✓

CFU-based

Challenge
Detect 1 CFU/ml

Example: *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	34.48	
	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	31.19	
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	

Limit of Detection (LOD_{95}) – Bacteria

currently tested

recommended extension

Species (CFU-based)	Classification	Strain no.	LOD_{95} (CFU/ml)	Acceptance passed?
Bacillus subtilis	Gram pos.	ATCC® 6633	25	✓
Clostridium sporogenes	Gram pos.	ATCC® 19404	25	✓
Pseudomonas aeruginosa	Gram neg.	ATCC® 9027	5	✓
Staphylococcus aureus	Gram pos.	ATCC® 6538	10	✓
Streptococcus pyogenes	Gram neg.	ATCC® 19615	99	✓
Bacterioides vulgatus	Gram neg.	ATCC® 8482	2,5	✓
Escherichia coli	Gram neg.	ATCC® 8739	10	✓
Pseudomonas protegens	Gram neg.	ATCC® 17386	10	✓
Bacillus cereus	Gram pos.	ATCC® 10876	5	✓
Enterococcus faecalis	Gram pos.	ATCC® 29212	99	✓
Kocuria rhizophila	Gram pos.	ATCC® 9341	10	✓
Staphylococcus epidermidis	Gram pos.	ATCC® 12228	99	✓
Serratia marcescens	Gram neg.	ATCC® 14756	50	✓
Propionibacterium acnes	Gram pos.	ATCC® 11827	25	✓

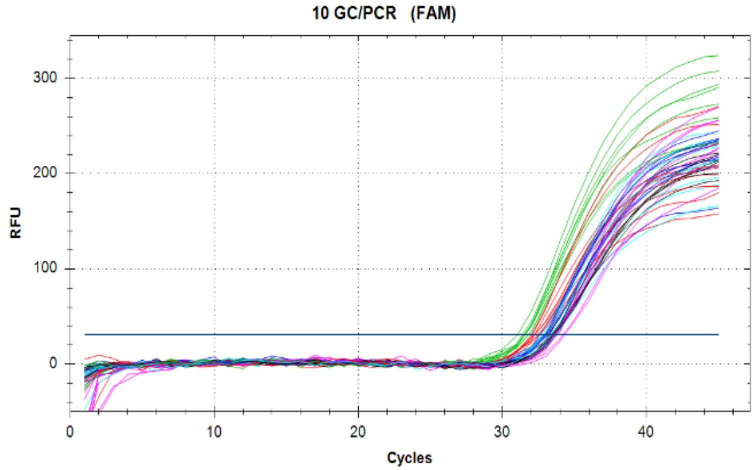
CFU-based

Limit of Detection (LOD$_{95}$) – Bacteria

recommended extension

Species (GC-based)	Classification	Strain no.	LOD$_{95}$ 10 GC/PCR	Acceptance passed?
Bacteroides fragilis	Gram neg.	ATCC® 25285	10	✓
Enterobacter cloacae	Gram neg.	ATCC® 13047	10	✓
Klebsiella pneumoniae	Gram neg.	ATCC® 13883	10	✓
Serratia marcescens	Gram neg.	ATCC® 13880	10	✓
Clostridium perfringens	Gram pos.	ATCC® 13124	10	✓
Yersinia enterocolitica	Gram neg.	ATCC® 27739	10	✓

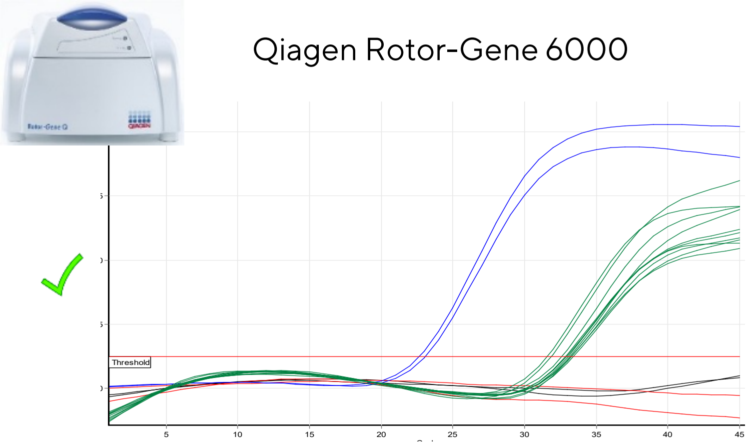
GC-based



Robustness and Ruggedness

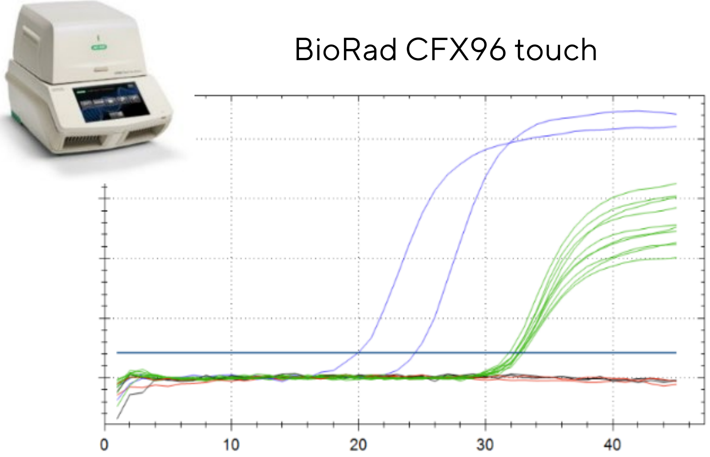
qPCR Device Comparability

Spiking 99 CFU/ml of the species with the highest LOD_{95}
Clostridium sporogenes (*LOD_{95}* = 25)
Candida albicans (*LOD_{95} = 50)*
Acceptance criterion 8/8 positive

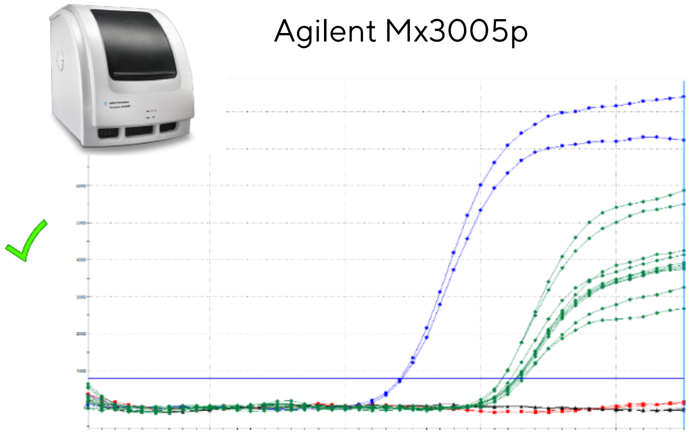

Qiagen Rotor-Gene 6000

✓

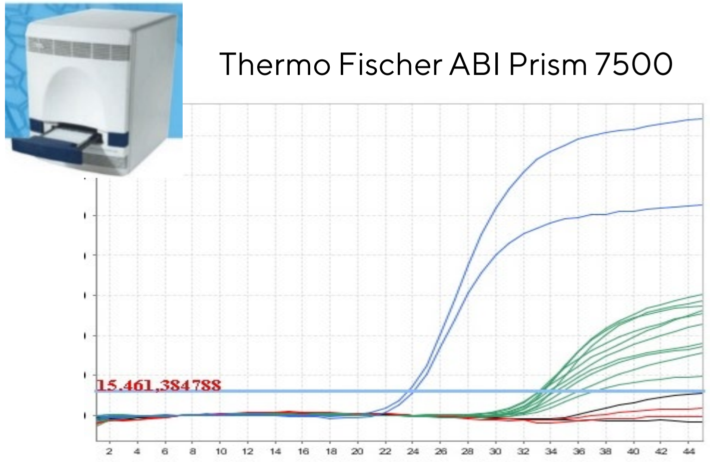
PC
NTC
NEC
Extracts


BioRad CFX96 touch

✓


Agilent Mx3005p

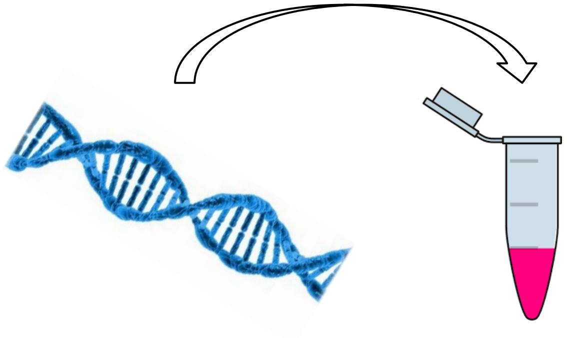
✓


Thermo Fischer ABI Prism 7500

✓

False-Positives due to Free Microbial DNA?

Challenge
Discriminate live and dead microorganisms



E. coli DNA spiked into DMEM
↓
DNA-extraction
↓
qPCR

failed		passed							
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
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

Low risk of false-positive results

Robustness and Ruggedness

Is the Cell Culture Medium DNA-free?

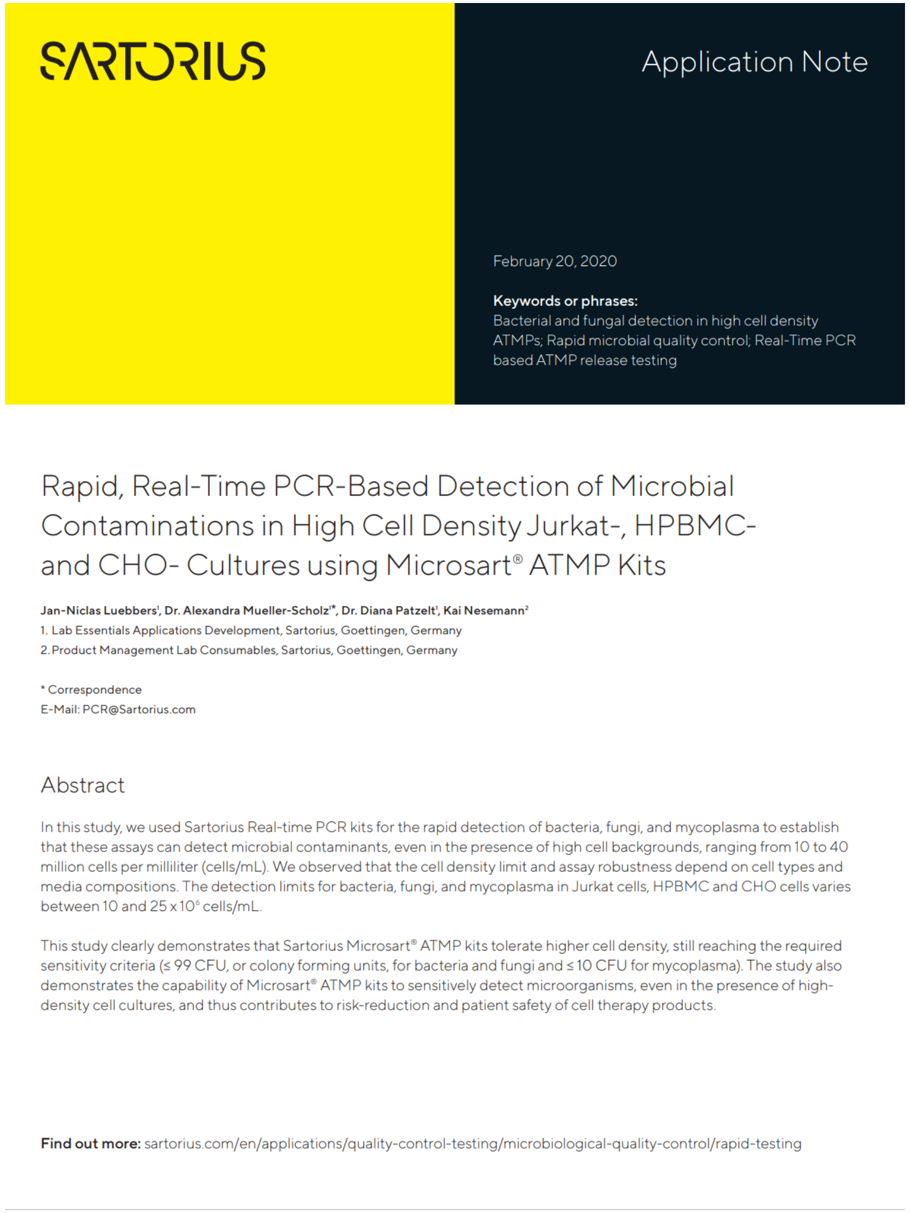
Cell culture medium without spike acceptance criterion
> 95 % negative

Fungi detection	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%

Low risk of false-positive results

Tolerance of Eukaryotic Cell Background

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



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Application Note

February 20, 2020

Keywords or phrases:
Bacterial and fungal detection in high cell density ATMPs; Rapid microbial quality control; Real-Time PCR based ATMP release testing

Rapid, Real-Time PCR-Based Detection of Microbial Contaminations in High Cell Density Jurkat-, HPBMC- and CHO- Cultures using Microsart® ATMP Kits

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Abstract

In this study, we used Sartorius Real-time PCR kits for the rapid detection of bacteria, fungi, and mycoplasma to establish that these assays can detect microbial contaminants, even in the presence of high cell backgrounds, ranging from 10 to 40 million cells per milliliter (cells/mL). We observed that the cell density limit and assay robustness depend on cell types and media compositions. The detection limits for bacteria, fungi, and mycoplasma in Jurkat cells, HPBMC and CHO cells varies between 10 and 25 x 10^6 cells/mL.

This study clearly demonstrates that Sartorius Microsart® ATMP kits tolerate higher cell density, still reaching the required sensitivity criteria (≤ 99 CFU, or colony forming units, for bacteria and fungi and ≤ 10 CFU for mycoplasma). The study also demonstrates the capability of Microsart® ATMP kits to sensitively detect microorganisms, even in the presence of high-density cell cultures, and thus contributes to risk-reduction and patient safety of cell therapy products.

Find out more: sartorius.com/en/applications/quality-control-testing/microbiological-quality-control/rapid-testing

Comparability to Compendial Sterility Test

Sartorius

qPCR-based detection

?
=

L+S external contract lab

classical sterility test according to EP 2.6.1. and USP<71>

2x LOD_{95} LOD_{95} ½ LOD_{95}

all 6 mandatory species

- *Bacillus subtilis*
- *Clostridium sporogenes*
- *Pseudomonas aeruginosa*
- *Staphylococcus aureus*

- *Candida albicans*
- *Aspergillus brasiliensis*

recommended extension

- *Streptococcus pyogenes*
- *Pseudomonas protegens*

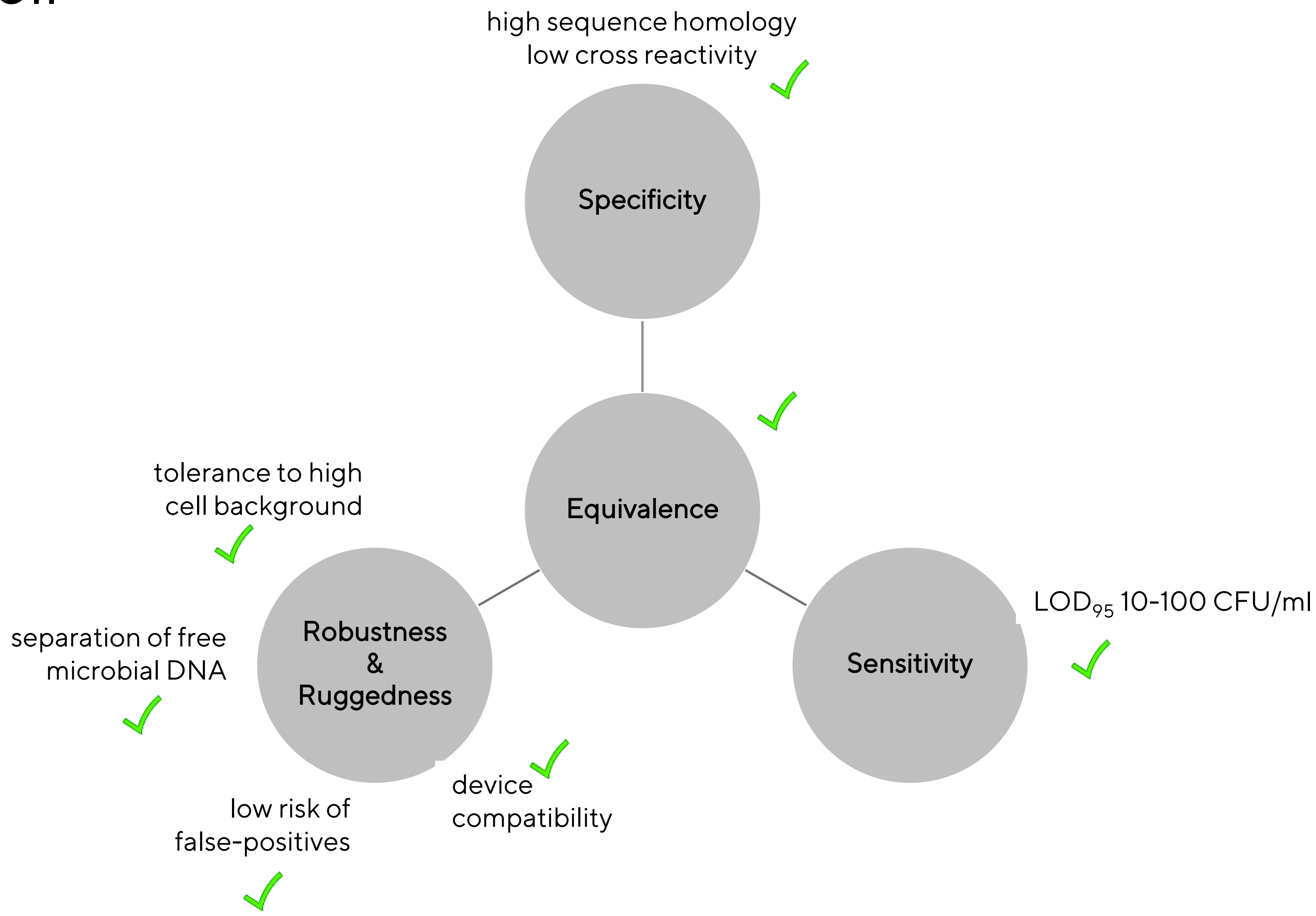
Comparability to Compendial Sterility Test

	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	34.23	35.47	*B. subtilis*	*B. subtilis*	*B. subtilis*
	33.23	34.32	34.38			
Staphylococcus aureus	35.42	35.77	36.56	*S. aureus*	*S. aureus*	*S. aureus*
	34.13	35.67	39.90			
Clostridium sporogenes	34.20	34.87	35.45	*C. sporogenes*	*C. sporogenes*	*C. sporogenes*
	34.10	33.43	35.61			
Pseudomonas aeruginosa	36.40	36.74	37.22	*P. aeruginosa*	*P. aeruginosa*	Negative
	36.22	37.96	No Cq			
Streptococcus pyogenes	34.89	35.53	36.55	*S. pyogenes*	*S. pyogenes*	*S. pyogenes*
	35.09	35.93	35.88			
Pseudomonas protegens	34.14	34.38	36.52	Gram – Oxidase +	Gram – Oxidase +	Gram – Oxidase +
	33.28	34.51	35.61			

Comparability to Compendial Sterility Test

	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	32.27	32.96	*C. albicans*	*C. albicans*	Negative
	31.94	32.12	33.96			
Aspergillus brasiliensis	34.38	37.06	34.94	*A. brasiliensis*	*A. brasiliensis*	*A. brasiliensis*
	32.40	33.17	34.20			

Validation Results in a Nutshell



high sequence homology
low cross reactivity ✓

Specificity

Equivalence ✓

tolerance to high
cell background ✓

separation of free
microbial DNA ✓

Robustness
&
Ruggedness

low risk of
false-positives ✓

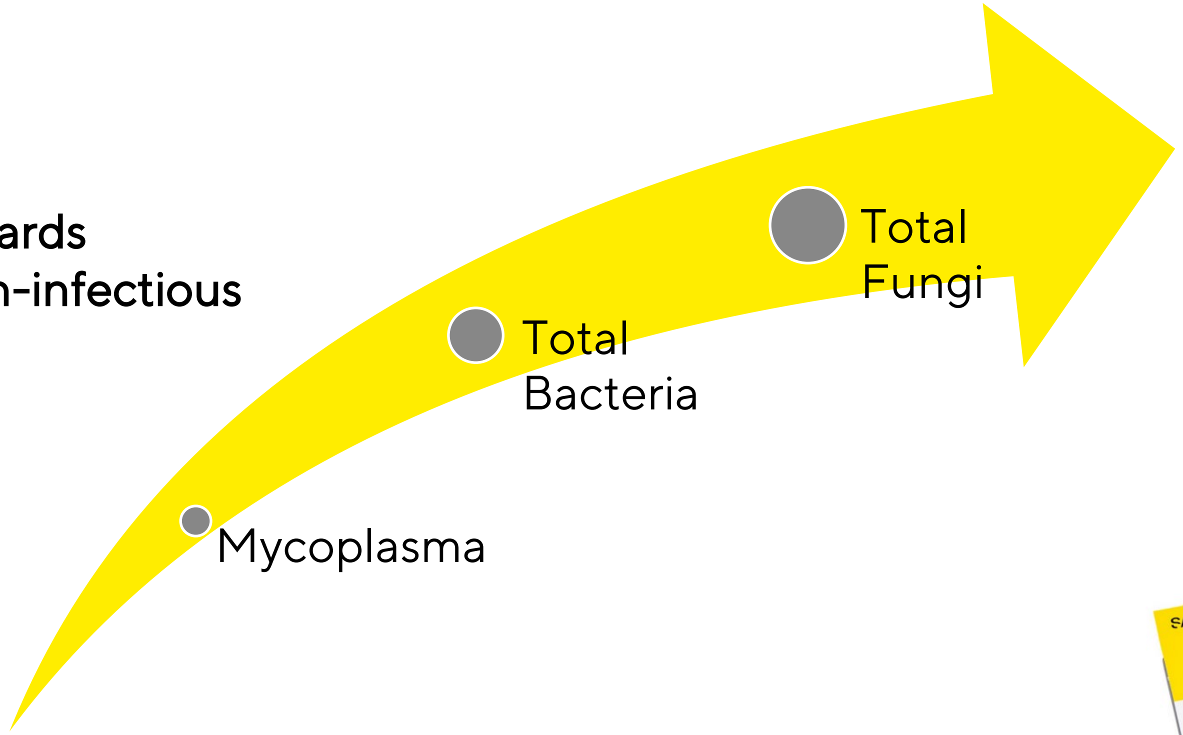
device
compatibility ✓

Sensitivity

LOD_{95} 10-100 CFU/ml ✓

PCR@Sartorius.com

- DNA extraction
- qPCR reagents
- **CFU Validation standards prequantified and non-infectious**



Mycoplasma

Total Bacteria

Total Fungi

Thank you.

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