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Eppendorf Mastercyclers Save Time and Cost in PCR

Dutt Way Wong[1], Jenny Lau[1], Yee Min Khor[1], Arora Phang[2]
[1] Eppendorf East Asia Regional Center, Kuala Lumpur, Malaysia
[2] Eppendorf AG, Hamburg, Germany

Abstract

Thermal cyclers available in the market are of different ramp rates. One of the more obvious advantages of high ramp rates is faster completion of PCR, allowing higher work productivity. However, ramp rates alone do not contribute to faster PCR completion. This study compares the time taken to complete a 3-step and 2-step GC-rich PCR by five different thermal cyclers. Reported herein is also the cost saving aspect by reducing reagent volume. An ideal thermal cycler would allow users to save both time (faster PCR completion) as well as cost (reproducible results at lower volume) in reducing optimization effort and in subsequent routine runs. Eppendorf Mastercycler® nexus GSX1 and nexus gradient are found to be able to amplify difficult templates across a wide range of enzymes at high speed, high reproducibility and low cost.

Introduction

Scientific advancement has made Polymerase Chain Reaction (PCR) work less time consuming and more cost effective. The gradient function has made possible testing of different temperatures in one single PCR. Thermal cyclers with increasingly higher ramp rates are being introduced to the market one after another. Fast consumables and fast reagents are pushing the completion time of a PCR ever shorter. With certain difficult applications however, such advancements elicit less excitement as it often comes with vastly higher cost.
PCR amplification of GC-rich templates such as the promoter region of a gene [1] requires higher melting temperatures due to the formation of secondary structures. The higher denaturation and annealing temperatures requirement limits the advantage conferred by high cycler ramp rates, as the heat needs to be transferred to the liquid reagent. Hence, time saving strategies for this application generally comes in the form of combining annealing and extension steps (also known as 2-step PCR) [2], cutting the time needed for transition between steps as the temperatures are closer to each other. What is less known is that ramp rates are not the whole story behind the speed of a thermal cycler [3]. Total run time for PCR differs between thermal cyclers, and not only as a function of heating and cooling ramp rates. Efficient control of heating and cooling affects both the speed as well as the performance of a thermal cycler, which is critical to producing reliable and reproducible results. This study aims to compare differences between 3-step and 2-step PCR of a GC-rich target for five thermal cyclers in terms of amplification efficiency, reproducibility, PCR reagent cost and total run time.

Materials and Methods

Three commercial enzyme kits were used: i-Taq DNA polymerase (iNtRON Biotechnology), KAPA2G Robust DNA Polymerase (KAPA Biosystems) and AmpliTaq Gold® 360 DNA polymerase (ATG360; Applied Biosystems). PCR reaction master mix was prepared according to the protocols supplied by the manufacturers for reaction volume set-up of 5 µL or 10 µL per reaction, respectively, in 0.2 mL Eppendorf PCR tubes. The amount of enzyme used was based on each manufacturer's recommendation. The vessels were placed in wells that have the corresponding gradient temperatures in each cycler. BAIP3 forward (5'-AGTG-CATGGAGGCGGACC-3') and reverse primers (5'-GC-CAAGAAGCCCCTTGTGAG-3'), 100 ng of Human Genomic DNA, Female (Promega) and 4 % final concentration of dimethyl sulfoxide (DMSO) were added to the master mix. PCR was carried out on five 96-well thermal cyclers listed in Table 1. For each of the thermal cycler models, the tests were performed on only one unit of each device.

Table 1: List of thermal cyclers in this study

Thermal cycler	Max block heating rate
Eppendorf Mastercycler® nexus GSX1	5°C/s
Eppendorf Mastercycler® nexus gradient	3°C/s
Applied Biosystems Veriti™ Fast	5°C/s
Life Technologies SimpliAmp™	4°C/s
Bio-Rad T100	4°C/s

Cycling conditions are listed in Table 2. The annealing temperatures used were in the range of 48.8°C to 61.2°C for Eppendorf Mastercycler® nexus GSX1, nexus gradient and T100, and 49°C to 60°C for Veriti and SimpliAmp in 3-step PCR (Table 3). This range is based on the common ±5°C rule of the predicted melting temperature for BAIP3 primers, which was 54°C. Combined annealing and extension temperature in 2-step PCR was between 58°C to 68°C. This range was chosen based on the nature of BAIP3 which contains high-GC, as higher temperature is required for specificity. For Eppendorf cyclers, the "Standard" temperature control mode was used while the volume of reaction was changed accordingly for the other manufacturers' cyclers. Total run time for each reaction was recorded based on the different cycling conditions of each of the enzyme used. The cost of enzyme per run was calculated based on listed price and the amount of enzyme used in each of the reaction run. Amount of enzyme was reduced proportionally according to 5 µL and 10 µL reaction volumes and the cost was calculated based on these amounts.

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Table 2: Cycling conditions of each DNA polymerase

	Step	3-step		2-step	
		Temperature	Duration	Temperature	Duration
i-Taq					
1	Initial denaturation	95°C	5 min	95°C	5 min
2 (30 cycles)	Denaturation	95°C	15 s	95°C	15 s
	Annealing	[a]Gradient °C	30 s	[b]Gradient°C	60 s
	Extension	72°C	30 s		
3	Final extension	72°C	1 min	72°C	1 min
KAPA2G					
1	Initial denaturation	95°C	5 min	95°C	5 min
2 (30 cycles)	Denaturation	95°C	15 s	95°C	15 s
	Annealing	[a]Gradient °C	30 s	[b]Gradient°C	45 s
	Extension	72°C	30 s		
3	Final Extension	72°C	1 min	72°C	1 min
ATG360					
1	Initial denaturation	95°C	10 min	95°C	10 min
2 (30 cycles)	Denaturation	95°C	30 s	95°C	30 s
	Annealing	[a]Gradient °C	30 s	[b]Gradient°C	60 s
	Extension	72°C	60 s		
3	Final Extension	72°C	7 min	72°C	7 min

[a,b] Please refer to table 3.

Table 3: Annealing temperatures for 3-step and 2-step PCR optimization

Thermal cycler	Maximum number of gradient temperatures	Annealing temperature used											
		1	2	3	4	5	6	7	8	9	10	11	12
		Set 1											
Mastercycler nexus GSX1	12 columns	48,8	49,2	49,9	51,1	52,7	54,2	55,8	57,3	58,9	60,1	60,8	61,2
Mastercycler nexus gradient	12 columns												
Veriti	6 zones*	49	50	51	52	53	54	55	56	57	58	59	60
SimpliAmp	3 zones*	49	50	51	52	53	54	55	56	57	58	59	60
T100	8 rows	48,8	49,6	51,1	53,5	56,3	58,6	60,1	61,1				
		Set 2											
Mastercycler nexus GSX1	12 columns	58,4	59,9	62	64	66	68						
Mastercycler nexus gradient	12 columns												
Veriti	6 zones*	58	60	62	64	66	68						
SimpliAmp	3 zones*	58	60	62	64	66	68						
T100	8 rows	58	60,1	62,2	64,7	66,8	68,2						

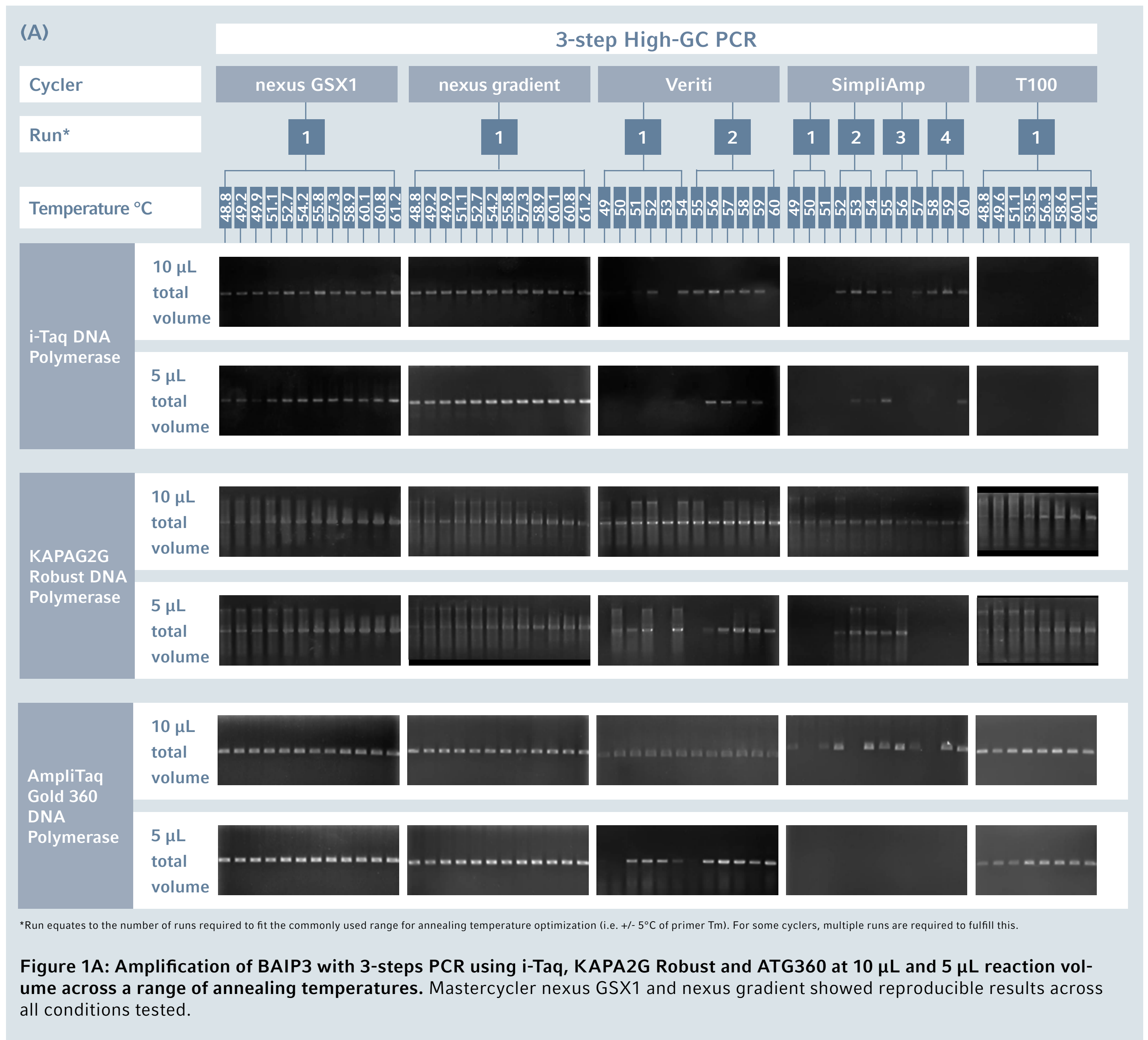
*Due to the limited number of permissible gradient temperatures per cycler, multiple PCR runs were performed to match the number of temperatures used in Mastercyclers.

Results and Discussion

The objective of this study is to investigate the amplification efficiency of thermal cyclers in amplifying GC-rich templates, especially when cycling conditions deviate from manufacturers' recommendation and lower volumes are used. Total PCR run time can be reduced by combining the annealing and extension steps, thus reducing the time taken by the cycler to ramp up and down when transitioning between the two steps. Reagent cost can be reduced by using less specialized and lower cost reagents in combination with common additives such as DMSO, as well as lower reagent volume per reaction.

In different combinations of the aforementioned scenarios, most amplification across the cyclers in this experiment produced single clean bands, with minimal smearing and multiple bands in some of the lanes. The presence of smearing could also be due to the efficiency of the cyclers, where some cyclers may not reach the set temperature or overshooting of temperatures, hence affecting amplification efficiency. In addition, some PCR reagent kits are optimized and validated based on certain cyclers, thus may follow specific heating or cooling rates. In this experiment, minimal variation of parameters was used. Hence, further optimization such as decreasing the concentration of starting template, increasing annealing temperature or adjusting holding time of the cycling steps may lead to better PCR result in all cyclers. However, as the final goal of this study is reduction in effort, time and cost, further optimization effort is contrary to purpose of this study.

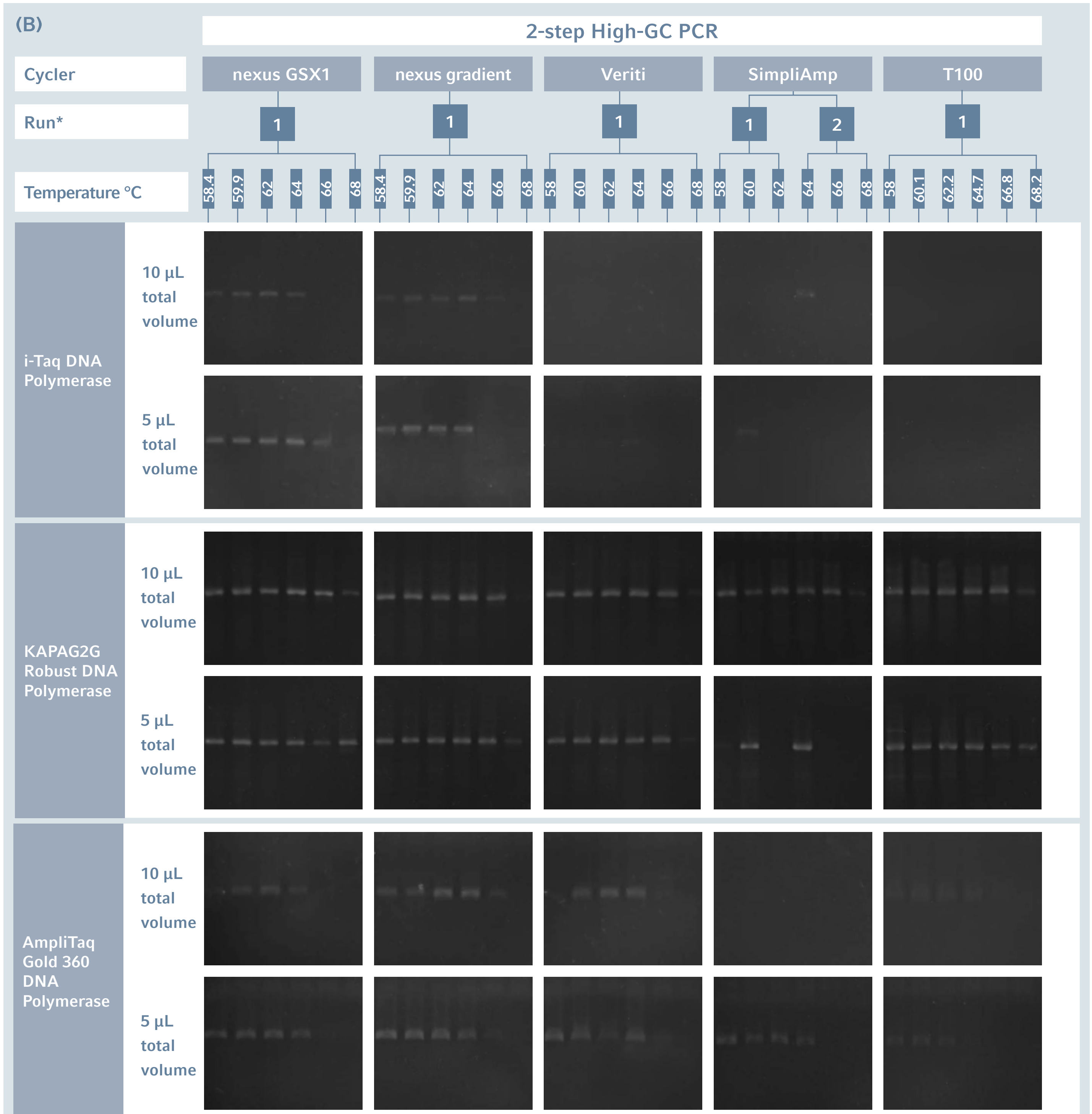
Amplification of BAIP3 on five thermal cyclers was compared using both 3-steps (Figure 1A) and 2-steps (Figure 1B) PCR programs with 3 different enzymes, starting with 10 µL reaction volume, and further reduced to 5 µL. For 3-step PCR, successful amplification was observed for Mastercycler nexus GSX1 and nexus gradient. However, amplifications with Veriti, SimpliAmp and T100 were not entirely successful with the range of annealing temperatures used in this study. We observed random negative amplifications, which could be attributed to high evaporation rate especially at 5 µL reaction volumes, leading to partial or complete loss of reaction mix.



*Run equates to the number of runs required to fit the commonly used range for annealing temperature optimization (i.e. +/- 5°C of primer Tm). For some cyclers, multiple runs are required to fulfill this.

Figure 1A: Amplification of BAIP3 with 3-steps PCR using i-Taq, KAPA2G Robust and ATG360 at 10 µL and 5 µL reaction volume across a range of annealing temperatures. Mastercycler nexus GSX1 and nexus gradient showed reproducible results across all conditions tested.

Based on the 3-step PCR results, we then moved on to 2-step PCR, which combines annealing and extension steps into a single step using higher range of temperatures. While all cyclers were able to yield good PCR amplifications at 10 µL reaction volume with KAPA2G enzyme using this strategy, only Mastercycler nexus GSX1 and nexus gradient maintained positive amplification with all 3 enzymes, and even when the volume was reduced to 5 µL (Figure 1B). In comparison, Veriti, SimpliAmp and T100 all showed partial amplification success depending on the enzyme used. Hence, one would require more optimization effort with these cyclers to get comparable results.



*Run equates to the number of runs required to fit the commonly used range for annealing temperature optimization (i.e. +/- 5°C of primer Tm). For some cyclers, multiple runs are required to fulfill this.

Figure 1B: Amplification of BAIP3 with 3-steps PCR using i-Taq, KAPA2G Robust and ATG360 at 10 µL and 5 µL reaction volume across a range of annealing temperatures. Mastercycler nexus GSX1 and nexus gradient showed reproducible results across all conditions tested.

The gradient temperature spans used in this study were chosen based on a general rule of thumb, which is a set of temperatures ±5°C from the predicted melting temperature (54°C for BAIP3 primers). The nature of GC-rich primers leads to non-specific binding to template; hence multiple products are possible across different temperatures. Thus, gradient function in a thermal cycler is important to test multiple temperatures in one run (Table 3). Different thermal cyclers would operate using different gradient technology or format (12 horizontal columns or 8-vertical rows across 96-block). Eppendorf thermal cyclers uses the 12 horizontal columns strategy, allowing users to concurrently test 12 different temperatures in each gradient mode. This allows users to test more temperatures during optimization and hence eliminate the need to set up another run which requires additional preparation and waiting time. Meanwhile, SimpliAmp requires four different PCR runs to provide comparable 12 different annealing temperatures; T100 uses 8 vertical rows strategy and can only run eight different annealing temperatures; and Veriti can run 6 different temperatures in a single run, thus requiring 2 separate PCR runs to yield comparable amount of information as a cycler with 12 temperature gradient.

Table 4: Comparison of total run times between 3-step and 2-step PCR (for 12 different annealing temperatures) of 10 µL reaction volume for each enzyme based on published maximum ramp rate for each thermal cycler.

Thermalcycler	Max ramp rate published	Time completion (mins)					
		i-Taq		KAPA2G		ATG360	
		3-Step	2-step	3-Step	2-step	3-Step	2-step
Nexus GSX1	5°C/s	60	56	58	51	87	76
Nexus Gradient	3°C/s	73	67	68	51	98	84
Veriti Fast[1]	5°C/s	160	131	148	114	208	170
SimpliAmp[2]	4°C/s	277	251	248	216	324	315
T100[3]	4°C/s	114	102	101	96	117	94

[1]The run time for Veriti was a sum of 2 runs as one run can only accommodate 6 different annealing temperatures.
[2]The run time for SimpliAmp was a sum of 4 runs as one run can only accommodate 3 different annealing temperatures.
[3]Each run of T100 only allows testing of 8 different annealing temperatures in one run.

Table 4 shows comparison of run time taken for each cycler to complete a gradient PCR program, giving a range of amplification results spanning 8 or 12 different annealing temperatures. As expected, 2-step PCR was completed in lesser time than 3-step PCR for all the cyclers. However, the time difference between the two programs is highly dependent on the cycler in question. Some cyclers (e.g. Veriti and T100) reported a big difference between the two programs while others (e.g. Eppendorf Mastercycler nexus GSX1) showed only a few minutes' difference, indicating optimal temperature control. This is possibly due to several factors, such as how efficiently a thermal cycler handle the temperature transition between steps or how a manufacturer measures and publishes the ramp rates. Small fluctuations/ differences in total run time may also be due to environmental influence (e.g. ambient temperature). However, fast run times are only important provided PCR reactions produce desired results. For all enzymes and PCR strategies, Mastercycler nexus GSX1 and nexus gradient used the shortest time to complete a PCR. This not only increases the productivity of a laboratory by freeing up the thermal cycler for more usage per day, it also increases sharing convenience between members of the same lab.

Table 5: Cost of enzyme used per 5 µL and 10 µL reaction volumes in USD based on listed price

Enzyme	Amount of enzyme/ 5 µL reaction	Price/ 5µL reaction	Amount of enzyme/ 10 µL reaction	Price/ 10µL reaction
i-Taq[1]	0.5U	0.06	1U	0.12
KAPA2G Robust	0.1U	0.06	0.2U	0.12
ATG360	0.125U	0.13	0.25U	0.25

[1]Price for i-Taq was converted from listed price in MYR to USD

Finally, the cost of reactions based on listed price was calculated for both 5 µL and 10 µL reactions in Table 5. However, even though the total cost of reactions between thermal cyclers that use the same amount of reaction (e.g. comparable 12 annealing temperatures) can be considered as the same, there exist a big gap in total time required for completing a PCR program (Table 4). For example, at the same reagent cost, the average run time for SimpliAmp was almost 4 times more than Mastercycler nexus gradient, the cycler with the lowest published ramp rate. Also, the reduction of reaction volume to 5 µL did not yield consistent amplification in comparison to 10 µL reactions in SimpliAmp, thus users can only use a minimum of 10 µL reaction volume. With KAPA2G and ATG360, amplifications were more or less consistent between 10 µL and 5 µL reaction volumes. However, with i-Taq (a locally available and inexpensive enzyme for general PCR applications), only Mastercycler nexus GSX1 and nexus gradient yielded consistent amplifications. A thermal cycler that can reproducibly yield results at lower reaction volume enables users to save on reagent cost per reaction. A thermal cycler that is robust in producing results with various enzymes of differing price range would reduce user dependency on expensive enzyme formulations. Hence, taking all optimization efforts into consideration, resources saving (cost, time, effort) needs to be balanced with quality of results (reproducibility) as final assessment of a thermal cycler quality.

Conclusion

The results showed that Eppendorf Mastercycler nexus GSX1 and nexus gradient are robust in supporting amplification of GC-rich template in a wide range of enzymes, even at low reaction volume whilst allowing flexibility to choose between 3-step or 2-step protocols depending on the run. Eppendorf Mastercycler nexus GSX1 and nexus gradient are able to accommodate 12 gradient temperatures in a single run; this enables results in the shortest time possible as a wide range of annealing temperatures can be tested per run. With the right thermal cycler, a user can save time and money resources without necessarily resorting to costly optimization strategies even for difficult applications such as amplification of GC-rich templates.

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Literature

[1] Jones, P. A., & Takai, D. (2001). The role of DNA methylation in mammalian epigenetics. Science, 293(5532), 1068-1070.
[2] Holt, S. E., Norton, J. C., Wright, W. E., & Shay, J. W. (1996). Comparison of the telomeric repeat amplification protocol (TRAP) to the new TRAP-eze telomerase detection kit. Methods in Cell Science, 18(3), 237-248.
[3] Gerke, N. (2013). Comparative run time evaluations of PCR thermal cyclers. Eppendorf Application Note 274.

Ordering information

Description	International Order no.	North America Order no.
Mastercycler® nexus GSX1, 230 V/50 – 60 Hz	6345 000.010	6345000028
Mastercycler® nexus GSX1e*, 230 V/50 – 60 Hz	6347 000.017	6347000025
Mastercycler® nexus gradient, 230 V/50 – 60 Hz	6331 000.017	6331000025
Mastercycler® nexus gradient eco*, 230 V/50 – 60 Hz	6334 000.018	6334000026
PCR tubes, 0.2 mL, PCR clean, colorless	0030 124.332	951010006

* To run a Mastercycler® nexus with the suffix »eco« or »e«, a Mastercycler® nexus model without such a suffix is needed. Up to 2 units with the suffix »eco« or »e« can be connected to a Mastercycler® nexus without such a suffix.

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