Low-Volume Amplification of DNA Using the C1000™ Thermal Cycler

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Introduction

PCR amplification is a powerful tool used in a variety of molecular biology techniques, such as cloning, gene expression profiling, site-directed mutagenesis, and genetic mapping. For most applications, PCR is performed in 20–50 µl reaction volumes, which provide ample product for post-PCR analysis and manipulation. However, for PCR-based screening applications, only enough DNA needs to be produced to adequately test for the presence of a DNA fragment after the reaction. In these applications, there are clear advantages to performing reactions in smaller volumes, as the miniaturization of PCR allows increased throughput and reduced reagent costs.

In many cases, low-volume PCR (1–5 µl) can yield variable results due to problems with thermal uniformity and accuracy of many thermal cyclers, as well as poor reaction vessel sealing.

The Bio-Rad 1000-series thermal cycling platform (comprising the full-featured C1000 and the basic S1000™ thermal cyclers) has been engineered for extremely uniform and reliable thermal performance. The 1000-series thermal cyclers have six independently controlled thermal electric units to maintain temperature uniformity at all times during the run, even during ramping. The average ramp rates, combined with a 10 sec settling time, deliver fast run times while also maintaining excellent thermal accuracy and uniformity. These instruments are ideally suited for successful PCR in low volumes.

We evaluated the ability of the C1000 thermal cycler equipped with a 384-well reaction module to successfully amplify four target sequences from human genomic DNA in 1, 2, 5, and 10 µl reaction volumes. We demonstrated robust and reproducible amplification of the four genomic DNA targets at all of the reaction volumes tested.

Methods

All PCR amplifications were performed on a C1000 thermal cycler equipped with a 384-well reaction module using Hard-Shell® thin-wall 384-well skirted PCR plates sealed with Microseal® 'B' adhesive seal (Bio-Rad Laboratories, Inc.). The melting temperature (T_m) of each primer (Table 1) was determined using the Ta Calc feature of the Protocol Autowriter available on the C1000 thermal cycler.

Reactions were prepared using iQ™ supermix (Bio-Rad), which is supplied as a 2x ready-to-use solution with optimal concentrations of reaction components, 0.25 µM of each primer, and human genomic target concentrations of 1,000 or 2,000 human genome equivalents per µl (K562 DNA, Promega Corporation). Reaction components were assembled as a master mix, then dispensed in the desired volumes into the appropriate reaction plate wells. Three replicate reactions were performed for the 2, 5, and 10 µl volumes for each of the amplicons. Six replicate reactions were prepared for the 1 µl volume. Control reactions that contained all of the reaction components except the target DNA were performed for each volume. The 1 and 2 µl reaction volumes were manually pipetted using a P2 Pipetman (Gilson, Inc.). Calibration of the pipets was checked with microcapillary Drummond pipets (Sigma-Aldrich Co.). The plate was sealed with Microseal 'B' adhesive seal and spun at 750 x g for 2 min prior to and after thermal cycling.

The protocol used for amplification included the following steps: 95°C for 3 min; 35 cycles of 95°C for 10 sec, 55°C for 20 sec, and 72°C for 30 sec; 72°C for 10 min; 4°C hold. The lid temperature was set to 100°C. The annealing temperature was 55°C (2.5°C below the average T_m of the primer pair with the lowest T_m, β-globin-261-60). The final 10 min incubation at 72°C was used to ensure significant amplicon reassociation. The reaction module adjustable lid was rotated approximately two-thirds turns past the touch point to provide appropriate sealing pressure for the Microseal 'B' adhesive seal.

Table 1. Target genes with primer and amplicon characteristics.

Target Gene	Amplicon Name	Amplicon Size, bp	Primer Sequence*	Primer T_m	Average T_m of Primer Pair
β-globin	β-globin-261-60	261	F: GAG GAG AAG TCT GCC GTT AC R: GAC AGA TCC CCA AAG GAC TC	56.5 58.5	57.5
Mannose receptor binding protein	TIP-171	171	F: TTT TCC AGC ATC CAC TCC TTC R: GGC TTT CTC AGT GAT TCC AGG	61.3 61.0	61.2
Glyceraldehyde phosphate dehydrogenase	GAPDH-494-60	494	F: CTG GAG CCT TCA GTT GCA G R: GAA GAT GGT GAT GGG ATT TC	59.7 56.9	58.3
Amelogenin	AMEL	106–112	F: CCC TGG GCT CTG TAA AGA ATA GTG R: ATC AGA GCT TAA ACT GGG AAG CTG	62.7 62.6	62.7

* F, forward; R, reverse.

A set of loading dye solutions was prepared at various concentrations. After thermal cycling, appropriate volumes of the loading dye solutions were added to the reactions such that 5 µl at 1x loading dye concentration (containing 0.1% Orange G and 3% glycerol in 1x PCR buffer) could be loaded onto the gel (Table 2). Orange G was used as a gel loading dye because it migrates in these gels well ahead of the 50 bp size marker. The traditional loading solution dye, xylene cyanole, migrates with 200–300 bp of DNA and can lessen the apparent intensity of the ethidium bromide fluorescence of comigrating DNAs.

Table 2. Preparation of samples for loading onto gel.

	Loading Dye*			
Reaction Volume, µl	Stock Concentration	Volume Added, µl	Final Concentration	Volume Loaded Onto Gel, µl
10	5x	2.5	1x	5
5	5x	1.25	1x	5
2	1.4x	5	1x	5
1	1.2x	6	1x	5

* 1x loading dye solution = 0.1% Orange G, 3% glycerol, 50 mM KCl, 10 mM Tris-HCl, pH 8.0.

Analysis of amplified DNA fragments was performed using 3% ReadyAgarose™ 96 Plus precast gels in TBE buffer containing ethidium bromide (0.5 µg/ml) (Bio-Rad). For each reaction, 5 µl of PCR product loading dye mix was loaded onto the gel, and electrophoresis was performed at 60 V for 40 min. An AmpliSize® molecular ruler 50–2,000 bp ladder (Bio-Rad) was used to determine amplicon sizes. Gels were imaged using the Molecular Imager® Gel Doc™ XR system (Bio-Rad).

The yield of each amplification reaction was measured by fluorogenic intercalation of Hoechst 33258 dye (Sigma-Aldrich Co.) using the DyNA Quant 200 fluorometer (Hoefer, Inc.).

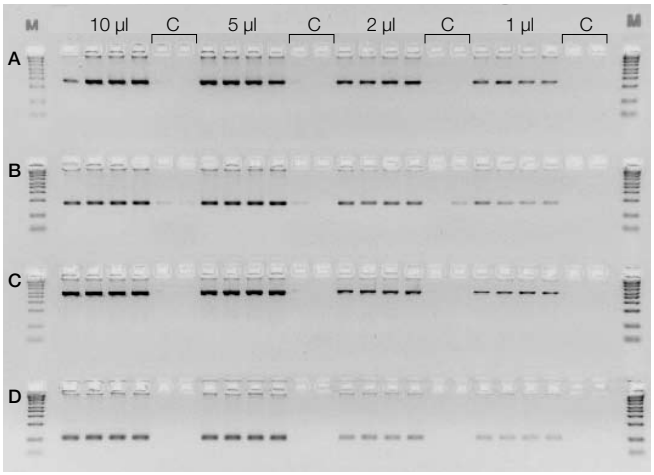

Fig. 1. Ethidium bromide fluorescence gel image of the reaction products. After adding loading dye solution to 1x (see Table 2), 5 µl of the resulting mix was run on a 3% agarose TBE gel and imaged with the Molecular Imager Gel Doc XR system (3.5 sec exposure). **A**, β-globin, 261 bp; **B**, TIP-171, 171 bp; **C**, GAPDH, 494 bp; **D**, AMEL, 106–112 bp. Lanes marked C are control reactions (no target). All other reactions began with ~1,000 human genomes/µl. M, molecular ruler.

Results and Discussion

All of the reactions performed using 1, 2, 5, and 10 µl reaction volumes amplified a DNA fragment of the expected size (Table 1) in sufficient quantities to be visible on a gel (Figure 1).

Although all of the reactions produced sufficient specific products to see on the ethidium bromide-stained gel, we wanted to obtain a more quantitative understanding of the relationship between reaction volume and PCR yield. To this end, additional reaction volume sets were run and the concentration of dsDNA products was determined for each reaction volume. We analyzed three replicates of the 2, 5, and 10 µl reactions, and six replicates of the 1 µl reactions for each. The yields of amplified double-stranded PCR product ranged between 6 and 23 ng/µl (Table 3 and Figure 2). These values are consistent with the gel image (Figure 1) and with the detection limits of dsDNA on this agarose/ethidium bromide gel, which are in the range of 1–5 ng per band.

Table 3. Amplicon yields expressed in ng/µl.

	10 µl Reactions*			5 µl Reactions*			2 µl Reactions*			1 µl Reactions**		
	Average	Range	SD	Average	Range	SD	Average	Range	SD	Average	Range	SD
Amplicon												
β-globin-261-60	14.7	14–16	1.16	13.7	13–15	1.16	12	11–13	1	10.3	10–12	1.16
TIP-171	8.7	6–11	0.58	9.7	9–11	1.16	7.3	6–8	1.16	8	6–12	3.06
GAPDH-494-60	21.3	19–23	1.53	19.3	19–20	0.58	16	15–17	1	16.7	16–18	1.16
AMEL	15.5	14–17	1.53	14	13–15	1	12.3	12–13	0.58	10.5	8–14.7	3.40

* n = 3.

** n = 6.

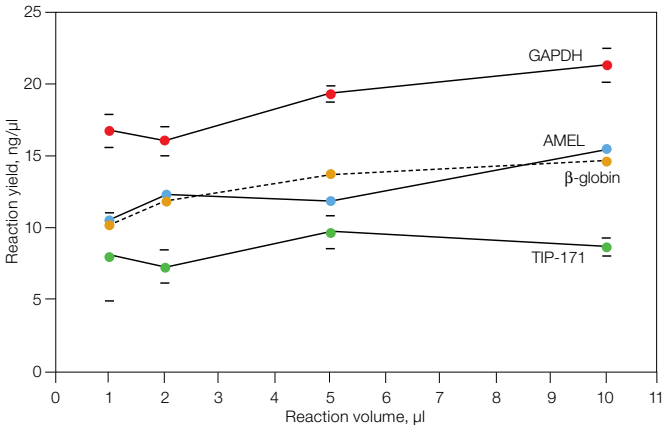

Fig. 2. Average concentrations of PCR products at four reaction volumes as determined by fluorogenic intercalation of Hoechst 33258 dye. For all volumes, three replicates were measured, except the 1 µl reactions, where six replicates were measured. The bars on the GAPDH and the TIP-171 data are ±1 standard deviation. They are not shown for the AMEL and the β-globin data for clarity, but are of the same magnitude as those shown.

For at least three of the four amplicons there appears to be a trend toward slightly lower yields as the reaction volume decreases. This phenomenon could be due to reduction in reaction efficiency due to slight evaporative losses. The data indicate that in the 1 and 2 µl reaction volumes, the yield reduction varies for each amplicon and ranges from about 15 to 30% compared to the 10 µl reactions. However, for all amplicons tested, the yields in the 1 and 2 µl reactions are sufficient to produce the specific and easily visible bands in an agarose/ethidium bromide gel.

Evaporation is a major concern when performing PCR amplifications in low volumes. Even when a tight seal is created using an adhesive sealer, small reaction volumes may be significantly affected by residual evaporation that takes place until the saturation vapor pressure is reached in the reaction vessel. The small volume of the wells in the 384-well plate minimizes evaporative loss from the reaction. The use of Microseal 'B' adhesive seal combined with proper pressure from the reaction module lid results in no visible loss of reaction volume at the end of the thermal cycling protocol. If higher sensitivity in gel analysis is required to detect smaller fragments or amplicons that do not amplify as efficiently, more sensitive fluorescent dyes, such as PicoGreen (Ahn et al. 1996) or SYBR® Green I, can be used to stain the gels.

Ways to potentially increase reaction yields at all volumes include starting with higher initial target concentrations, increasing primer concentrations, and increasing the number of repeat cycles in the protocol, especially if the initial target concentration is low (for example, <100 targets/µl).

Conclusions

We demonstrated that the C1000 thermal cycler equipped with a 384-well reaction module can accurately and reproducibly amplify target sequences from genomic DNA in as little as 1 µl reaction volumes. We showed that even fragments in the 100 bp size range can be amplified in quantities sufficient to be easily detectable on an agarose/ethidium bromide gel.

Reducing the total reaction volume used for a PCR assay can greatly reduce reagent costs in high-throughput experiments. Success in miniaturization of reaction volumes depends on precise thermal control of the thermal cycler, stability of the reagents at small scales, superior sealing of reaction vessels to prevent evaporation, and the ability of the reaction to withstand residual evaporation.

The precise temperature control of the 1000-series thermal cyclers allows fast temperature ramping while maintaining tight uniformity across the heating block throughout the ramp. This results in reaching the target temperature and beginning the incubation time more quickly, for faster overall run times. The C1000 and the S1000 reaction modules have fully adjustable heated lids that snap tight when locking. The lid is designed to lock below a housing lip that provides strong and even downward force on the block, thereby minimizing evaporative sample loss and supporting low-volume PCR amplifications.

Reference

Ahn SJ et al. (1996). PicoGreen quantitation of DNA: effective evaluation of samples pre- or post-PCR. Nucleic Acids Res 24, 2623-2625.

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