Consumable Effects on Low Volume TaqMan® Assays for Quantitative PCR on Roche LightCycler® 480

Thomas Bergmann[1], Axel Möhrle[2], Andreas Dahl[1]

[1] Max-Planck-Institut für Molekulare Genetik, Abt. Lehrach, Ihnestr. 63-73, 14195 Berlin

[2] 4titude® Deutschland, Sickingenstr. 26, 10553 Berlin

INTRODUCTION

Probe based chemistries such as TaqMan® assays are an important tool in numerous molecular biology research and diagnostic applications. Due to considerable costs for fluorescent TaqMan® probes a reduction of reaction volumes leads to significant cost savings per assay.

In our group we work on assay miniaturisation down to nanoliter scales in order to facilitate higher throughput and shortening of workflow time. While nanodispensing technology and thermal cycling formats are well advanced, standard PCR plates have limited the smallest possible reaction volume mainly due to evaporation issues.

In this work we evaluated different 384well PCR plates with both adhesive and heat seals in a standard PCR in order to find a combination of consumables with optimal tightness. This might allow us to run nanoliter volume assays in a standard 384well format. Consumables were tested on standard thermal cyclers such as the PTJ 200 (MJ Research) and the LightCycler® 480 from Roche for real-time applications.

„The FrameStar® plates led to significantly better results and reduced evaporation than standard PCR plates."

Dr. Andreas Dahl

FRAMEstar®

- ✓ Avoiding evaporation and sample loss
- ✓ Reducing costly reagent consumption
- ✓ Cost-effective consumables for Roche LightCycler® 480

EXPERIMENTS AND RESULTS

1) Comparison of different PCR plates and sealing methods

We compared the existing options for 384well cycler formats, namely standard 384well PCR plates (ABgene TF-0384) versus two component 384well plates with polycarbonate frame and polypropylene tubes (4titude® "FrameStar®" 4Ti-0384). The plates were sealed with adhesive seals (4titude® 4Ti-0500) or heat seals (4titude® 4Ti-0541) using the 4titude® semi-automated 4s2™ heat sealing instrument (4titude® 4Ti-0650).

For automated liquid handling the iNL10 STACCATO nanodispenser (Caliper LS) was used with a variation in dispensed volumes below 3% at 200nl.

In four plates 500, 1000 and 2000nl of 2% tartrazine solution was dispensed into adjacent wells across the plate (64 replicates each). After sealing and spinning 5s at 4000rpm the plates were cycled using a standard PCR protocol (10min at 95°C, 40x [15sec at 95°C, 20sec at 60°C, 25sec at 72°C], 15°C holding temperature) in a PTJ 200 (MJ Research).

After cycling, the average percentages of wells without evaporation were calculated. Evaporation was judged as absence of colour, as tartrazine is only visible in solution. Results are shown in Table 1:

Table 1

	Standard PCR Plate nl	% of wells with detectable liquid	Two component PCR plate nl	% of wells with detectable liquid
Heat Sealing	500	90,63	500	97,268
	1000	99,61	1000	100
	2000	98,44	2000	100
Adhesive Sealing	500	81,97	500	96,83
	1000	80,74	1000	100
	2000	82,34	2000	100

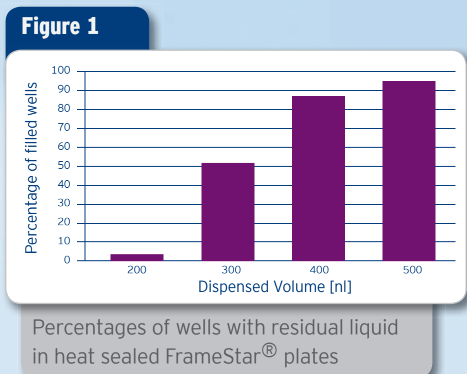
Sealing efficiencies with standard and FrameStar® PCR plates using adhesive or heat sealing

2) Estimation of Evaporation Loss

To determine the lowest possible volume in a 384well PCR plate and to estimate the evaporation loss 200, 300, 400 and 500nl of a 2% tartrazine solution were dispensed into a 384well Framestar® plate (each volume in 256 wells). The plate was heat sealed using 4Ti-0541 Clear Seal for real-time PCR and the 4s2™ heat sealing instrument.

Figure 1 shows the percentages of the wells with remaining tartrazine solution after PCR:

Figure 1



Percentages of wells with residual liquid in heat sealed FrameStar® plates

At 500nl over 95% of the wells show tartrazine still in solution, at 400nl almost 90% of the wells remain filled. At 300nl half of the wells are empty. 200nl volumes are almost completely evaporated. Thus, we conclude the average loss of water during a standard PCR to be between 100 and 300nl.

3) Effects on TaqMan® assay perfomance

The TaqMan® assay reaction mix contained 1x Universal TaqMan® PCR Mastermix from Applied Biosystems, 1x primer and probe concentration (RNAse P Detection Reagents Kit), deionized water and template in varying amounts. Template was a 423 bp fragment of the RNAseP gene in a stock concentration of 10ng/µl. The RNAse P TaqMan®assay targeted the middle part of the PCR fragment.

In a first test of a 1.000– and 10.000–fold template dilution 500nl, 1µl, 2µl and 3µl of the complete reaction mixes were dispensed into the FrameStar® LightCycler® 480 PCR plates. After sealing with the 4titude® adhesive Optical Seal for quantitative PCR (4titude® 4Ti–0560) thermal cycling was performed using the LightCycler® 480 (Roche) with the following protocol: 10min at 95°C, 40x [10sec at 95°C, 40sec at 60°C].

In Table 2a/b the obtained Cp values for the different volumes and concentrations (all 32 replicates) in two FrameStar® plates are shown. Surprisingly, the Cp values remain very stable at different reaction volumes. The number of dropouts correlates with the values seen in the test above. Values, which were more than 1 cycle away from the last Cp in the ranked list, were regarded as outlier and thus removed from the data set.

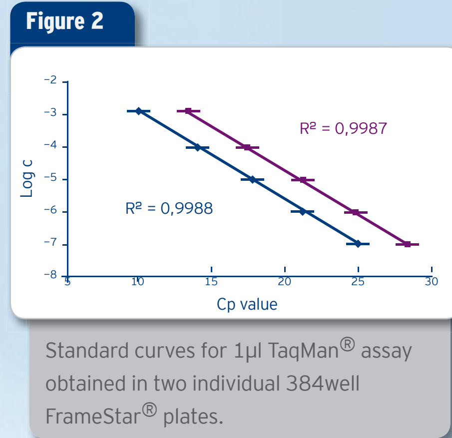
Across all volumes the obtained Cp values show surprisingly little variation, which is comparable to the variation in conventional volumes of 10µl. This uniformity of results was very promising.

We therefore investigated whether these low volumes could also be used if the setup was performed by manual pipetting.

4) Test of 1µl TaqMan® based real–time PCR with manual pipetting

Across a concentration gradient of 5 orders of magnitude with two different 384well plates standard curves were constructed and are shown in Figure 2.

Figure 2



Standard curves for 1µl TaqMan® assay obtained in two individual 384well FrameStar® plates.

Both plates showed comparable assay efficiencies of 81 and 82% as can be seen from the equal slope of both graphs.

The coefficient of variation across all concentrations was between 2 and 5% for both plates. From comparison to experiment 3 using the more precise nanodispenser it can be seen that the main cause for variation is likely to be manual pipetting. However, linear regression analysis with R = 0,999 shows that even with manual pipetting TaqMan® based chemistry performs robust and leads to sufficient results in real–time PCR across 5 orders of magnitude.

Table 2a

Volume	Concentration	Cp Average	Cp median	STDEV	CV	Dropouts
0,5µl	1:10^3	14,36	14,37	0,29	2,0 %	9 %
	1:10^4	17,73	17,76	0,29	1,6 %	13 %
1µl	1:10^3	14,55	14,64	0,22	1,5 %	0 %
	1:10^4	17,85	17,91	0,17	0,9 %	6 %
2µl	1:10^3	14,64	14,68	0,19	1,3 %	0 %
	1:10^4	17,92	17,97	0,17	0,9 %	3 %
3µl	1:10^3	14,61	14,72	0,35	2,4 %	0 %
	1:10^4	17,93	17,95	0,16	0,9 %	0 %

Table 2b

Volume	Concentration	Cp Average	Cp median	STDEV	CV	Dropouts
0,5µl	1:10^3	14,65	14,71	0,22	1,5 %	0 %
	1:10^4	17,95	18,03	0,21	1,2 %	0 %
1µl	1:10^3	14,82	14,82	0,06	0,4 %	0 %
	1:10^4	18,03	18,08	0,16	0,9 %	0 %
2µl	1:10^3	14,83	14,83	0,05	0,4 %	0 %
	1:10^4	18,06	18,12	0,18	1,0 %	3 %
3µl	1:10^3	14,81	14,83	0,11	0,7 %	0 %
	1:10^4	18,04	18,09	0,18	1,0 %	0 %

Cp Values and Standard Deviations in two individual FrameStar® PCR plates a and b with adhesive sealing

DISCUSSION AND CONCLUSIONS

Crucial prerequisites for lowering the volume of PCR based assays below 5µl are the hermetic sealing of the reaction chamber, adequate thermal cycling and in case of real–time applications appropriate optics for accurate monitoring. In this work we present improvements in tightness of sealing and reduction of assay volumes. The minimal volume we found for robust assays in standard SBS format PCR plates was 500nl.

Here the FrameStar® plates led to significantly better results and reduced evaporation than standard polypropylene PCR plates. The two component design of these plates feature a robust frame in which soft polypropylene PCR tubes are inserted. This robust polycarbonate frame does not expand during thermal cycling, which contributes to a better integrity of the seal. We found this for both heat and adhesive sealing materials.

The generated real–time data were very reproducible along a wide range of volumes, although at a volume of 500nl a dropout rate of 10% has to be expected. The LightCycler® 480 (Roche) showed sufficient detection abilities down to 500nl, although volumes below 3µl are not supported by the company. In our real–time PCR experiment we found a good sensitivity and therefore we expect this to be sufficient for more robust applications such as SNP genotyping as well.

We could perform real–time PCR in 500nl in standard SBS format 384well plates with standard lab equipment for PCR. Only for liquid handling at that volume range a nanodispenser was used. Although there are limitations to precise manual pipetting below 2µl, the manually pipetted real–time PCR data for 1µl volumes were still reproducible and surpassed our expectations.

In summary, for our low volume real–time PCR assays the optimal combination we found were the 4titude® two component "FrameStar®" 384well PCR plates in combination with heat sealing. Interestingly, the robust two component design also provided significant tightness advantages using adhesive seals.

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