How to Achieve Ultimate PCR Optimisation

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The new Eppendorf Mastercycler X50 is the only thermal cycler in the market equipped with the innovative 2Dgradient function. PCR optimisation, typically of the annealing temperature using gradient function is an established technique. Optimisation of the denaturation temperature is less commonly done and typically limited to applications dealing with complex or GC-rich DNA templates.

This is mainly due to the high amount of effort required to obtain useful optimal result from the combination of denaturation and annealing conditions. The 2D-gradient function reported herein allows optimisation of both denaturation and annealing temperatures in just one PCR run. This provides users with rich amount of information in the least amount of time and effort, thus greatly shortening the scientific research process.

Introduction

Since the inception of PCR, the technique has gone through numerous evolution steps. Similarly, the thermal cycler, a device designed to carry out PCR, has evolved from a simple heating device to one with numerous functions that allows PCR to be performed more efficiently. Perhaps one of the most powerful innovations in the thermal cycler is the gradient function. This function directly targets the fundamental principle of PCR, that the annealing step in PCR is primer-dependent and the correct temperature for this step is very ambiguous and hard to predict. Determination of the correct annealing temperature generally involves much trial and error and this fine-tuning can be very time-consuming. Thermal cyclers with gradient function are able to simultaneously provide multiple different temperatures at a certain step. When used at the annealing step, this function can thus reduce the time and effort needed in optimising the annealing temperature of a primer [1, 2].

On the other hand, the denaturation step in PCR has less ambiguous working temperature, generally only deviating slightly from the temperature specified by the manufacturer. This is because most DNA will be completely denatured at 95°C and most enzymes have a maximum temperature tolerance around that temperature. However, while not as variable as primers, each DNA template has its own characteristic and hence a certain degree of variation is unavoidable. Complex DNA or DNA templates with rich GC content naturally require higher denaturation temperature. Thus, while PCR might be successful without optimising the denaturation step, the quality and yield of the PCR might not be optimal. An optimal PCR is thus to a smaller or larger degree also affected by the denaturation temperature used [2].

To date, it is possible to optimise the denaturation and annealing steps of a PCR system by doing two separate runs (by keeping either the denaturation or the annealing temperature constant while changing the other). To find the best combination of optimal denaturation and annealing temperatures, one would have to first run a gradient for the annealing temperature. Subsequently, for each of the annealing temperatures tested, a gradient is then repeated for the denaturation step. This would result in multiple PCR runs that is both time- and resource-consuming. With the introduction of the new Eppendorf Mastercycler X50 however, this difficulty can now be solved. This article will present a new innovative technique called the 2Dgradient that allows for the ultimate PCR optimisation with utmost ease and speed.

Materials and Methods

PCRBio Taq DNA polymerase (Nippon Genetics) and Human Genomic DNA (Roche®) were used for the following amplification. PCR reaction master mix containing 1X reaction buffer, 0.25U of enzyme, 0.2 µM of each primer and 20ng DNA template was prepared. 10 µl of the master mix was dispensed into each respective 96 wells of Eppendorf twin.tec® skirted PCR plates. Dispensing was carried out by Eppendorf *epMotion*® 5073. Plates were sealed with adhesive PCR film and PCR was carried out on Mastercycler X50s.

The following primers were used for amplification of the human ß-actin gene:

Forward primer: 5′- ATCGCCGCGCTCGTCGTC-3′
Reverse primer: 5′- TGGGTCATCTTCTCGCGGTTGG-3′

Cycling conditions are listed in *Table 1*. The PCR products were detected using GelRed™ (Biotium) following agarose gel electrophoresis and visualised using the Gel Doc XR+ (BioRad®).

Table 1: PCR condition with two concurrent gradient setting at denaturation and annealing steps.

Header (Eppendorf settings)	Lid	105 °C
	TSP/ESP	ON
	Lid auto-off	ON
	Temperature mode	Fast
Initial Denaturation		96 °C/5 min
Cycles: 35x	Denaturation	Gradient at 90-99 °C/20 s
	Annealing	Gradient at 52-72 °C/20 s
	Elongation	72 °C/30 s
Post-Cycle Elongation		72 °C/2 min
Storage	Hold	**4 °C**

Results and Discussion

The new 2D-gradient function of the Mastercycler X50 enables optimisation of both the denaturation and annealing temperatures in one PCR run. This was achieved through a matrix-style temperature set-up whereby the first gradient at denaturation step is set vertically while the second gradient at annealing step is set horizontally. This means that each of the eight rows of the thermal block has a different temperature at the denaturation step while each of the 12 columns of the thermal block has a different temperature at the annealing step.

Hence, for each denaturation temperature (T_D), 12 samples would be amplified at that temperature (e.g. wells A1–A12 would be subjected to 99°C T_D while B1–B12 would be subjected to 98.5°C T_D). After the denaturation step, samples under the same column would be subjected to the same annealing temperature (T_A), thus giving rise to 12 different T_A across the block (e.g. A–H1 would be subjected to 51.9°C T_A and A–H2 would be subjected to 52.3°C T_A). At the end of the completed PCR, the best combination of denaturation+annealing temperatures can then be determined (*Figure 1*).

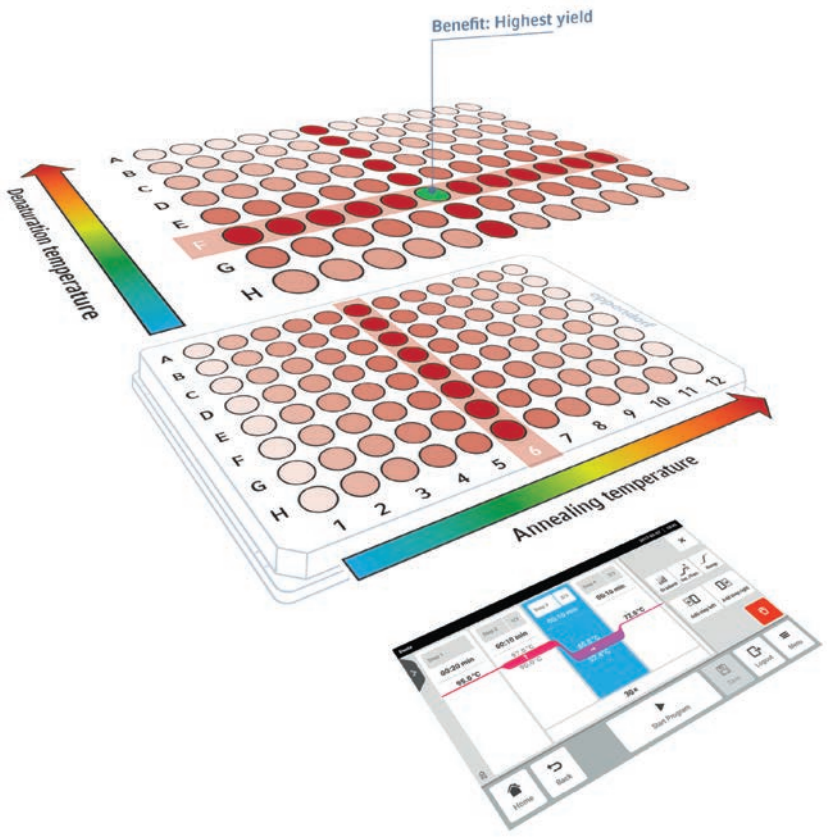

Figure 1: 2D-gradient function can be used in a matrix-style optimisation of both denaturation and annealing temperatures concurrently to find the optimal condition for highest PCR yield.

Optimal PCR result is defined by maximum yield of the specific amplicon of interest. Therefore, the aim of PCR is always first and foremost specificity followed closely by yield. While this can be primarily achieved through optimising the annealing temperature, there is no guarantee that the result obtained is the true 'optimal' result. It is always possible that the yield could be increased or the amount of non-specific product be reduced.

Figure 2 shows the result of the matrix-style optimisation technique of the 2D-gradient in amplifying the human ß-actin gene. This PCR system was chosen because of its temperature sensitive nature. Specific amplification will yield 484 bp fragments while sub-optimal condition will give rise to non-specific amplification visible as a 350 bp artefact in the gel.

Ordinarily, gradient optimisation is only performed for the annealing step at a fixed denaturation temperature at ca. 95°C. Taking the example from *Figure 2*, when 95.6°C is used, gradient result for annealing step showed that 65.9°C gives the best yield with small amount of non-specific product and at 70.5°C, only specific product will be obtained. Depending on the objective of the PCR, both of these temperatures can be considered 'optimal' conditions that are usually sufficient for most applications.

However, in certain cases such as low target copy number, a small difference in yield can be crucial to the application. In the example above, it can be clearly seen that 95.6°C is not an optimal T_D for this PCR system. By lowering the T_D to 93.4°C, the specific bands almost doubled in intensity. In addition, the results in this study showed that increasing T_D leads to decreasing non-specific amplification. For PCR systems where non-specific amplification is a problem, especially those with multiple bands, running a gradient at denaturation step would be especially beneficial. Hence the 2D gradient allows users to easily obtain a rich amount of information about the characteristic of their PCR system,

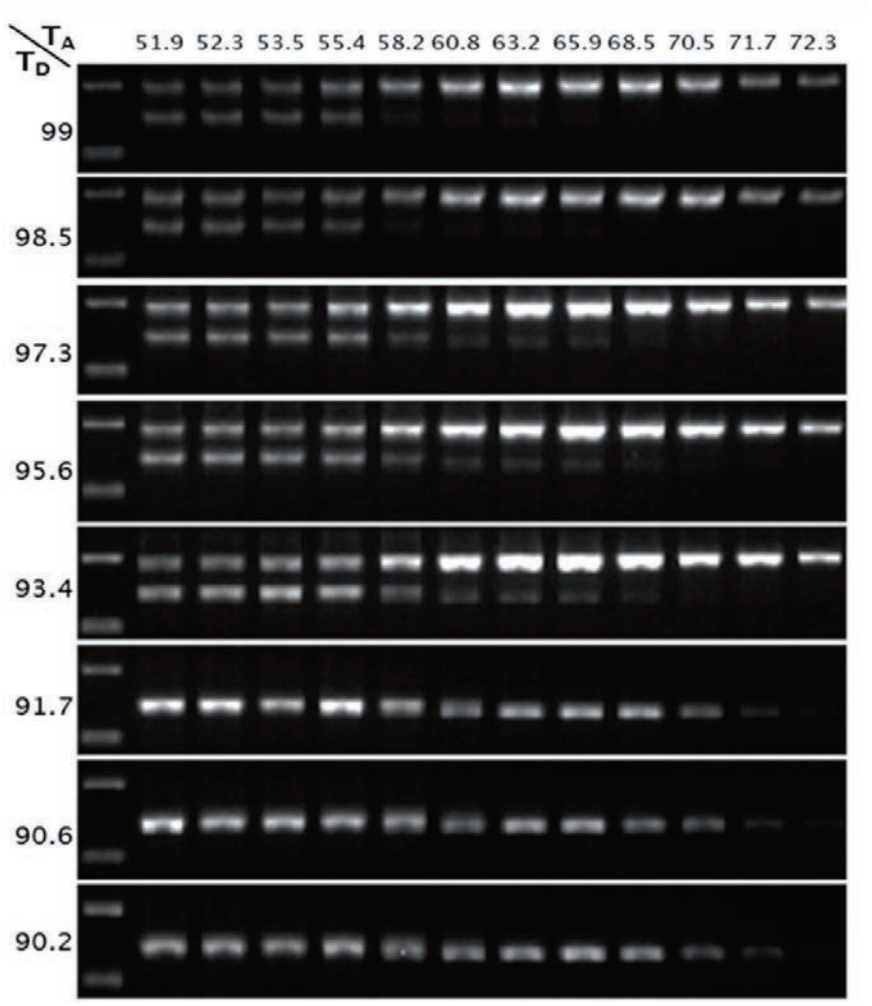

Figure 2: PCR optimisation of ß-actin gene with 2D gradient technique.

which in turn is beneficial for various application objectives such as increasing yield or resolving non-specific amplification problems.

Conclusion

The 2D-gradient function of the Mastercycler X50 allows users to simultaneously optimise both denaturation and annealing temperatures to determine the conditions for combined optimal yield and specificity for best PCR result. Not only does the convenience of this function allow users to save much time and effort in their optimisation work, it also has important implications for applications relating to low target copy number and GC-rich targets. In addition, this function is highly useful in troubleshooting non-specific amplification issues.

References

1. *Ong, W.K. (2010) Using the gradient technology of the Mastercycler® pro to generate a single universal PCR protocol for multiple primer sets. Eppendorf Application Note 220.*
2. *Gerke, N. (2013) Straightforward PCR optimization and highly flexible operation on the dual block thermocycler Mastercycler® nexus GX2. Eppendorf Application Note 289.*

Ordering information	Order no. International	Order no. UK/IRL
Mastercycler® X50s	6311 000.010	6311 000.045
Mastercycler® X50a	6313 000.018	6313 000.042
Mastercycler® X50p	6315 000.015	6315 000.040
Mastercycler® X50h	6316 000.019	6316 000.043
Mastercycler® X50l*	6303 000.010	6303 000.044
Mastercycler® X50r*	6305 000.017	6305 000.041
Mastercycler® X50t*	6306 000.010	6306 000.045
Mastercycler® X50i*	6301 000.012	6301 000.047
Accessories		
Ethernet cable, 5 m	6313 070.008	6313 070.008

* To operate this unit, it needs to be connected to a Mastercycler X50 s,a,p, or h. Up to 9 units can be connected to a Mastercycler X50 s,a,p, or h.