Applications

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Automated Quantitative PCR Set-up using the epMotion 5070 Workstation

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Introduction

The automation of diverse molecular biology methods has recently gained significance and is rapidly replacing classical manual protocols. Eppendorf has developed a product line that enables a streamlined and versatile approach to the automation of PCR-based applications [1].

In this report, the liquid handling station epMotion 5070, has been used for quantitative PCR (qPCR) set-up including the accurate pipetting of standard dilution series, as well as subsequent transfer of all reaction components to a reaction plate. This compact station provides an easy to operate system allowing precise and quick reagent handling of volumes down to 1 µl in a free jet mode.

For specific target amplification and signal detection, the Eppendorf RealMasterMix Probe was used, which is optimized for probe-hydrolysis based assays and features the patented HotMaster Taq DNA Polymerase facilitating automated reaction set-up at room temperature [2]. Two qPCR cycler platforms were used in the experiments: ABI Prism 7000 SDS and Bio-Rad iCycler iQ.

In summary, we report here automated protocols for quantitative PCR set-up using the Eppendorf liquid handling station epMotion 5070. By this means, the common pitfalls of manual set-up could be alleviated enabling a higher degree of reproducibility and precision.

Materials and methods

Reproducibility Assay
Primers and a FAM-labeled probe were commercially obtained (Applied Biosystems) and used to amplify and detect a segment of Rattus norvegicus beta-2-microglobulin (ß2M) gene (amplicon length not provided). The qPCR reaction and oligonucleotide sequence details are summarized in the Table 1.

Prior to automated reaction set-up, the epMotion 5070 worktable was cleaned with a 10% bleach solution, bi-distilled water and 70% ethanol. The 1x reaction mix contained all primers, probe (Applied Biosystems) and RealMasterMix Probe (Eppendorf) in a volume of 45 µl per reaction. Five microliters from the cDNA dilution series were added per reaction as template for a final reaction volume of 50 µl.

Cross-well Contamination Assay
Primers and a FAM-labeled probe were designed to amplify and detect a 78 bp segment of the human beta-2-microglobulin (ß2M) gene. The qPCR reaction and oligonucleotide sequence details are summarized in Table 1.

Prior to automated reaction set-up, the epMotion 5070 worktable was cleaned with a 10% bleach solution, bi-distilled water and 70% ethanol. The 1x reaction mix contained the final reaction concentration of primers, probe (TriLinks, USA), and RealMasterMix in a volume of 20 µl per reaction. 5 µl from the plasmid dilution series were added per reaction as template for a final reaction volume of 25 µl.

The Reaction Plate was heat-sealed, vortexed briefly and then cycled either on the Bio-Rad iCycler iQ or the ABI Prism SDS 7000 (data not shown). The detailed epMotion set-up procedure is summarized in Table 3. The reaction plate layout is shown in Figure 3B.

More information can be obtained online under www.epmotion.com.

Table 1: Oligonucleide sequences and qPCR reaction conditions

Experiment	Gene Name	Probe & Primers Sequence with T_M (°C)		Cycling Conditions # Cycles	Temp °C	Time min	Reaction Conditions
Reproducibility	Rattus norvegicus beta-2-microglobulin	Applied Biosystems TaqMan Gene Expression Assay Rattus norvegicus Endogenous Control: beta-2-microglobulin. Non-primer limited. FAM-labeled (4331182)		1 x	95	2:00	● 1x RealMasterMix Probe (Eppendorf) ● 1x Taq Man Gene Expression Assay incl. probe & primers (ABI) ● 5 µL cDNA Dilution **50 µl reaction vol.**
				50 x	95	0:15	
					60	1:00	
Contamination Survey	Homo sapiens beta-2-microglobulin	Forward	GGAATTGAT TTGGGAGAG CATC (55.2)	1 x	95	2:00	● 1x RealMasterMix Probe (Eppendorf) ● 0.2 µM each primer and probe ● 50 ng Human genomic DNA (Promega, G147A) or MBGW **25 µl reaction volume**
		Probe	[FAM]-AGTGTGACT GGGCAGATC ATCCACCTTC -[BHQ1] (65.5)	50 x	95	0:20	
					56	0:10	
		Reverse	CAGGTCCTG GCTCTACAA TTTACTAA (57.9)		68	0:30	

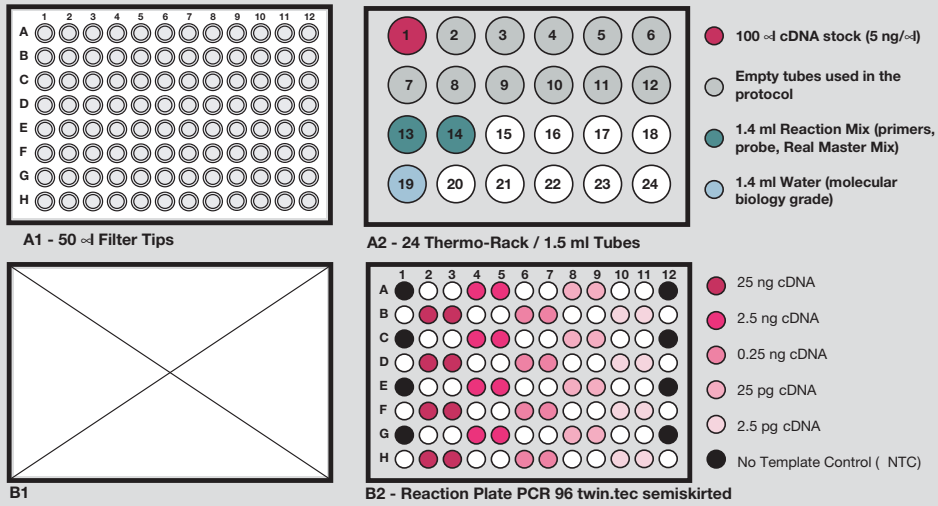

Fig. 1: ep*Motion* 5070 worktable and reagents layout

Table 2: Automated qPCR set-up procedures for the reproducibility assay

Reagent Transferred	Source	Destination	Comments
270 µl Water	A2 tube 19	A2 tubes 2-6	
450 µl Reaction Mix	A2 tubes 13-14	A2 tubes 7-12	
30 µl cDNA stock	A2 tube 1	A2 tubes 1-6	1:10 dilution series of cDNA stock; tube 6 contains water and is used as NTC in further steps
50 µl cDNA dilution series	A2 tubes 1-6	A2 tubes 7-12	Each dilution is transferred to the corresponding tube it the second row (with Reaction Mix) resulting in 500 µl mix containing all reaction components
50 µl all component reaction mix	A2 tubes 7-12	B2 Reaction Plate	Reaction mix containing all components is mixed and multidispensed in a semi-random pattern (Figure 1, position B2)

Table 3: Automated qPCR set-up procedures for the cross-well contamination survey

Reagent Transferred	Source	Destination	Comments
90 µl water	A2 Wells F-H Columns 6-7	B2 Wells F-H Columns 2-5	Water is dispensed to the Set-up Plate for serial dilution
5 µl water	A2 Wells A-E Column 8	B2 Wells A-E Column 9	Water is dispensed to the Reaction Plate for the No Template Control
20 µl Reaction Mix	A2 Columns 9-12	B2 Columns 1-12	Reaction Mix is transferred from the Set-up Plate to the Reaction Plate
5 µl plasmid template	B1 Columns 1-8	B2 columns 1-8	DNA template is transferred column-wise from the Template Plate to the Reaction Plate
50 µl plasmid stock	A2 Wells F-H Column 1	A2 Wells F-H Columns 2-5	Serial dilution of the plasmid stock 1:10
5 µl plasmid dilution	A2 Wells F-H Columns 2-5	B2 Wells F-H Columns 9-12	Standard dilutions of the template are transferred from the Set-up Plate to the Reaction Plate

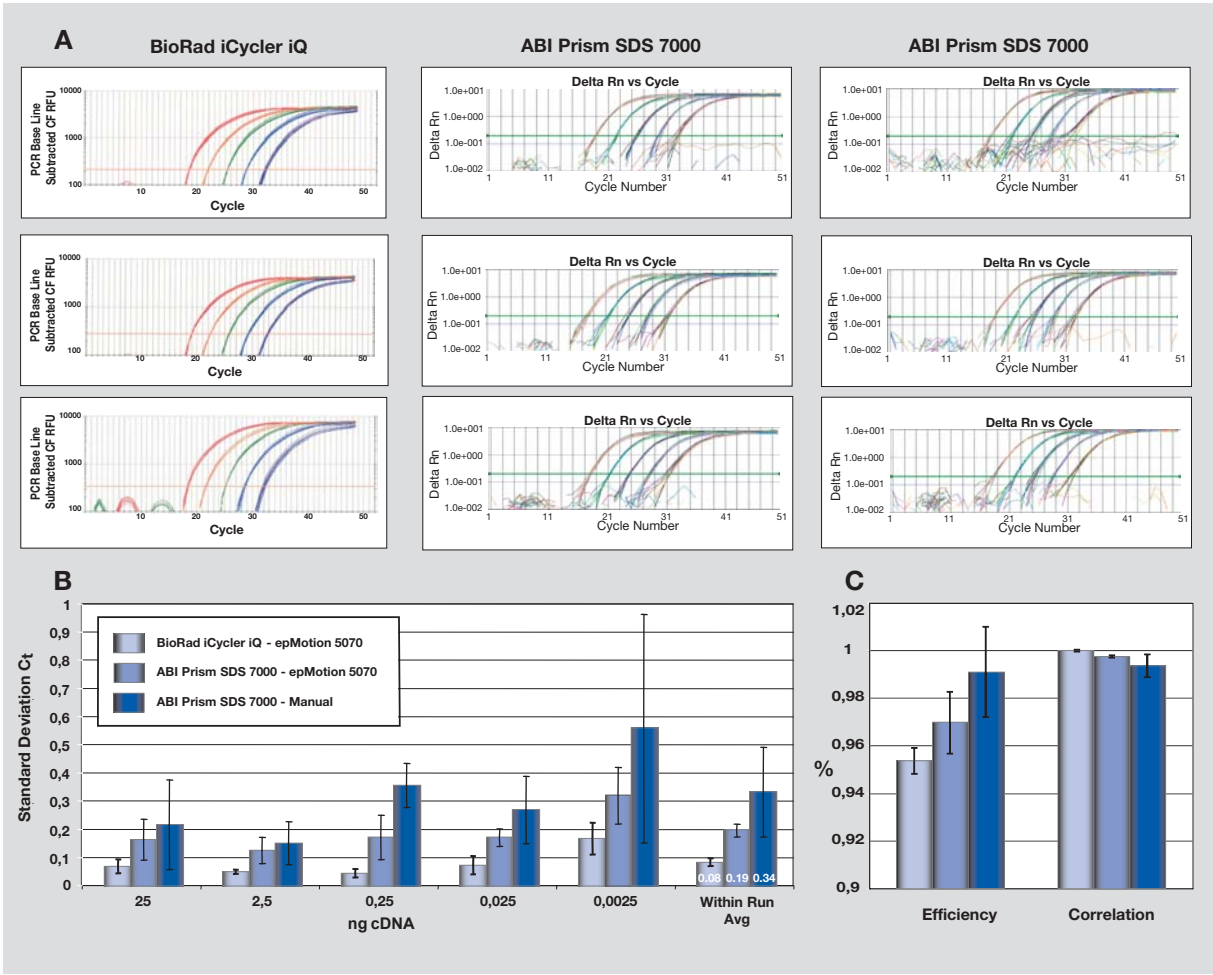

Fig. 2: Reproducibility of qPCR reaction set-up using epMotion 5070. The amplification plots of manual versus automated set-up are compared in A. The average C_t standard deviations (SD) for each template concentration are presented in B. The respective average C_t SD across all sample replicates (8 replicates x 5 concentrations) is depicted as All Run Avg. The PCR reaction efficiencies and correlation coefficients of the standard curves are depicted in C.

Results

Reproducibility Assay: Five point standard curve on two cycler platforms: Bio-Rad iCycler and ABI 7000 SDS

The manual and automated assays were compared and their overall performance, replicate reproducibility and standard curve parameters were assessed.

The amplification plots for the automated set-up show high reproducibility within each replicate series and across the three separate runs on each platform (Figure 2A). The automated set-up showed a lower standard deviation both within and between runs as compared to the manual set-up (Figure 2B). The average C_t standard deviation between all samples (All Run Avg, Figure 2B) was 0.083 C_t for the Bio-Rad/epMotion, 0.196 C_t for the ABI/epMotion and 0.333 C_t for the ABI/manual set-up, respectively. Furthermore, smaller standard deviation in the reaction efficiencies as well as better standard curve correlation coefficients were obtained while setting up the reactions automatically (Figure 2C).

Cross-well contamination assay: 60 reactions alternating between DNA positive and negative controls – standard curve

In the cross-well contamination experiment the template was added individually to each well instead of creating a replicate mix with all components. This type of set-up is more representative of a screening assay, where each well or set of replicates is expected to contain a different template preparation. Since it involves more pipetting steps, it is generally more prone to errors and may show larger pipetting deviations. We observed no contamination between adjacent wells during automated reaction set-up using epMotion 5070 (Figure 3A) and the amplification plots show high reproducibility (std dev = 0.23 C_t) between all Positive Control sample replicates. Noteworthy, none of the NTC samples showed any amplification signal (Figure 3A) along all 50 cycles of the reaction. The average standard deviation across the four dilutions was 0.14 Ct. The standard curve generated in this experiment showed high efficiency (99.8%) and correlation coefficient (0.998).

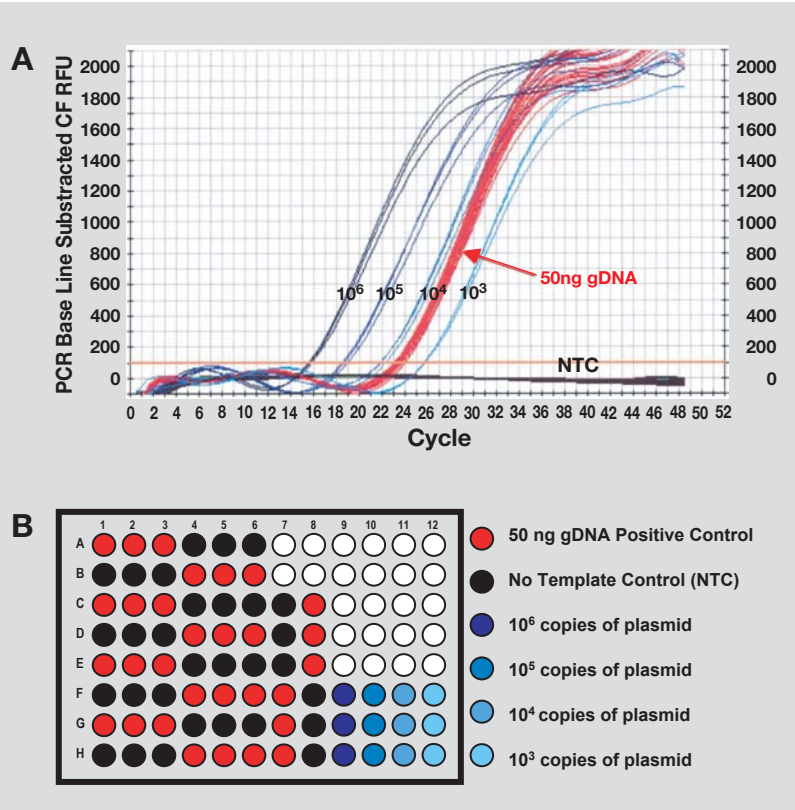

Figure 3: Cross-well contamination survey of epMotion 5070 qPCR set-up. Amplification plots (A) and corresponding reaction plate layout are presented (B). The samples and their plots are shown in corresponding colors.

Conclusions

In this study, we report the use of a versatile and compact liquid handling station - epMotion 5070 - for automated qPCR set-up. The overall assay performance, the reproducibility of the replicates as well as the characteristics of the standard curves indicate that the automated set-up can provide better results than manual protocols.
In particular, we were able to demonstrate very low standard deviation and lack of contamination, which provides significant advantages for most molecular biology and clinical research applications employing Real Time qPCR. These results indicate that reproducible data can be reliably achieved and that the influence of user-errors can virtually be eliminated. The system can perform complex set-up tasks in short times and helps to achieve precise target quantification on a routine basis. Furthermore, high reproducibility and precision opens the door to reduced sample replicate numbers and lower reaction volumes – helping to reduce costs and increase sample throughput. Once programmed, the whole protocol can be easily executed thereby reducing the hands-on steps required for qPCR set-up.
As an additional benefit, the pipetting tools can easily be changed, the method files modified, and the labware adapted to accommodate various different assay designs, offering the user an easy to use and flexible pipetting system for routine liquid handling tasks.

Literature

[1] Apostel F. (2003), Facilitating PCR set-up via an automated liquid handling system. International Biotechnology Laboratory; 12: 1-2

[2] Grutt J., Westberry R., Goodrich J., Peters L., Halcome J., Huit G. (2004), Eppendorf RealMasterMix Probe – A Novel System for Quantitative PCR with Target-specific Probes. Application Note No. 82, Eppendorf AG, 05/04

Ordering information

Products used in this application	International Ordering No.	North American Ordering No.
epMotion 5070	5070 000.000	960000005
Dispensing Tool TS 50	5280 000.010	960001010
Dispensing Tool TS 300	5280 000.037	960001028
Dispensing Tool TM-8 50	5280 000.215	960001044
Dispensing Tool TM-8 300	5280 000.231	960001052
epMotion Tips 300 Filter (15x96)	0030 003.977	960050061
epMotion Tips 50 Filter (15x96)	0030 003.950	960050029
Thermoblock 96	5075 766.000	960002083
Thermorack 24 (1,5 ml and 2 ml)	5075 771.004	960002075
Thermoadapter Frosty	5075 789.000	960002300
Tub Holder	5075 754.002	960002148
30 ml Reservoirs (50)	0030 126.505	960051009
Twin.tec PCR Plate 96, semi-skirted (25)	0030 128.575	951020303
Safe-Lock 1.5 ml, individually sealed, 100 pcs	0030 121.589	22600028
Safe-Lock 2.0 ml, individually sealed, 100 pcs.	0030 121.597	22600044
RealMasterMix Probe 2,000 reactions at 50 µl	0032 002.460	954160017
RealMasterMix Probe ROX 2,000 reactions at 50 µl	0032 002.470	954160114
Heat Sealer	5390 000.024	951023078
Heat Sealing Film (10x10)	0030 127.650	951023060
epMotion Editor complete Version	5075 014.009	960000269

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