Applications

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Low volume real-time PCR on the Mastercycler® ep *realplex*

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Abstract

This Application Note provides a comparative overview of real-time PCR reactions at 20 µl, 10 µl and 5 µl reaction volumes, each generated on the Mastercycler ep *realplex* real-time PCR system. SYBR® Green I- as well as TaqMan® probe-based assays were performed, and the reaction setup was done with the aid of the ep*Motion*® 5070 automated pipetting system.

It is shown that real-time PCR reactions with small reaction volumes can successfully be performed on the Mastercycler ep *realplex*: The reaction efficiencies obtained for the presented assays, including the lowest volume reactions, were comparably good and the C_t values obtained from the 10 µl- and 5 µl- reactions were only slightly higher than those detected for the 20 µl setups.

Introduction

While PCR setups of 50 µl or 25 µl used to be customary, there is nowadays a trend towards carrying out PCR in lower reaction volumes [1].

One of the advantages when working with reduced volumes is that a correspondingly lower amount of template is needed to achieve a particular DNA template concentration in the reaction sample (Table 1). This is of particular importance when only small amounts of template are available, for example in gene expression studies using degraded biological starting materials or when working with forensic sample material [1].

As an additional benefit, faster run times can be achieved by reducing the temperature holding times in the PCR protocol, since the programmed temperatures are transferred to the sample more quickly in a smaller reaction volume. However, in many cases the main reason for reducing the reaction volume is to minimize reagent costs. These reagents, in particular the kits and the fluorescence-labeled DNA probes, comprise a significant share of total cost during routine use of real-time PCR. Therefore, depending on the sample throughput of the laboratory, significant savings can be achieved by reducing the reaction volume.

Materials and Methods

With real-time PCR, two different targets were amplified:

1 PCR target: 108 bp, fragment of Lambda DNA
Forward primer (600 nM): cgcacaggaactgaagaatg,
Reverse primer (300 nM): ccgtcgagaatactggcaat,
Lambda TaqMan probe (labeled with FAM, 200 nM): tgtactttcgtgctgtcgcggatcg
Template: Lambda DNA (Roche)

2 PCR target: 80 bp, fragment of human SRY gene
Forward primer (300 nM): gcgacccatgaacgcatt,
Reverse primer (300 nM): agtttcgcattctgggattctct,
TaqMan probe (labeled with FAM, 200 nM): tggtctcgcgatcagaggcgc Template: Human Genomic DNA (Roche)

For each PCR reaction SYBR Green I- and TaqMan probe-based assays were carried out and each setup was done with 20 µl, 10 µl, and 5 µl reaction volumes.

For pipetting the reaction setup, a Mastermix (consisting of 5Prime RealMasterMix, SYBR Green and primer or 5Prime RealMasterMixProbe, TaqMan probe and primer) and the highest template DNA concentration were each initially prepared manually for each reaction (Table 1). In subsequent steps, the reaction samples were pipetted with the ep*Motion* 5070 automated pipetting system.

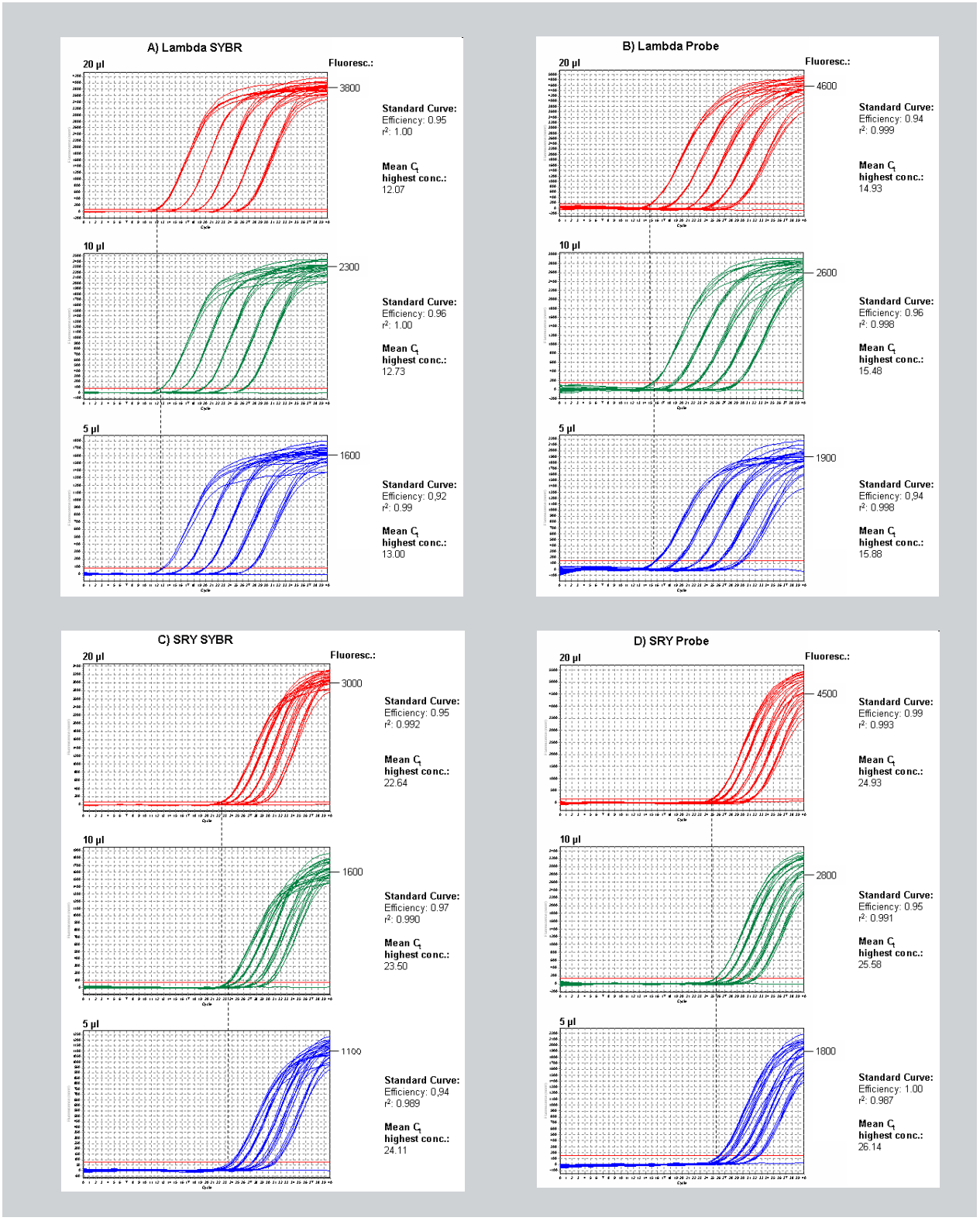

Figure 1: Selected amplification plots and parameters of the 20 µl, 10 µl und 5 µl Lambda (A, B) and SRY (C, D) assays.

The ep*Motion* was programmed to dispense Mastermix into the corresponding wells of an Eppendorf twin.tec PCR Plate 96 skirted, to pipette a dilution series starting with the highest template concentration and then to add template DNA to the appropriate wells:

20 µl total reaction volume: 12 µl Mastermix + 8 µl template DNA
10 µl total reaction volume: 6 µl Mastermix + 4 µl template DNA
5 µl total reaction volume: 3 µl Mastermix + 2 µl template DNA

A 5-step serial dilution with five replicates each was pipetted to generate a standard curve that permitted comparison of the PCR results. While the Lambda DNA standard samples were diluted ten-fold, the standard samples for human genomic DNA were diluted three-fold.
A negative control was included in each assay by substituting water for template DNA.

The Eppendorf twin.tec PCR Plates 96 skirted were sealed with Eppendorf Heat Sealing Film and then subjected to a short spin (approx. 500 x g) prior to the run.
The PCR setup for all assays was carried out with the same ep*Motion* 5070 automated pipetting system and all real-time PCR runs were performed on the same Mastercycler ep *realplex*^{4} S device (Table 2).

Results

The amplification plots of the presented assays are comparably steep for all reaction volumes (Figure 1).
Absolute fluorescence intensity is lower at the smaller reaction volumes. However, even in the case of the lowest fluorescence intensity (5 µl SRY SYBR assay) the analysis can be performed easily with adjusted scaling.
PCR efficiencies of the shown assays, calculated from the C_t values of the standard samples, differ only minimally among the various reaction volumes of the particular PCR system. The r^2-coefficients of the Lambda assays do not show a decline at smaller reaction volumes (≥ 0.99 for all). The r^2-values of the 5 µl setups for the SRY assays are, with values of 0.989 and 0.987, slightly below the 10 µl and 20 µl setups (Figure 1).

Table 1: Highest template concentration and its appropriate template amount for the 20 µl, 10 µl and 5 µl reaction volumes

Reaction Setup	Lambda		SRY	
	highest template conc. [cop./µl]	template amount [cop./rxn]	highest template conc. [ng/µl]	template amount [ng/rxn]
20 µl	$2.5x10^5$	$5x10^6$	1.215	24.3
10 µl		$2.5x10^6$		12.15
5 µl		$1.25x10^6$		6.075

When considering all assays shown in Figure 1, the mean replicate C_t values of the 10 µl reaction samples are, at the highest template concentration level, between 0.55 and 0.86 higher compared to the 20 µl reactions. For the 5 µl setups an increase of between 0.27 and 0.61 relative to the 10 µl assays was observed.

Discussion

The selected assays may indicate that there is a small loss in sensitivity for the low volume qPCR assays. The C_t values of the 10 µl reactions were only slightly higher than those detected for the 20 µl setups (Figure 1). One reason for the small shift of the C_t value at lower volumes could be explained by the fact that due to the smaller amount of template used (Table 1), the increase of PCR product amount during the course of the reaction was delayed accordingly. Since the intensity of the detected fluorescence signal is proportional to the amount of PCR product, a delayed increase in the fluorescence signal at constant instrument detection sensitivity is observed. For many applications, this small C_t value shift does not pose a problem. However, this should be taken into account particularly when working with small amounts of template (low copy PCR).
The reaction efficiencies obtained for all assays shown in Figure 1, in particular those with low reaction volumes, were comparably good with values above 0.92.
The reactions presented here were all performed successfully using the same reagents, concentrations and reaction vessels. The Eppendorf twin.tec PCR Plate 96 skirted used in this study has got a relatively low total reaction volume of 150 µl/well. Also, by sealing the plate with an Eppendorf Heat Sealing Film, the risk of evaporation is almost eliminated, which is of particular importance when working with small reaction volumes. It must be assumed that not all reaction conditions are equally suited for the establishment of small reaction volumes. In order to assess this, reduced-volume PCR reactions should be evaluated in direct comparison to larger volumes, prior to the routine use of small reaction volumes [2].
The integrity of the data fit to the theoretical line of the standard curve is described by the r^2-coefficient [3].

Table 2: PCR programs on the Mastercycler ep *realplex*^{4} S

Reaction	PCR program			
	95 °C	95 °C	60 °C	melting curve (default settings)
Lambda	2 min	10 s	40 s	only with SYBR
SRY	2 min	10 s	30 s	only with SYBR
		40 cycles		

Lower r^2-coefficients of the presented SRY 5 µl setups may indicate that the lower reaction volumes put higher demands on liquid handling accuracy during PCR setup (Figure 1). In order to ensure reproducible results when routinely working with small volumes, it is recommended to employ an automated PCR setup, such as the ep*Motion* automated pipetting system. These results demonstrate that real-time PCR with small reaction volumes can successfully be performed on the Mastercycler ep *realplex*. With the aid of the ep*Motion* automated pipetting system, up to 75% savings in reagent costs can be achieved compared with 20 µl real-time PCR reactions.

References

[1] Leclair B, Sgueglia JB, Wojtowicz PC, Juston AC, Fregeau CJ, Fourney RM. STR DNA typing: increased sensitivity and efficient sample consumption using reduced PCR reaction volumes. J Forensic Sci 2003; 48(5):1001-1013.

[2] Thomson E, Vincent B. Reagent volume and plate bias in real-time polymerase chain reaction. Analytical Biochemistry 2005; 337: 347-350.

[3] Dorak T (ed.). Real-time PCR. Taylor & Francis Group; 2006.

Ordering information

Product	Description	Order no. International	Order no. North American
Mastercycler® ep *realplex*^{2}	with aluminum block		
	and two emission filters	6300 000.507	950020202
Mastercycler® ep *realplex*^{2} S	with silver block		
	and two emission filters	6300 000.604	950020211
Mastercycler® ep *realplex*^{4}	with aluminum block		
	and four emission filters	6302 000.504	950020300
Mastercycler® ep *realplex*^{4} S	with silver block		
	and four emission filters	6302 000.601	950020318
ep*Motion*® 5070	Liquid Handling Workstation	5070 000.000	960000005
twin.tec PCR Plate 96 skirted	25 pcs	0030 128.648	951020401
Heat Sealing Film	10 x 10 pcs	0030 127.650	951023060
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