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Notes & Tips

Reagent volume and plate bias in real-time polymerase chain reaction

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When optimal conditions are employed, real-time polymerase chain reaction (PCR) is a sensitive and accurate technique enabling the quantification of low-copy-number transcripts [1]. However, as with conventional PCR, small variations in initial reaction conditions are amplified exponentially and can significantly affect results [2]. Uniform reaction conditions are therefore essential to achieve accuracy and reproducibility during transcript quantification. Reagent costs are considerable, and in a high-throughput setting a reduction in the reagent volume used in each reaction would significantly reduce the cost of real-time PCR. However, the effect of reduced volume on the accuracy of results in a plate-based system has not, to our knowledge, been examined. Here we show that lower reagent volumes can reduce reproducibility by enhancing a bias in results across a plate.

Total RNA was isolated from confluent A549 lung epithelial type II cells (ATCC, Manassas, VA) using the TRIzol method (Invitrogen Canada, Burlington, ON) and quantified using the RiboGreen Reagent and Kit (Molecular Probes, Eugene, OR). RNA was reverse transcribed using MuLV reverse transcriptase and random hexamers (Applied Biosystems, Mississauga, ON) according to the manufacturer's instructions. Primers for human endothelin-1 (NM_001955), the gene encoding a potent vasoconstrictor peptide relevant in a number of cardiovascular pathologies [3], were designed using Vector NTI (Informax), and double-desalted primers were ordered from Invitrogen. Annealing conditions were optimized, and high reaction efficiency (90%) was confirmed using a dilution series of A549 cDNA over a 80- to 0.625-ng range. We compared three reagent mixes, a homemade reagent mix, the same mix with 1.5% Triton X-100 (Fisher Scientific, Ottawa, ON), and a commercial mix (iQ SYBR Green Supermix; Bio-Rad Laboratories Canada, Mississauga, ON) at three volumes: 50, 25, and 12.5 μL. Master mixes of the reagents were prepared to minimize differences in reagent composition. The composition of the homemade SYBR Green reagent mix was as follows: 3 mM $MgCl_2$, 200 μM each dNTP, 0.025 U/μL AmpliTaq Gold, 1× GeneAmp PCR buffer (Applied Biosystems), a 1:100,000 dilution SYBR Green I (Molecular Probes), and water. An equal concentration of cDNA (0.67 ng/μL) and 200 nM each primer were added to each reagent mix. To compare the effect of volume reduction on the PCR reaction, 12.5- or 25-μL volumes were pipetted into alternating wells on the same plate as 50-μL volumes thus ensuring that conditions were equivalent for both volumes. This procedure was conducted on separate plates for both the homemade SYBR Green reagent mixes and the commercial iQ SYBR Green Supermix. To compare the homemade mix to the commercial mix, equal volumes of each reagent mix were pipetted into alternating wells on the same plate. All reactions were performed in the iCycler iQ spectrofluorometric thermal cycler (Bio-Rad) using the 96-well heating block module as follows: 40 cycles of 15 s at 95 °C (denaturation), 15 s at 62 °C (annealing), and 30 s at 72 °C (elongation). Fluorescence was monitored at every cycle during the elongation step by the optical unit of the iCycler and associated software (Bio-Rad). The background, threshold value, and threshold cycles (the cycle at which the amplification curve crosses the threshold value) were determined automatically by the iCycler iQ software. SigmaPlot (SPSS, Chicago, IL) was used for graphical display of results averaged from replicate plates for each reagent mix at the three volumes tested.

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The results indicated a plate bias in which the amplification curves resulting from reactions in wells located on the periphery of the plate crossed the threshold later than those located closer to the middle (Fig. 1). The effect was most pronounced for the 12.5-μL volume (Figs. 1A, D, and G), followed by the 25-μL volume (Figs. 1B, E, and H) and the 50-μL volume (Figs. 1C, F, and I) of each reagent mix. Similarly, the standard deviation among wells and the difference between results from inner and results from outer wells increased at lower volumes (Table 1). Importantly, both homemade and commercial mixes exhibited similar standard deviation among inner wells, indicating that the homemade mix was of sufficient quality to permit reproducible results in the region of the plate least affected by the plate bias. However, the commercial reagent mix appeared to be less sensitive to the plate bias than the homemade mix (compare Figs. 1A–C to G–I), with the 50-μL volumes of the commercial preparation virtually abolishing the bias (Fig. 1I). Reagent volume also appeared to affect the later progression of the reaction, as inspection of the amplification curves revealed greater spread among the replicates at the lower volumes than at the 50-μL volume, even when using the commercial reagent mix

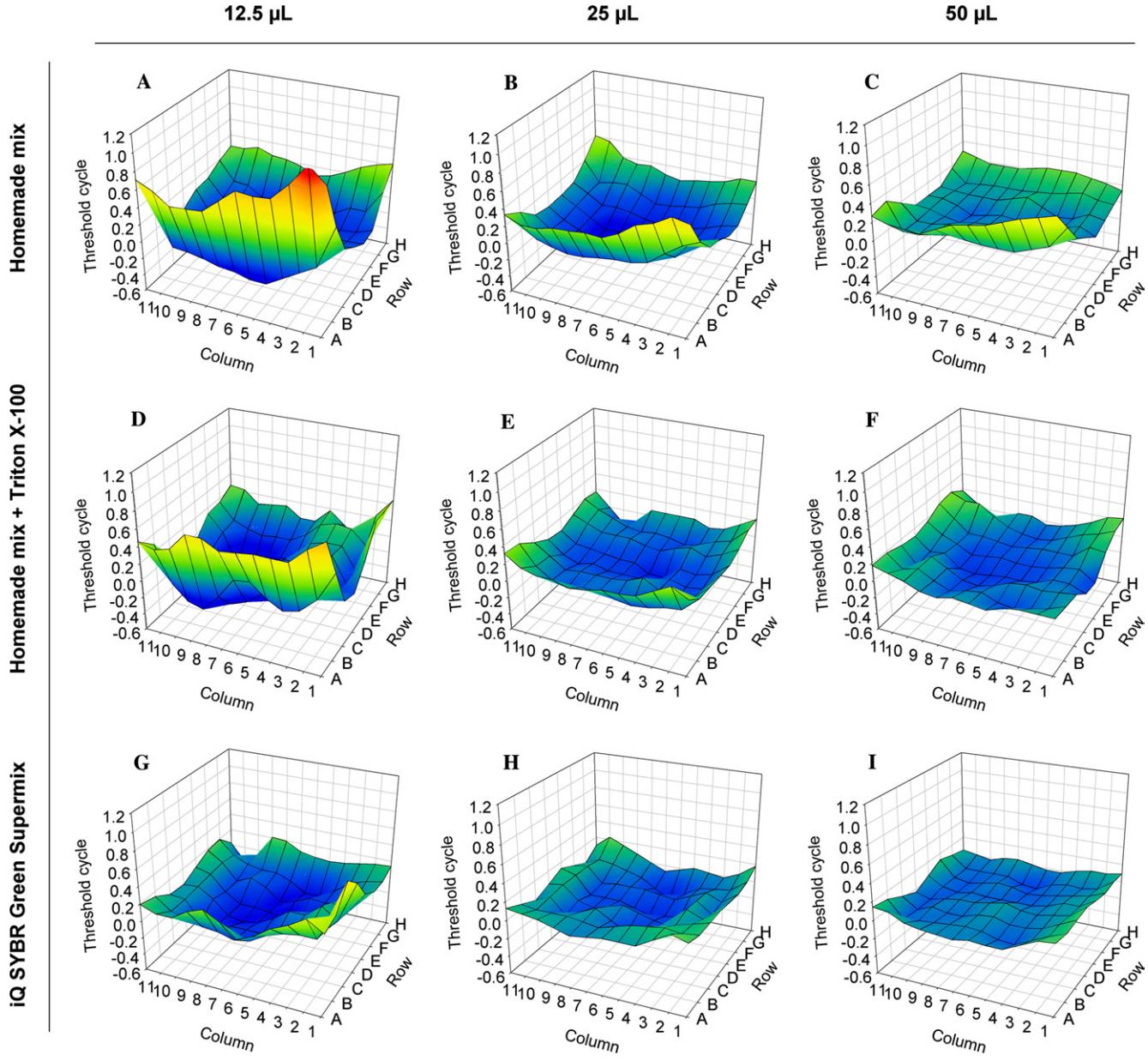

Fig. 1. Threshold cycle distribution across 96-well plates for 12.5 μL (A, D, G), 25 μL (B, E, H), and 50 μL (C, F, I) of assay volume, using the homemade SYBR Green mix (A, B, C), the homemade mix plus Triton X-100 (D, E, F), or the iQ SYBR Green Supermix (G, H, I) reagents. The threshold cycle is expressed as the difference between the value for an individual well and the global mean of all threshold cycles. All plots represent the average of two to four individual PCR plates.

Table 1
Comparison of the effect of reagent volume on average threshold cycle (Ct), standard deviation (SD), and coefficient of variance (CV) for replicate real-time PCRs across a plate using a homemade SYBR Green mix, the homemade mix plus Triton X-100, and iQ SYBR Green Supermix (Bio-Rad)

	12.5 (μL)			25 (μL)			50 (μL)		
	Average Ct	SD	CV (%)	Average Ct	SD	CV (%)	Average Ct	SD	CV (%)
Homemade SYBR Green mix									
All wells	23.76	0.42	1.77	24.16	0.27	1.12	23.83	0.18	0.76
Outer wells[a]	24.13	0.46	1.91	24.39	0.27	1.09	23.96	0.20	0.83
Inner wells[b]	23.50	0.15	0.64	23.95	0.10	0.42	23.72	0.09	0.38
Difference outer–inner	0.63			0.44			0.23		
Homemade mix plus 1.5% Triton X-100									
All wells	23.42	0.39	1.67	23.38	0.17	0.73	23.40	0.14	0.60
Outer wells[a]	23.71	0.42	1.77	23.51	0.16	0.68	23.46	0.16	0.68
Inner wells[b]	23.18	0.24	1.04	23.28	0.13	0.56	23.32	0.09	0.39
Difference outer–inner	0.53			0.23			0.15		
iQ SYBR Green Supermix									
All wells	22.85	0.25	1.09	23.02	0.13	0.56	22.92	0.09	0.39
Outer wells[a]	23.03	0.28	1.22	23.09	0.11	0.48	22.96	0.11	0.48
Inner wells[b]	22.70	0.17	0.75	22.94	0.10	0.44	22.90	0.07	0.31
Difference outer–inner	0.33			0.15			0.06		

All values represent the average of two to four independent plates.
[a] Outer wells are defined as rows A and H and columns 1 and 12.
[b] Inner wells are defined as all but the outermost two rows and columns.

(see Supplemental material, Fig. 1). Plates were centrifuged briefly following the PCR run and the content of each well was weighed. Reagent volumes were uniform across the plate, indicating that the plate bias was not due to reagent losses or pipetting error (data not shown).

We observed increased droplets above the level of the reagent mix in the wells located on the edge of the plate, possibly due to increased evaporation and condensation in the outer wells due to slight differences in temperature above the heated block. Addition of Triton X-100 (1.5% v/v [4]) to our homemade mix to wet well surfaces and help prevent beading improved performance by reducing the plate bias (Figs. 1D–F), suggesting that reagent composition can be altered to lessen to some extent the plate bias. The iCycler employs a solid block to uniformly heat all wells and a heated roof to minimize evaporation (iCycler product literature; Bio-Rad). The 96-well PCR plate sits on the heated block such that the bottom half of each well (a 100-μL volume) is encircled by the metal block. The top half of the plate rises above the solid block and is therefore exposed to the air. There is presumably a slight loss in the temperature of the outer wells, since they are more exposed to the surrounding air than are the wells within the plate. Increased evaporation and condensation of water on the walls of outer wells will affect reagent concentration in the reaction mix in the bottom of the wells, which may well affect the kinetics of the reaction or fluorescence readings. Lower reagent volumes would be expected to be more sensitive to fluctuations in conditions and thus more subject to interference.

We show here that using low reagent volumes as a cost-saving measure may introduce variability in the data and eventually reduce power within statistical analyses, which can be compensated only by increasing sample size. Furthermore, differences in reagent composition may affect the degree of plate bias. The bias detected in this study at the lowest volume tested would correspond to an average 60% difference in gene expression if one were comparing samples on the outer wells to samples on the inner wells. Such a bias can have relevance in gene expression studies. For example, inhalation of air pollution in experimental animals results in a 60–70% increase in mRNA expression of lung endothelin-1, associated with physiologically relevant changes in the peptide [5]. Therefore, for experiments using low reagent volumes, it may be advisable to use the center wells only and avoid the peripheral wells. For experiments with low sample numbers, the center wells can be used rather than distributing samples on one side of the plate or evenly across the plate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2004.10.047.

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