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Simplifying Progress

Autolus

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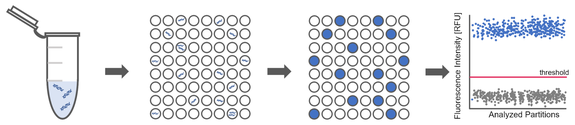
# Next Generation digital PCR Technology for Sterility Testing of Cell and Gene Therapy Products

## 1. Abstract

Sterility testing is crucial for the release of cell therapy products, as microbial contamination can be fatal for recipients. The current compendial sterility test requires 14 days for most microbes to ultimately rule out any contamination. However, for cell-based therapeutics, particularly autologous cell therapies for critically ill patients, the time to result is a significant factor, to reduce the vein-to-vein-time.
In this poster, we will present the first digital (droplet) PCR-based approach for sterility testing which is intended for release of cell and gene therapy products, elucidate the critical steps, and highlight the many benefits of this approach. We will address the handling of background signals of a bacteria digital (droplet) PCR experiment and explain how to clearly differentiate between the background and real positive signals. We will demonstrate how the groundbreaking digital (droplet) PCR technology allows a new level of precision in rapid sterility testing. We propose an open discussion on this advanced method and its potential for QC testing and release.

## 2. Digital PCR technology

Digital PCR (dPCR) is a new PCR method for the detection and quantification of DNA. dPCR is based on the principle of separating one real-time PCR reaction into multiple individual reactions. As a result of the separation, some partitions contain DNA targets, while others do not. Data acquisition is realized after PCR amplification (endpoint). Based on the real-time PCR principle, the fluorescence intensity increases with increasing number of amplicons. After data read out, the software calculates the concentration of the target sequence using Poisson's statistic.

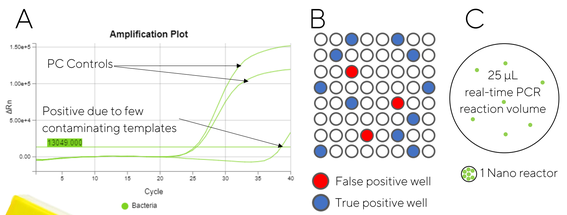



## 3. New Sartorius dPCR-based detection kit: Cyclus® dPCR Tool Box Bacteria Fungi

The Cyclus® dPCR Tool Box Bacteria Fungi kit is optimized for use with dPCR instruments. It consists of a Bacteria Primer/Probes Mix (red cap) for the detection of bacterial contaminants, a Fungi Primer/Probes Mix (orange cap) for the detection of fungal contaminants, an Internal Control (yellow cap) sufficient for 24 extraction controls, a Positive Control (green cap) for functional control of the bacteria and fungi assays, and PCR grade Water (white cap) for the No Template Control (NTC) and for volume replenishment. One kit is sufficient for testing up to 10 samples simultaneously using the QIAcuity® dPCR System from QIAGEN or the QX200™ ddPCR System from BioRad. It has been successfully tested with both nanoplate-based and droplet-based digital PCR. However, it is also conceivable that the kit is functional with other dPCR systems, such as the Absolut Q™ from ThermoFisher or the Nio®+ from Stilla.


## 4. Stable, Robust, and Sensitive

Bacterial DNA is ubiquitous and thus a challenge for all DNA-based detection methods. Even if an assay is free of bacterial DNA, lab, working materials and operators are part of the equation. If one template copy of bacterial DNA enters a real-time PCR reaction, it sooner or later will amplify and turn the reaction positive (**A**). This is the reason why most commercially available real-time PCR assays use Ct cut-off values and do not run until enzyme collapse. This can limit the detection of difficult-to-detect targets which would require additional amplification cycles in the real-time PCR. Such microbes are only detectable at increased CFU levels.
By introducing the DNA extract into a digital PCR reaction with 20 to 26 K cavities, possible contaminants are isolated in individual nano reactors (**B**). Those contaminants will lead to positive signals but remain isolated even with increased cycle numbers. The overall experiment remains valid, taking the positive signals of the background contamination as a Limit of Blank (LoB). Additional cycling is not required even though it would be possible now. This is due to the much smaller volume of the digital reaction chamber. In the small reaction volume, the resulting signal stays concentrated and is not diluted by the large volume of a real-time PCR reaction (**C**). Thus, one template in a digital cavity reaches the signal intensity of a 25 µL real-time reaction already after about 25 cycles. Consequently, the additional 15 cycles serve to amplify difficult targets thereby increasing sensitivity and improving the Limit of Detection (LoD).




# Results

## 5. Exploring the limits

The Cyclus® dPCR Tool Box Bacteria Fungi kit detected EP 2.6.27 microbes with a concentration of about 10 CFU in 1 mL DMEM+5%FBS matrix with 0.5 Mio. HEK cells. Parallel growth experiments with the spiking materials revealed that the CFU counts were frequently below the targeted 10 CFU. This highlights the potential of the new system and its ability to detect 10 CFU or even below.

| | Sample (0.5 Mio. HEK cells/mL) | Detection concentration [cp/µL] Set 1 | Set 2 | Set 3 | Set 4 | Considered positive if >1 cp/µL Considered inconclusive if <1 cp/µL and >0.5 cp/µL |
|---|---|---|---|---|---|---|
| Bacteria | No Template Control | 0.21 | | 0.05 | | Negative |
| | Negative Extraction Control | 0.52 | 0.11 | 0.21 | 0.27 | Negative |
| | *Pseudomonas aeruginosa* 10 CFU/mL (Ø4.6)* | 5.81 | 5.68 | 10.86 | 9.20 | Positive |
| | *Staphylococcus aureus* 10 CFU/mL (Ø6.3)* | 17.66 | 19.14 | 10.80 | 11.03 | Positive |
| | *Clostridium sporogenes* 10 CFU/mL (Ø7.7)* | 1.90 | 2.40 | 2.02 | 1.86 | Positive |
| | *Cutibacterium acnes* 10 CFU/mL (Ø4.6)* | 7.83 | 11.88 | 10.80 | 10.54 | Positive |
| | *Bacillus subtilis* 10 CFU/mL (Ø8.9)* | 1.74 | 1.27 | 1.43 | 1.06 | Positive |
| | *Kocuria rhizophila* 10 CFU/mL (Ø7.7)* | 2.89 | 2.88 | 3.32 | 3.87 | Positive |
| | *Streptococcus pyogenes* 10 CFU/mL (Ø7.6)* | 0,85 | 0,97 | 0,38 | 0,76 | Inconclusive |
| | Positive Control | Confirmed positive | | | | Positive |
| | Sample (0.5 Mio. HEK cells/mL) | Detection concentration [cp/µL] Set 1 | Set 2 | Set 3 | Set 4 | Considered positive if >0.2 cp/µL |
| Fungi | No Template Control | 0 | 0 | 0 | n.d. | Negative |
| | Negative Extraction Control | 0 | 0.06 | 0.05 | 0 | Negative |
| | *Candida albicans* 10 CFU/mL (Ø3.2)* | 0.92 | 0.53 | n.d. | n.d. | Positive |
| | *Aspergillus brasiliensis* 10 CFU/mL (Ø6.2)* | 0.98 | 0.92 | 1.81 | 1.07 | Positive |
| | Positive Control | Confirmed positive | | | | Positive |

Measurements were performed using QIAcuity® dPCR. DMEM + 5% FBS and 0.5 Mio. HEK cells were used as matrix. The viable cell count was determined on agar plates under culture conditions optimal for the respective organism. *The average viable colony count (colony forming units) is shown in parentheses. n.d. = no data

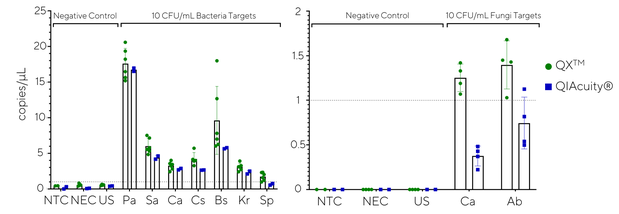
## 6. Detecting 10 CFU within a CAR-T product with 10 Mio. T-cell per 1 mL

The Cyclus® dPCR Tool Box Bacteria Fungi kit was successfully used in combination with the droplet digital PCR system from BioRad. 10 CFU of all EP 2.6.27 microbes could be successfully detected in a CAR-T matrix with ~10 million immune cells.

| | Sample (10 Mio. CAR-T cells/mL) | Detection concentration (cp/µL) Run 1 | Run 2 | Run 3 | Run 4 | Reportable result based on cut-off value (>1 cp/µL) |
|---|---|---|---|---|---|---|
| Bacteria | No Template Control | 0,34 | 0,66 | 0,67 | 0,54 | Negative |
| | Negative Extraction Control (PBS) | 0,43 | 0,56 | 0,56 | 0,52 | Negative |
| | Un-spiked sample | 0,62 | 0,86 | 0,74 | 0,36 | Negative |
| | *Pseudomonas aeruginosa* 10 CFU/mL | 19,31 | 20,24 | 18,85 | 23,78 | Positive |
| | *Staphylococcus aureus* 10 CFU/mL | 7,9 | 5,65 | 8,01 | 6,27 | Positive |
| | *Clostridium sporogenes* 10 CFU/mL | 3,85 | 3,75 | 10,2 | 3,96 | Positive |
| | *Cutibacterium acnes* 10 CFU/mL | 5,73 | 6,38 | 4,35 | 3,17 | Positive |
| | *Bacillus subtilis* 10 CFU/mL | 9,47 | 7,01 | 9,09 | 7,19 | Positive |
| | *Kocuria rhizophila* 10 CFU/mL | 3,4 | 3,3 | 4,02 | 3,92 | Positive |
| | *Streptococcus pyogenes* 10 CFU/mL | 1,11 | 2,15 | 2,06 | 1,75 | Positive |
| | Positive Control | 159,76 | 160,46 | 205,84 | 222,31 | Positive |
| Fungi | No Template Control | 0 | 0 | 0,04 | 0 | Negative |
| | Negative Extraction Control (PBS) | 0 | 0,04 | 0,04 | 0 | Negative |
| | Un-spiked sample | 0,53 | 0,77 | 0 | 0,47 | Negative |
| | *Candida albicans* 10 CFU/mL | 2,16 | 2,37 | 1,22 | 1,13 | Positive |
| | *Aspergillus brasiliensis* 10 CFU/mL | 2,81 | 5,24 | 2,54 | 3,07 | Positive |
| | Positive Control | 147,6 | 147,6 | 146,16 | 210,77 | Positive |

Data determined with the QX200™ droplet digital PCR (ddPCR) with 10 CFU spikes in 1 mL containing 10 million T-cells.

Comparative analysis of spiked T-cell samples showed that the sensitivity is independent of the used digital PCR technology. Both the QX™ System from BioRad and the QIAcuity® System from QIAGEN were able to identify all germs at 10 CFU.




NTC = No Template Control
NEC = Negative Extraction Control
US = Un-spiked Matrix
Pa = *Pseudomonas aeruginosa*
Sa = *Staphylococcus aureus*
Ca = *Cutibacterium acnes*
Cs = *Clostridium sporogenes*
Bs = *Bacillus subtilis*
Kr = *Kocuria rhizophila*
Sp = *Streptococcus pyogenes*

NTC = No Template Control
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US = Un-spiked Matrix
Ca = *Candida albicans*
Ab = *Aspergillus brasiliensis*

The dotted line marks the concentration of 1 cp/µL

## 7. Discussion

Switching from growth-based to molecular-based sterility testing is challenging. Rapid molecular-based methods usually did not reach comparable sensitivity and robustness levels up to now. The presented data demonstrate that all relevant compendial germs can be detected at 10 CFU per mL. This excellent result begs the question of whether it is possible to detect even less than 10 CFU. This is certainly possible for some germs. However, some aspects need to be considered.
First point to consider, is the limit of blank (LoB), which allows to obtain usable results even in the presence of low levels of bacterial background DNA contamination. Absence of background contamination or at least a very low contamination level will automatically lead to an increased sensitivity of the test.
The second and more critical point to consider, is the risk of unprecise distribution of CFUs at very low spike concentration levels. For example, when trying to add an exact number of 10 CFU with a 100 µL spike to 900 µL of a test matrix, 12% of spiked samples will receive less than 7 CFU when considering the Poisson distribution. That is why variations are frequently observed when measuring 10 CFU per mL. Consequently, it is challenging to stably measure less than 10 CFU, and this applies also to growth-based methods.

**Comment:** The Microsart® ATMP Extraction Kit is recommended for sample extraction.

For further information or support contact: **PCR@sartorius.com**