

Efficient qPCR Setup Without Cross Contamination Using the epMotion® Family of Automated Liquid Handling Systems

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Abstract

This application note shows that the epMotion family of automated liquid handling systems is ideal for reliable and reproducible setup of sensitive real-time quantitative PCR (qPCR) assays in daily routine, without the risk of cross contamination. The complete workflow from Master Mix preparation to 96-well PCR plate setup was done with the automation system. Cross contamination assay in chess

board pattern was performed according to FDA guidance. Runs on two independent epMotion systems with 2 plates each on 3 days are compared in this document. The assay reproducibility was excellent with low coefficients of variation for both intra and inter-plate comparisons of C_t values. Automated assay setups were carried with the epMotion 5073 and the 5075 systems.

Introduction

Based on the standard PCR combined with a fluorescent reporter monitoring, the real-time quantitative PCR (qPCR) allows to amplify, detect and quantify the targeted molecule in a single step. Over the last few decades, this molecular biology technology has progressively become a reference method commonly used in research for a wide range of applications including gene expression studies, DNA damage measurement or SNP genotyping. By combining excellent sensitivity, great specificity, low contamination risk, performance and speed, the real-time PCR also rapidly appeared as the perfect alternative to conventional culture-based or immunoassay-based methods used for diagnosing infectious diseases [1]. Moreover, because it is compatible with automated liquid handling systems, qPCR can be regarded

as a high throughput method. Indeed, manual qPCR setup is time-consuming, tedious and requires lots of practice. In theory, PCR is quite robust, but in reality, minor variations can lead to large changes in the overall amount of amplified product. The most probable source of imprecision is the operator itself. Three different individuals used same pipettes, Master Mix, template, and machine to quantify the same target and found initial copy numbers ranging from 2.7×10^3 to 8.7×10^5 [2]. When a large number of samples has to be processed in a short time, automating qPCR setup can be the solution to increase the throughput while the quality of results is maintained.

Besides issues with reproducibility, as with traditional PCR, real-time PCR reactions can also be affected by nucleic acid contamination, leading to false positive results. The three possible sources of contamination are: cross-contamination between samples, contamination from laboratory equipment and carryover contamination by amplified products from previous qPCRs. Precautions can be taken to reduce the risk of contamination: uracil DNA glycosylase (UDG) can be used to prevent DNA carryover contamination between reactions, materials can be systematically decontaminated, and sepa-

rate workstations for each step of the qPCR process can be designated to create an efficient workflow. Those measures do not eliminate the major cause of false positive results: the accidental contamination with positive samples during liquid handling. By automating qPCR setups, the challenge is consequently to guarantee a reproducible qPCR setup as well as the absence of cross-contamination. This application note shows the possibility to fully automate a real-time qPCR setup either on the Eppendorf epMotion 5073 or on the epMotion 5075 automated liquid handling systems.

Materials and Methods

qPCR Assay

- > Specific primers, 2 µM (Eurogentec®)
- > TaqMan® probe labelled in 3' with BHQ1 and with FAM in 5', 2 µM (Eurogentec)
- > qPCR Master Mix Plus (Eurogentec, order no.: RT-QP2X-03) containing dNTP/dUTP, Hot GoldStar®, UNG, MgCl₂, stabilizers and ROX passive reference
- > *Acinetobacter baumannii* Genomic DNA (extracted from ATCC® Strain 17978)
- > Eppendorf twin.tec® real-time PCR plates 96, semi-skirted (Eppendorf, order no.: 0030 132.505)
- > HeatSealing PCR Film (Eppendorf, order no.: 0030 127.838)
- > Mastercycler® ep realplex instrument (Eppendorf)
- > HeatSealer S100 (Eppendorf, order no.: 5391 000.001)

Real-time qPCR assay developed for *Acinetobacter baumannii* (*A. baumannii*) is composed of two specific primers and one TaqMan probe specific for the target. Each reaction was carried out in a total volume of 25 µL containing 12.5 µL of qPCR Master Mix Plus, 50 nM of each specific primer, 50 nM of the TaqMan probe, water and sample. The mix was subjected to the following thermal conditions: 2 minutes at 50 °C (UNG incubation), 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

Automation

- > epMotion P5073c for automated PCR set-up, 100–240 V/50–60 Hz, with CleanCap, EasyCon-control panel, Software epBlue™ and PCR-Assistants, TS 50, TS 300, Thermoblock and Rack 24 accessories (Eppendorf, order no.: 5073 000.002)
- > epMotion 5075mc with CleanCap configuration option including UV lights and HEPA filters (Eppendorf, order no.: 5075 000.305 and 5075 751.607)

- > TS50 single-channel dispensing tool (Eppendorf, order no.: 5280 000.010)
- > TS300 single-channel dispensing tool (Eppendorf, order no.: 5280 000.037)
- > Thermorack for 24x1.5/2 mL Safe-Lock Tubes (Eppendorf, order no.: 5075 771.004)
- > Height adapter 40 mm (Eppendorf, order no.: 5075 755.009)
- > epT.I.P.S.® Motion 50 µL Filter, sterile (Eppendorf, order no.: 0030 015.215)
- > epT.I.P.S. Motion 300 µL Filter, sterile (Eppendorf, order no.: 0030 015.231)
- > RNA/DNA LoBind microcentrifuge tubes, 2 mL (Eppendorf, order no.: 0030 108.078)
- > RNA/DNA LoBind microcentrifuge tubes, 1.5 mL (Eppendorf, order no.: 0030 108.051)

For each epMotion instrument used, two methods have been programmed using the PCR assistant. The goal of the first method is to generate the final Master Mix containing forward and reverse primers, TaqMan probe, qPCR Master Mix Plus and water. The second method is dedicated to the qPCR reaction setup: 20 µL of the final Master Mix is firstly dispensed into the real-time PCR 96-well plates and followed by the sample addition (5 µL). For both methods, epMotion surfaces and tools are cleaned using a DNA decontamination solution. UV-lights and HEPA filters are started one hour before using the automate. The UV-lights stopped automatically after 15 minutes. Worktables of both epMotion instruments are equipped as shown in figures 1 and 2.

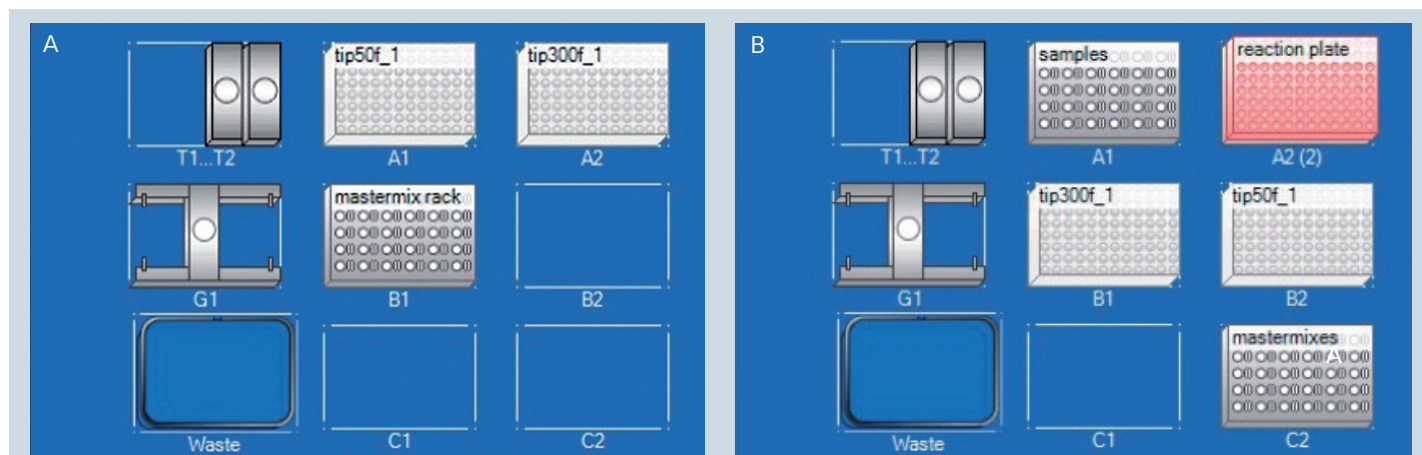


Figure 1: epMotion P5073 worktable for Master Mix preparation (A) and for qPCR setup (B)

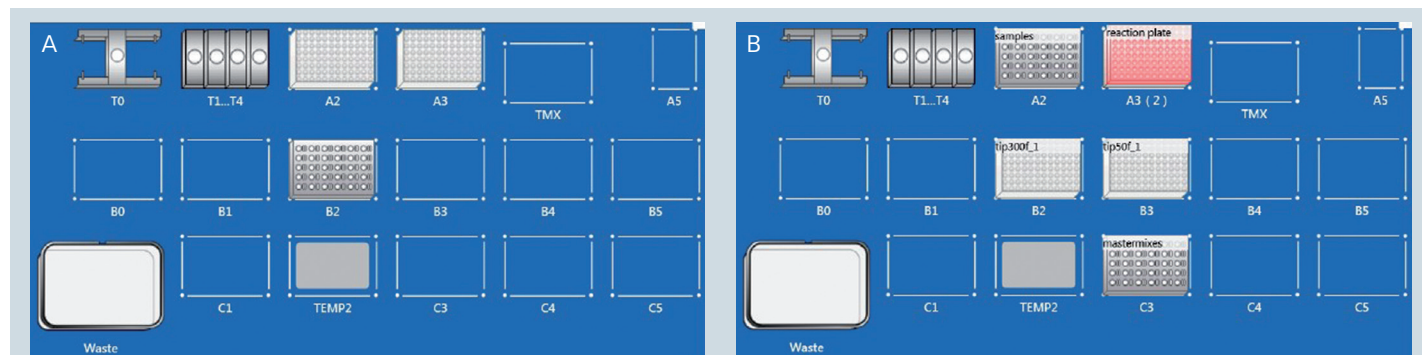


Figure 2: epMotion 5075m worktable for Master Mix preparation (A) and for qPCR setup (B)

Results and Discussion

To evaluate the risk of cross contamination when an epMotion liquid handling system is used, a very sensitive qPCR assay developed for *Acinetobacter baumannii* (*A. baumannii*) has been selected. Performances of this assay are demonstrated by generating a standard concentration curve of *A. baumannii* genomic DNA (gDNA). This curve shown in figure 3A corresponds to a series of 10-fold dilutions of target tested from 10^6 copies to 1 copy including a negative control using water.

Parameters used to determine the assay efficiency are the detection limit, the dynamic range (indicated by the slope value) and the R^2 value (figure 3B). A slope of -3.3 reflects an efficiency of 100 % while an R^2 value above 0.99 indicates the good curve linearity and provides confidence in correlating two values. Finally, the assay sensitivity is ensured as 1 copy of genomic DNA can be detected.

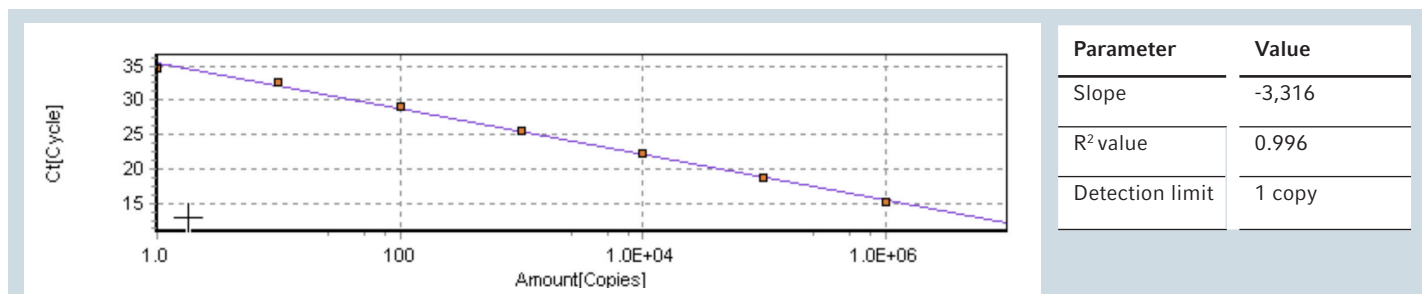


Figure 3: Standard curve generated by using a 10-fold dilution series of *A. baumannii* genomic DNA (A) and efficiency parameters evaluated (B)

Cross contamination

Even if the risk of contamination is considerably reduced with real-time PCR compared to conventional PCR, contamination remains a concern - especially for forensic laboratories. The main causes of false positive results are the accidental contamination of samples or reagents with positive samples (cross-contamination). The sample transfer to the PCR plate represents one of the most critical steps. Care must be taken to avoid the creation of aerosol or droplet. The FDA provides information to the industry to establish the performance of devices intended for pathogen detection. In order to demonstrate that cross-contamination will not occur with the device, this guideline proposes to use high positive samples in series alternating with negative samples. The FDA also suggests that at least five runs with alternating high positive and negative samples should be performed [3]. Based on those recommendations and in order to demonstrate that epMotion systems can be used for a complete qPCR set-up without cross-contamination, 48 high positive samples (containing 10^5 copies of *A. baumannii* gDNA) and 48 negative samples (containing water) have been dispensed in the 96-well PCR plate in a chessboard pattern (figure 4). One plate was processed per run. Two runs were performed per day and the complete experiment has been carried out during three different days. The complete procedure (Master Mix preparation and qPCR setup) was executed on two epMotion models: either on epMotion P5073 or on epMotion 5075.

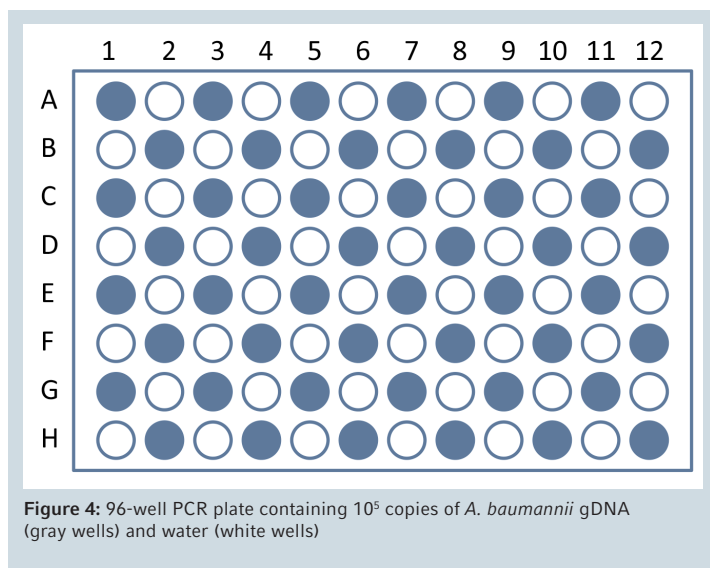
Table 1: Number of negative samples in which *A. baumannii* gDNA is detected when the complete PCR setup is performed with epMotion P5073 (A) and epMotion 5075 (B)

epMotion P5073					
Day 1		Day 2		Day 3	
Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2
0/48	0/48	0/48	0/48	0/48	0/48

epMotion 5075					
Day 1		Day 2		Day 3	
Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2
0/48	0/48	0/48	0/48	0/48	0/48

Reproducibility

Reproducibility is a key component of real-time PCR assay reliability. When a large number of samples are analyzed in 96-well format, the assay must be reproducible enough to allow the data comparison between samples, plates and separate runs. The assay reproducibility when an epMotion automate is used for qPCR setup was assessed by comparing results generated from 48 positive samples containing a known gDNA amount. As illustrated on figure 5, the complete process is highly reproducible. The mean intra-plate C_t value is very consistent with a coefficient of variation never exceeding 1.5 %.



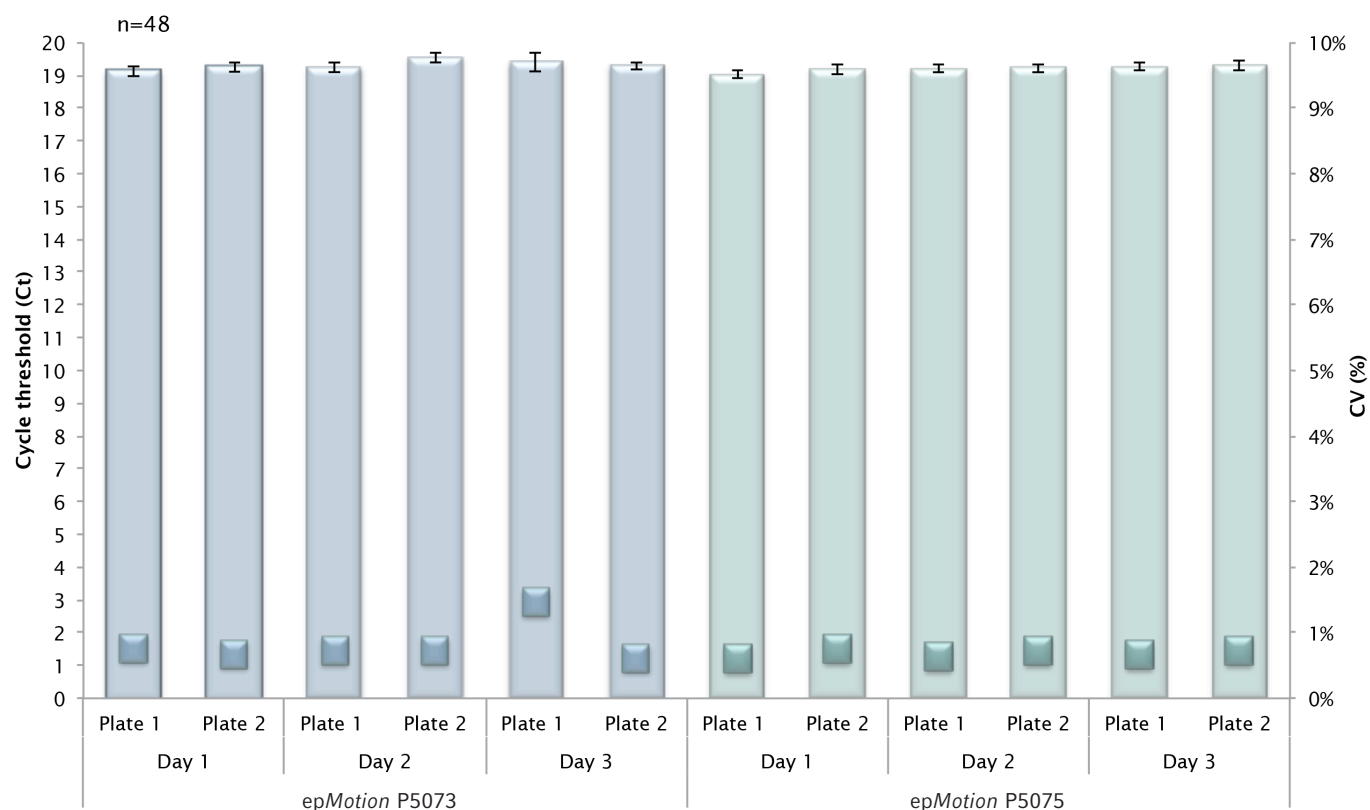


Figure 5: *A. baumannii* qPCR results. Mean intra-plate C_t value, standard deviation and coefficient of variation (CV) are calculated for each plate containing 48 positive samples.

Both epMotion models offer comparable reproducibility as the epMotion P5073 gives an average inter-plate C_t value of 19.31 ± 1.11 % while a mean inter-plate C_t value of 19.19 ± 0.80 % is obtained with the epMotion 5075.

Conclusion

In the present Application Note, we demonstrate the capability of the epMotion liquid handling system to automate a complete real-time quantitative PCR assay from Master Mix preparation to 96-well PCR plate setup. A cross contamination study was carried out and showed that qPCR setup can be completed by the epMotion without contamination. The assay reproducibility was also assessed by comparing results generated from positive samples containing a known gDNA amount and concluded to a highly consistent amplification with a CV lower than 1.5 %. The qPCR automation was equally successfully performed on two epMotion models: epMotion P5073 and epMotion 5075 allowing customer to select the most suitable automated solution to his needs. Those results clearly indicate that for scientists interested

in a low to a medium-throughput analysis, epMotion workstations can represent a perfect solution. By reducing human intervention and thanks to an accurate pipetting system, epMotion systems provide superior assay reproducibility without cross contamination, ensuring reliable results.

Literature

- [1] Espy M.J., Uhl J.R., Sloan M., Buckwalter S. P., Jones M.F., Vetter E.A., Yao J.D.C., Wengenack N.L., Rosenblatt J.E., Cockerill III F.R. and Smith T.F. Real-Time PCR in Clinical Microbiology: Applications for Routine Laboratory Testing. *Clinical Microbiology Reviews* 2006; 19:165-256.
- [2] Bustin S.A. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems *Journal of Molecular Endocrinology* 2002; 29:23-39.
- [3] Establishing the Performance Characteristics of In Vitro Diagnostic Devices for the Detection or Detection and Differentiation of Human Papillomaviruses. *Guidance for Industry and Food and Drug Administration Staff*. 2011.

Ordering information

Description	Order no. international
epMotion® P5073 for automated PCR set-up, 100–240 V/50–60 Hz, with CleanCap, EasyCon-control panel, Software epBlue™ and PCR-Assistants, TS 50, TS 300, Thermoblock and Rack 24 accessories	5073 000.002
epMotion® 5075mc with CleanCap configuration option including UV lights and HEPA filters	5075 000.305
TS50 single-channel Dispensing tool	5280 000.010
TS300 single-channel Dispensing tool	5280 000.037
Thermorack for 24x1.5/2 mL Safe-Lock Tubes	5075 771.004
Height adapter 40 mm for pipet tips	5075 755.009
epT.I.P.S.® Motion 50 µL Filter, sterile	0030 015.215
epT.I.P.S.® Motion 300 µL Filter, sterile	0030 015.231
RNA/DNA LoBind microcentrifuge tubes , 2 mL	0030 108.078
RNA/DNA LoBind microcentrifuge tubes , 1.5 mL	0030 108.051
Eppendorf twin.tec® real-time PCR plates 96 , semi-skirted	0030 132.505
HeatSealing PCR Film	0030 127.838
HeatSealer S100	5391 000.001

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