Effect of increasing reaction volumes on PCR

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Total reaction volume affects the efficiency of heating and cooling on the Spartan DX™. It was found that 20 µl is the ideal reaction volume, although volumes from 10-50 µl may be used.

Introduction

Due to the high heat capacity of liquids, the volume of a PCR reaction can affect the rate of temperature transition and maximum/minimum temperatures achieved during cycling. Temperature differences may have effects on the efficiency of template denaturation, primer annealing, polymerase extension and the formation of non-specific products (Ref 1). Since different primers and templates have different melting temperatures, the effect of reaction volume on liquid temperature may impact each reaction differently.

The purpose of this study was to determine the effect of reaction volume on real-time PCR with the Spartan DX.

Material and Methods

DNA extraction

DNA was extracted from clinical isolates of Methicillin-resistant *Staphylococcus aureus* (MRSA). For each isolate, 4-5 medium-sized bacterial colonies were re-suspended in 100 µl of lysis buffer (50 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, pH 8) with 2 µl of 1 mg/ml lysostaphin (Sigma-Aldrich, Cat No. l7386). The samples were incubated at 37°C for 30 min. Following this incubation, 5 µl of 20 mg/ml Proteinase K (Sigma-Aldrich, Cat. No. p2308) was added to the mixture and the tubes were shaken at 50°C for 1 h. The tubes were then incubated at 100°C for 10 min to inactivate the Proteinase K. Samples were adjusted to a concentration of 435 ng/µl and stored at 4°C.

Determination of actual reaction liquid temperature

Two thermocouples (Omega, Part#5SRTC-TT-T-40-72) were used to measure the reaction liquid temperature. Specifically, a thin-wall 0.2 ml polypropylene tube (Axygen) was altered in the following manner: a hole 1 mm in diameter, was drilled into the top of the tube, two thermocouples were threaded through the hole, and the tips of the thermocouples were positioned 1-2 mm from the bottom and just below the meniscus (Fig 1). The tube was then filled with 10, 20, 35, and 50 µl of distilled water and topped with mineral oil (see Columns 1 & 2 in Table 3). Table 2 lists parameters for the cycling program. Temperature data from the thermocouples were recorded with a Data Logger (Fluke, 54II Thermometer).

Real-time PCR

Oligonucleotide primers were designed against the *mecA* gene for methicillin resistance and the *16S rRNA* gene. For the *mecA* gene, the forward primer sequence was 5'- tcc agg aat gca gaa aga cca a -3', and the reverse primer was 5'-ggc caa ttc cac att gtt tcg -3'. The expected amplicon size was 92 bp. For the *16S* gene, the forward primer sequence was 5'-cga aag cgt ggg gat caa ac-3', and the reverse primer was 5'-ccc agg cgg agt gct taa tg-3'. The primers were designed with Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The expected amplicon size was 125 bp.

Components of the real-time PCR amplification mixture are listed in Table 1. SYBR® Green I dye (Invitrogen, Cat. No. 57563) was used for fluorescent detection. Reaction volumes were 10, 20, 35, and 50 µl which were topped with 10, 15, 20, or 28 µl of mineral oil (Biotools, Cat. No. 20.032) to prevent evaporation. Samples were loaded into 0.2 ml thin-wall, flat-cap PCR tubes (Axygen, Cat. No. PCR 02C), and real-time PCR was performed with the Spartan DX. Table 2 shows

Component	Final amount
10X PCR Reaction Buffer (No $MgCl_2$) (Invitrogen)	1 X
$MgCl_2$ (Applied Biosystems)	2.5 mM
dNTP mix (Invitrogen)	0.125 mM
Taq DNA polymerase (Biotools)	1 U
SYBR Green I (Invitrogen)	0.5 X
PCR primers (Sigma-Aldrich)	0.5 µM
Template DNA	1.5 µg
Sterile water	
Total reaction volume	See ***Table 4***

Table 1. Components of amplification mixture.

Step	Temperature	Time	Cycles
Initial denaturation	93.8°C	30 s	1
Denaturation	93.8°C	30 s	30
Annealing/extension	57.4°C	30 s	30

Table 2. Cycling parameters.

Liquid Volume (µl)		Temperature Gradient* (°C)		Expected PCR Product formed	
Reaction mix	Oil Overlay	Denaturation	Annealing/Extension	*mecA*	*16S*
10	10	3	4	yes	yes
20	15	5	5	yes	yes
35	20	5	6	yes	no
50	28	8	7	yes	no

Table 3. Temperature gradients measured during PCR cycling for various reaction volumes(* see Figure 1).

the cycling parameters for a two-temperature program that combines the primer annealing and extension steps.

DNA analysis

Fluorescence values were downloaded from the Spartan DX to a computer and graphed using Microsoft Excel®. Real-time results were confirmed by gel electrophoresis using 10 µl of the PCR amplification products.

Results

The setup shown in Figure 1 was used to determine the vertical temperature gradients in PCR tubes with increasing volumes. The observed gradient ranged from 3-8°C, and larger volumes had larger vertical gradients (Table 3). We used this as an indication that the average liquid temperature was decreasing as volume increased. To test if this observation would affect real-time PCR amplification we used two separate primer sets. We found that reaction volume only affected the PCR efficiency with the *16S* primer set (Table 3).

Discussion and Conclusion

Larger reaction volumes result in larger liquid temperature gradients and this can prevent successful PCR for some primers. In the case of the 16S primers, reaction volumes greater than 20 µl were unable to perform PCR with comparable efficiency to smaller volumes. The reason may be that the larger temperature gradients inhibited primer annealing and/or amplicon denaturation, resulting in inefficient PCR.

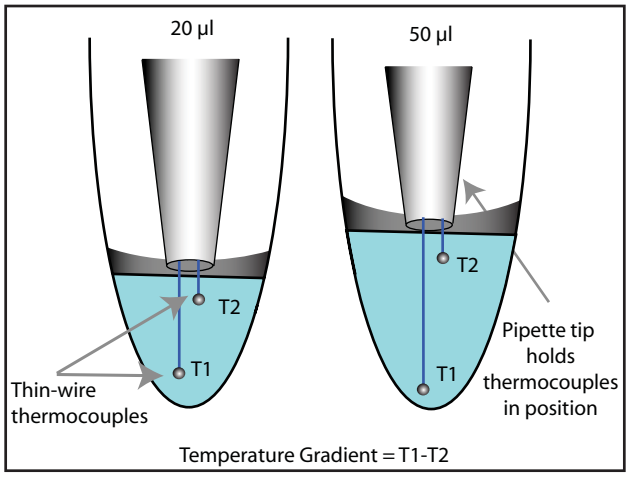

Figure 1. Diagram showing the thermocouple positions used to determine the Temperature Gradient across the tubes.

The 10 µl reactions gave lower fluorescent signals than larger volumes and the reason may be that the area of fluorescence is smaller with smaller volumes. This may have reduced the amount of fluorescent signal detectable by the Spartan DX's optical detection system.

In conclusion, 20 µl reaction volumes are ideal for the Spartan DX, although volumes from 10-50 µl may work depending on primer and amplicon characteristics.

References

1. Wittwer CT, Garling DJ. (1991). Rapid cycle DNA amplification: time and temperature optimization. *Biotechniques.* 10(1):76-83.