Applications

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Using the gradient technology of the Mastercycler® pro to generate a single universal PCR protocol for multiple primer sets

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Abstract

The possibility to perform PCR with different primer sets under the same PCR protocol is time saving and convenient to any lab with different research projects going on simultaneously. This Application Note shows how four different primer sets were optimized to a single PCR protocol with the gradient function of the Mastercycler® pro from Eppendorf. The results also reveal the importance to perform a gradient run on any primer set in order to find out the primers' workable temperature range and limitation.

Introduction

PCR has become a routine method in almost every biological laboratory. Typically, multiple users run their PCRs on the same thermal cycler in a laboratory. Because of this, the use of a fast ramping thermal cycler can improve opportunities for the users to share the same thermal cycler. Additionally, the utilization efficiency of a thermal cycler can be increased by running multiple PCR applications simultaneously on the same block and using the same PCR protocol.

With this objective in mind, this Application Note describes the possibility to run multiple primer sets under the same PCR protocol through the use of the temperature gradient technology developed by Eppendorf. The gradient property of the Eppendorf Mastercycler family of thermal cyclers is built upon the SteadySlope® technology (Fig. 1). The heating and cooling ramp rates across the gradient temperature of the block remain constant while each column on the heating block will reach its set temperature at a slightly different time. Therefore, this technology ensures identical heating and cooling rates when transferring the results of a gradient experiment to routine applications.

The opposite concept is realized in some thermocyclers of other manufacturers. An unsteady down-ramping leads to identical holding times in the temperature steps but results in different ramp rates between gradient and routine runs (Fig. 1).

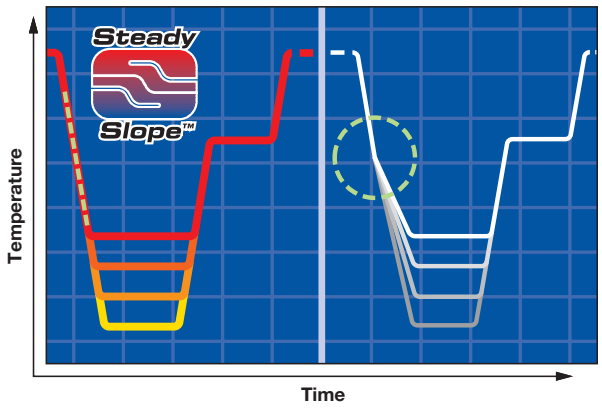

Figure 1: SteadySlope temperature gradient technology in comparison to an unsteady down-ramping behavior of thermocyclers of other manufacturers.

Materials und Methods

Gradient Optimization: Four sets of primers were identified for gradient optimization with the Eppendorf Mastercycler pro. Based on each primer's calculated melting temperature, a gradient temperature range from 55 – 67 °C was selected for the optimization process. The description of the primer sets are shown in Table 1.

Target Gene	Primer Sequence	Calculated melting temperature
β-globin (forward)	5'- GGTTGGCCAA TCTACTCCCAGG -3'	67.3 °C
β-globin (reverse)	5'- GCTCACTCAG TGTGGCAAAG -3'	64.3 °C
Sex related Y-chromosome gene (forward)	5'- CTCCGGAGAA GCTCTTCCTT -3'	63.1 °C
Sex related Y-chromosome gene (reverse)	5'- CAGCTGCTTG CTGATCTCTG -3'	62.9 °C
Human retinoblastoma gene (forward)	5' - CAGGACAGCG GCCCGGAG - 3'	67.6 °C
Human retinoblastoma gene (reverse)	5' - CTGCAGACGC TCCGCCGT - 3'	67.8 °C
Human tubulin gene (forward)	5' – ATGGACGTTGTC AGAAAGGAGGCT – 3'	68.3 °C
Human tubulin gene (reverse)	5' – GGGTACCCATC CCAGACCCAGT – 3'	69.6 °C

Table 1: Target gene, primer sequence and calculated melting temperature information for the primer sets tested. The melting temperatures were calculated using the OligoAnalyzer provided online by Integrated DNA Technologies under the following parameters: [Na^+] of 50 mM, 2.5 mM [Mg^{2+}], [dNTPs] of 0.1 mM and [Primer] of 400 nM for the β-globin primer set; [Na^+] of 50 mM, 2.5 mM [Mg^{2+}], [dNTPs] of 0.1 mM and [Primer] of 300 nM for the tubulin primer set; [Na^+] of 50 mM, 2.5 mM [Mg^{2+}], [dNTPs] of 0.2 mM and [Primer] of 200 nM for Sex related Y-chromosome (SRY) primer set; [Na^+] of 50 mM, [Mg^{2+}] of 2 mM, [dNTPs] of 0.4 mM and [Primer] of 100 nM for the human retinoblastoma primer set.

The reaction volumes were fixed at 20 µL. The β-globin [1], tubulin, human retinoblastoma and SRY reaction setup were done using the *Taq* DNA Polymerase kit (5 PRIME GmbH, Germany) whereas the Human retinoblastoma reaction setup was done using the TripleMaster PCR System (5 PRIME GmbH, Germany) with 5 % DMSO as an enhancer. Human genomic DNA obtained from male saliva (1 mL) using the i-Genomic CTB Mini Kit from iNtRON Biotechnology, Inc. (South Korea) was used in all the reaction setups. The reaction setups for the primer sets are shown by the tables 2, 3, 4 and 5.

Component	Final Concentration
10x *Taq* Buffer (2.5 mM Mg^{2+})	1x
dNTPs Mix (10 mM)	0.1 mM
β-globin forward (10 µM)	400 nM
β-globin reverse (10 µM)	400 nM
Human Template DNA (26 ng/µL)	26 ng
Taq DNA Polymerase (5 U/µL)	1 U
Molecular Biology Grade Water	--

Table 2: Reaction setup for β-globin [1].

Component	Final Concentration
10x *Taq* Buffer (2.5 mM Mg^{2+})	1x
dNTPs Mix (10 mM)	0.2 mM
Tubulin forward (10 µM)	300 nM
Tubulin reverse (10 µM)	300 nM
Human Template DNA (26 ng/µL)	26 ng
Taq DNA Polymerase (5 U/µL)	1 U
Molecular Biology Grade Water	--

Table 3: Reaction setup for Tubulin.

Component	Final Concentration
10x *Taq* Buffer (2.5 mM Mg^{2+})	1x
dNTPs Mix (10 mM)	0.2 mM
SRY forward (10 µM)	200 nM
SRY reverse (10 µM)	200 nM
Human Template DNA (26 ng/µL)	26 ng
Taq DNA Polymerase (5 U/µL)	1 U
Molecular Biology Grade Water	--

Table 4: Reaction setup for SRY (previously published in BioNews Nov 2002, [2]).

Component	Final Concentration
10x Tuning Buffer (incl. Mg^{2+})	1.6 x (4 mM)
DMSO (100 %)	5 %
dNTPs Mix (10 mM)	0.8 mM
Retinoblastoma forward (10 µM)	100 nM
Retinoblastoma reverse (10 µM)	100 nM
Human Template DNA (26 ng/µL)	26 ng
TripleMaster DNA Polymerase (5 U/µL)	2 U
Molecular Biology Grade Water	-

Table 5: Reaction setup for Human retinoblastoma (previously published in Application Note No. 65 [3] but with a modification to the primer concentration from 400 nM to 100 nM in this experiment).

The gradient optimization was done simultaneously for all the reaction setups using the running protocol shown at Table 6.

Cycle	Step	Temperature (°C)	Time (s)	Description
1x	1	95	2 min	Initial Denaturation
35x	2	95	15	Template Denaturation
	3	55 - 67	10	Primer Annealing
	4	72	30	Primer Elongation
1x	5	72	5 min	Final Elongation
1x	6	10	∞	Holding step

Table 6: Running protocol for all three primer sets with a gradient step introduced into the annealing step.

Based on the results of the gradient optimization for all four primer sets, a single common annealing temperature was selected to represent them. To check whether or not the slight time differences in the annealing step of the gradient assay have an influence on the results of a non-gradient assay a further setup was prepared. All 4 PCR systems were run with the determined common annealing temperature three times each with a slightly different holding time (Table 7).

Cycle	Step	Temperature (°C)	Time (s)	Description
1x	1	95	2 min	Initial Denaturation
35x	2	95	15	Template Denaturation
	3	67	10/13/15	Primer Annealing
	4	72	30	Primer Elongation
1x	5	72	5 min	Final Elongation
1x	6	10	∞	Holding step

Results and Discussion

The outcome of optimizing the annealing temperature under a single gradient experiment with the selected primer sets was successful. Under a single gradient range of 55 °C to 67 °C, all four primer sets displayed a different range of annealing temperatures that can successfully amplify their respective targets (Figure 2). With the SRY primer set, the specific target size of 201 bp was amplified in the annealing temperature range of 60.5 °C to 67.1 °C. A slight decrease in yield was seen at 67.1 °C for this primer set. As for the β-globin primer set, a temperature range of 55.2 °C to 67.1 °C was found to be able to amplify the specific target size of 536 bp. This particular primer set is highly robust showing a very specific amplification across the 12 gradient temperatures. The suitable range of annealing temperatures for the human retinoblastoma (RB) primer set is between 66.2 °C to 67.1 °C in order to amplify a specific target size of 180 bp. Lastly, the primer set for tubulin gene (Tub) showed an amplification range of 62.1 °C to 67.1 °C.

This experiment demonstrates the possibility to optimize a few primer sets using a single PCR protocol with a selected range of gradient temperatures. Additionally, it also shows the fact that all these primer sets have their own range of optimal annealing temperatures and are not limited to a single annealing temperature. Evidently, performing a proper gradient optimization with 12 different gradient temperatures is advantageous in the sense that the user can observe the gradual changes in the way the annealing temperature affects the primer-template binding complex. In other words, a proper gradient run divulges much more information to the user about a given primer set's binding behavior and robustness over a temperature range. Furthermore, in this experiment the user can clearly see a common range of annealing temperatures that will work for all four primer sets tested. Their common annealing temperature range is between 66.2 °C to 67.1 °C (Figure 2). Such convenience may not be apparent to the user if the user were to rely on a thermal block with six temperature zones. With 6-temperature zones thermal block, the user may miss certain temperatures and therefore may not see how the temperatures gradually affect the primer-template binding complex.

Table 7: Three PCR experiments were run with the four primer sets. Each PCR was using the determined common annealing temperature but running a different annealing holding time at 10 sec, 13 sec or 15 sec.

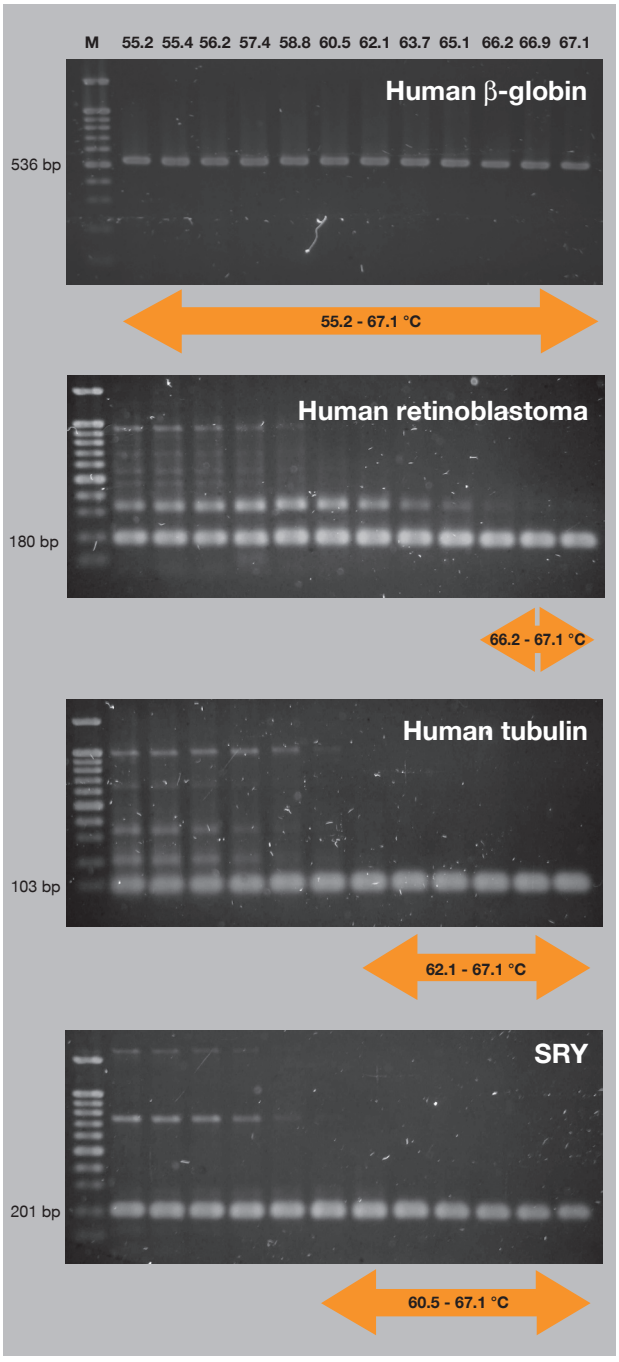

Figure 2: Gradient optimization of the 4 primer sets simultaneously under the same gradient temperature range of 55 °C to 67 °C.

Based on the results in Figure 2, there is a possibility to run all 4 primer sets under a single PCR protocol. Because of this, a second experiment was performed with a common annealing temperature of 67 °C. In this experiment, annealing times of 10 seconds, 13 seconds and 15 seconds were tested. The outcome of the results demonstrates the success of applying a single PCR protocol to all four primer sets by using a single annealing temperature at 67 °C (Figure 3). All four primer sets showed their respective target size between 10 to 15 seconds of annealing time.

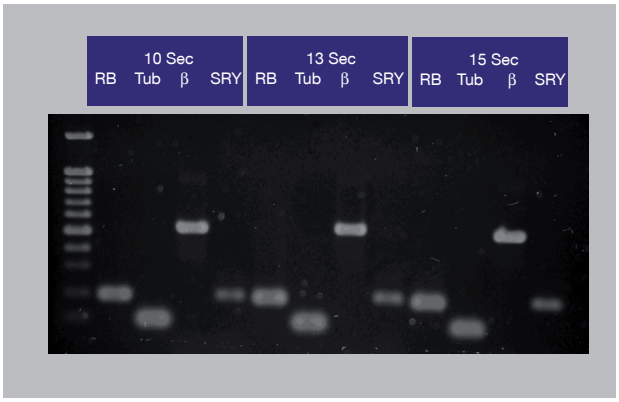

Figure 3: All 4 primer sets were run successfully under a single PCR protocol with an annealing temperature set at 67 °C. The same run was repeated 3 times, each at a different annealing time. The specificity of all the primer sets was maintained in all the annealing times tested.

Conclusion

Performing a single gradient optimization protocol on multiple sets of primer allows the user to identify the optimal temperature range for each primer set tested. In turn this will allow the user to identify a single universal PCR protocol that will amplify all the primer sets successfully, as long as there is an overlap of the optimal temperature range among them.

The SteadySlope Technology as a gradient feature of the Mastercycler pro ensures highly reproducible results. This Application Note has proven for the tested primer sets that the minor differences in the annealing times among the gradient temperatures in gradient mode did not affect the reproducibility of the results when a proper run was repeated again in normal mode. The 12-column gradient temperatures enable a wider temperature optimization and increase the overall visibility to the primer's performance over a selected temperature range. The gradient mode is not limited to the annealing step but can be applied to other steps (e.g. denaturation or extension) as well. Through the use of the gradient technology, users are provided with a deeper understanding about their primer sets and therefore allow them the possibility to generate a single universal PCR protocol to run their multiple primer sets simultaneously.

References

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

[2] George Halley, 5 Prime, Inc., Andrés Jarrin, Eppendorf AG and Melanie Persson, Eppendorf Instrumente. 2002. A comparison of specificity, processivity and sensitivity of different Hot Start PCR Systems. *BioNews Nov 2002*, Eppendorf AG.

[3] Scott Tarpinian, Jennifer Halcome, 5 Prime, Inc. and Andres Jarrin, Eppendorf AG. 2003. Using the TripleMaster PCR System for robust amplification of GC-rich DNA templates. *Application Note No. 65*, Eppendorf AG.

Ordering information

Product	Description	Order no. international	Order no. North America
Mastercycler pro (96 well aluminum block)	230 V / 50 - 60 Hz 120 V / 50 Hz, with US-plug	6321 000.019 6321 000.027	- 950030010
Mastercycler pro S (96 well silver block)	230 V / 50 - 60 Hz 120 V / 50 Hz, with US-plug	6325 000.013 6325 000.021	- 950030020
Mastercycler pro 384 (384 well aluminum block)	230 V / 50 - 60 Hz 120 V / 50 Hz, with US-plug	6324 000.010 6324 000.028	- 950030030
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