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Using the Mastercycler® X50 and Its 2D-Gradient to Increase Yield and Specificity of Your PCR

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Abstract

The discovery of the polymerase chain reaction (PCR) in 1983 led to the need for semi-automated devices to lessen the workload and improve reproducibility and productivity in the field of PCR. The development started with water bath based devices, led to Peltier heated devices, added a heated lid to reduce evaporation and to get rid of oil-overlays and subsequently to the invention of the annealing temperature gradient for easier optimization of PCR conditions. The latest addition to this line of inventions is the 2D-Gradient.

Here we show the use of the 2D-Gradient to eliminate unspecific signals and to increase product yield. The 2D-Gradient allows for a temperature gradient during the denaturation step AND the annealing step in the same PCR run. This allows for 96 different conditions in one PCR run and thus to quickly screen for the optimal temperature combination, which finally saves time and resources.

Introduction

PCR is, among others, routinely used in research, diagnostics and industrial area. This technique was discovered in 1983 and was at that time a tedious, time and resource consuming process [1,2]. Therefore, it is not surprising that the discovery of PCR was followed by the development of the first thermocycler [3]. These machines rapidly evolved from metal blocks heated and cooled by water to Peltier heated and cooled metal blocks [4]. Subsequently a heated lid [5] was added to get rid of the need to add mineral oil and still prevent evaporation. Optimizing a PCR protocol still proved to be tedious and time consuming. Every annealing temperature needed a separate PCR run. This quickly led to the development of the temperature gradient to run up to 12 different annealing temperatures in one go using a 96 well cycler [6].

The annealing temperature is the temperature that is classically optimized. Optimizing the denaturation temperature on the other hand is usually not a focus, since the impact of the annealing temperature is considered much bigger than the one of the denaturation temperature. However, different denaturation temperatures especially with GC-rich templates may lead to higher yield and thus should be kept in mind when talking about optimizing PCR. Our aim is to show that optimizing the denaturation temperature does yield benefits for many PCR reactions. The 2D-Gradient of the Mastercycler X50 offers for the first time a tool to optimize both temperatures at once thus providing 96 different conditions in a single PCR run (see figure 1).

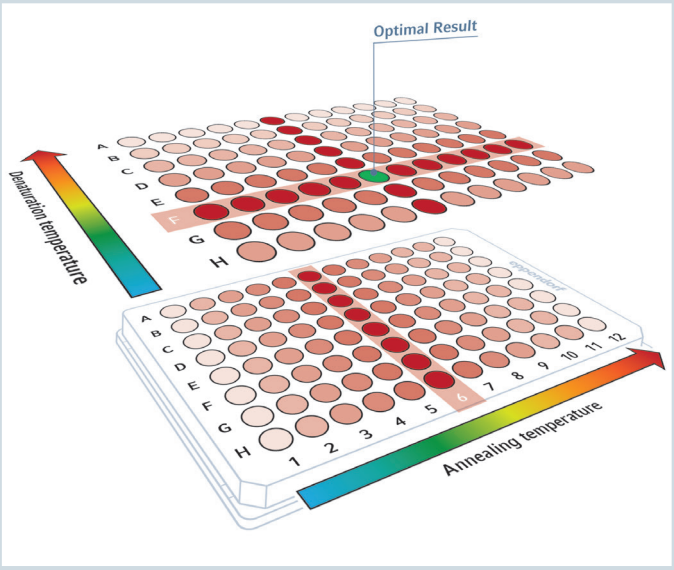

Figure 1: Principle of the 2D-Temperature-Gradient. The traditional gradient during the annealing step is depicted in the bottom part of the picture while the second dimension is added by applying a temperature gradient during the denaturation step, thus generating 96 different conditions across the PCR plate.

Here we show that modulating the denaturation temperature does indeed change the outcome of the PCR i.e. by increasing the specificity and / or yield of the PCR product. Three different targets that are used in the lab were optimized, namely one plasmid construct, a mouse originating gene and a zebrafish gene. We could show three different benefits with these three templates.

Firstly: An increase in specificity for the amplification of the gene plasmid construct.
Secondly: An increase in yield for the mouse originating gene.
Thirdly: We could show that changing the denaturation temperature leads to a failure of amplification of one of the two specific target products in a genetically modified zebrafish line.

The Eppendorf 2D-Gradient did proof to be the tool of choice for quickly optimizing PCR protocols and increasing yield and specificity of your PCR as well as to ensure that all relevant products are being amplified. We could also proof that modulating the denaturation temperature did indeed influence the PCR results.
One might argue that analyzing 96 different conditions is tedious, time and resource consuming as well, thus we decided to minimize the effort but maintaining an efficient number of different temperature combinations by using the presented pipetting and tempering scheme (see figure 2).

	Annealing Temperature / °C - Gradient 50-70°C horizontal											
	49.9	**50.3**	**51.5**	**53.4**	**56.2**	**58.8**	**61.2**	**63.9**	**66.5**	**68.5**	**69.7**	**70.3**
Denaturation Temperature / °C Gradient 98-90°C vertical												
98	49.9/98	50.3/98	51.5/98	53.4/98	56.2/98	58.8/98	61.2/98	63.9/98	66.5/98	68.5/98	69.7/98	70.3/98
97.5	49.9/97.5	50.3/97.5	51.5/97.5	53.4/97.5	56.2/97.5	58.8/97.5	61.2/97.5	63.9/97.5	66.5/97.5	68.5/97.5	69.7/97.5	70.3/97.5
96.5	49.9/96.5	50.3/96.5	51.5/96.5	53.4/96.5	56.2/96.5	58.8/96.5	61.2/96.5	63.9/96.5	66.5/96.5	68.5/96.5	69.7/96.5	70.3/96.5
95	49.9/95	50.3/95	51.5/95	53.4/95	56.2/95	58.8/95	61.2/95	63.9/95	66.5/95	68.5/95	69.7/95	70.3/95
93	49.9/93	50.3/93	51.5/93	53.4/93	56.2/93	58.8/93	61.2/93	63.9/93	66.5/93	68.5/93	69.7/93	70.3/93
91.5	49.9/91.5	50.3/91.5	51.5/91.5	53.4/91.5	56.2/91.5	58.8/91.5	61.2/91.5	63.9/91.5	66.5/91.5	68.5/91.5	69.7/91.5	70.3/91.5
90.5	49.9/90.5	50.3/90.5	51.5/90.5	53.4/90.5	56.2/90.5	58.8/90.5	61.2/90.5	63.9/90.5	66.5/90.5	68.5/90.5	69.7/90.5	70.3/90.5
90.2	49.9/90.2	50.3/90.2	51.5/90.2	53.4/90.2	56.2/90.2	58.8/90.2	61.2/90.2	63.9/90.2	66.5/90.2	68.5/90.2	69.7/90.2	70.3/90.2

Figure 2: Tempering scheme used in this work. The scheme depicts the sample positions in the thermoblock. Positions marked in blue were used to reduce the sample, work and analysis load from 96 samples to 31 samples. Temperature combinations stated in the boxes are in °C.

Materials and Methods

All experiments were performed on a Mastercycler X50s (Eppendorf) with the lid temperature set to 105°C, energy-saving mode on, the temperature mode set to standard and the block settings set to silver 96.
Eppendorf twin.tec® PCR Plates 96 (semi-skirted and skirted) were used in all experiments. Plates were sealed with Eppendorf PCR Film or Eppendorf Heat Sealing Film.

Plasmid located Gene A

The 10 µL reaction setup contained 1x in house Taq buffer (10 mM KCl, 10 mM $(NH_4)_2S0_4$, 20 mM Tris-HCl, 2 mM $MgS0_4$, 0.1% Triton X-100, pH 8.8), 200 µM dNTP's, 0.2 µM forward Primer Gene A (5'- AAG TTC ATC TGC ACC ACC G-3'), 0.2 µM reverse Primer Gene A (5'- TCC TTG AAG AAG ATG GTG CG-3'); 0.2 U in house produced Taq polymerase, 2.5 ng total template DNA.

Table 1: The following PCR program was used for amplification:

Initial Denaturation	95 °C	120 seconds
Cycles 35x	Gradient 98-90 °C	30 seconds
	Gradient 50-70 °C	30 seconds
	72 °C	30 seconds
Post Cycle Elongation	72 °C	60 seconds
Storage	15 °C	Hold

Temperature combinations used are depicted in the results part. PCR products were analyzed by Agarose Gel-Electrophoresis (1.5% agarose in 1x TAE, run for 45 min at 100 V in 1x TAE).

Mouse located Gene B11

PCR ReadyMix™ (Sigma-Aldrich® ordering number R4775), 3.2 µl ultrapure water, 0.4 µM forward Primer Gene B (5'- AAA GTC GCT CTG AGT TGT TAT -3'), 0.4 µM reverse Primer Gene B (5'- GGA GCG GGA GAA ATG GAT ATG -3'), 30 ng total genomic DNA (2-propanol purified).

Table 2: The following PCR program was used for amplification:

Initial Denaturation	95 °C	120 seconds
Cycles 35x	Gradient 98-90 °C	30 seconds
	Gradient 50-70 °C	30 seconds
	72 °C	60 seconds
Post Cycle Elongation	72 °C	60 seconds
Storage	15 °C	Hold

Temperature combinations used are depicted in the results part. PCR products were analyzed by Agarose Gel-Electrophoresis (1.5% agarose in 1x TAE, run for 45 min at 100 V in 1x TAE).

Zebrafish located Gene C

The 11 µL reaction setup contained 1x Phu buffer, 200 µM dNTP's, 0.5 µM forward Primer Gene C (5'- ACGATCAGATTTGCAGAGCAG -3'), 0,5 µM reverse Primer Gene C (5'- TGAGTACCATTTATGACCTCCTGAC -3'); 0.25% (v/v) DMSO; 0.2 U Phusion® DNA polymerase (NEB Order Number: M0530), 5 ng total genomic DNA.

Table 3: The following PCR program was used for amplification:

Initial Denaturation	98 °C	120 seconds
Cycles 35x	Gradient 98-90 °C	15 seconds
	Gradient 50-70 °C	20 seconds
	72 °C	20 seconds
Post Cycle Elongation	72 °C	300 seconds
Storage	15 °C	Hold

Temperature combinations used are depicted in the results part. PCR products were analyzed by Agarose Gel-Electrophoresis (2% agarose in 1x TAE, run for 45 min at 100 V in 1x TAE).

Results and Discussion

Optimizing a PCR can be a very resource consuming process, especially if the desired product is not appearing or if the bothering unspecific bands are not disappearing. One possible reason could be the lack of optimization of the denaturation temperature, which is usually considered to be "just hot". This work shows that modulating the denaturation temperature indeed does influence specificity, yield and in the extreme case also influences the presence or absence of specific bands.

Plasmid located gene A

The target amplicon is 179 bp in size and is present in different yields in all samples except one. Unspecific products with a size of approximately 1200 bp, 900 bp and 650 bp are present in almost all samples.
However, it is obvious that it is possible to eliminate these unspecific bands by modulating the denaturation temperature by one degree. The best result in this experiment is achieved with a denaturation temperature of 90,5°C and an annealing temperature of 56.2 °C. The yield here is comparable to the most intense specific product signal at 93 °C / 58.8 °C. However, the unspecific products are not present at 90.5 °C / 56.2 °C (compare figure 3). Thus, this experiment alone could show that modulating the denaturation temperature indeed can give the same yield but eliminate unspecific products. The originally used PCR program with a denaturation temperature of 94 °C showed the unspecific bands. Hence optimizing the PCR program using the 2D-gradient and changing the annealing temperature to 90-91 °C would result in a more specific PCR product.

Running this analysis on a comparable cycler without a 2D-Gradient option would have taken 8 times the time and resources since eight single PCR runs with different denaturation temperatures and a gradient in the annealing temperature would have been needed.

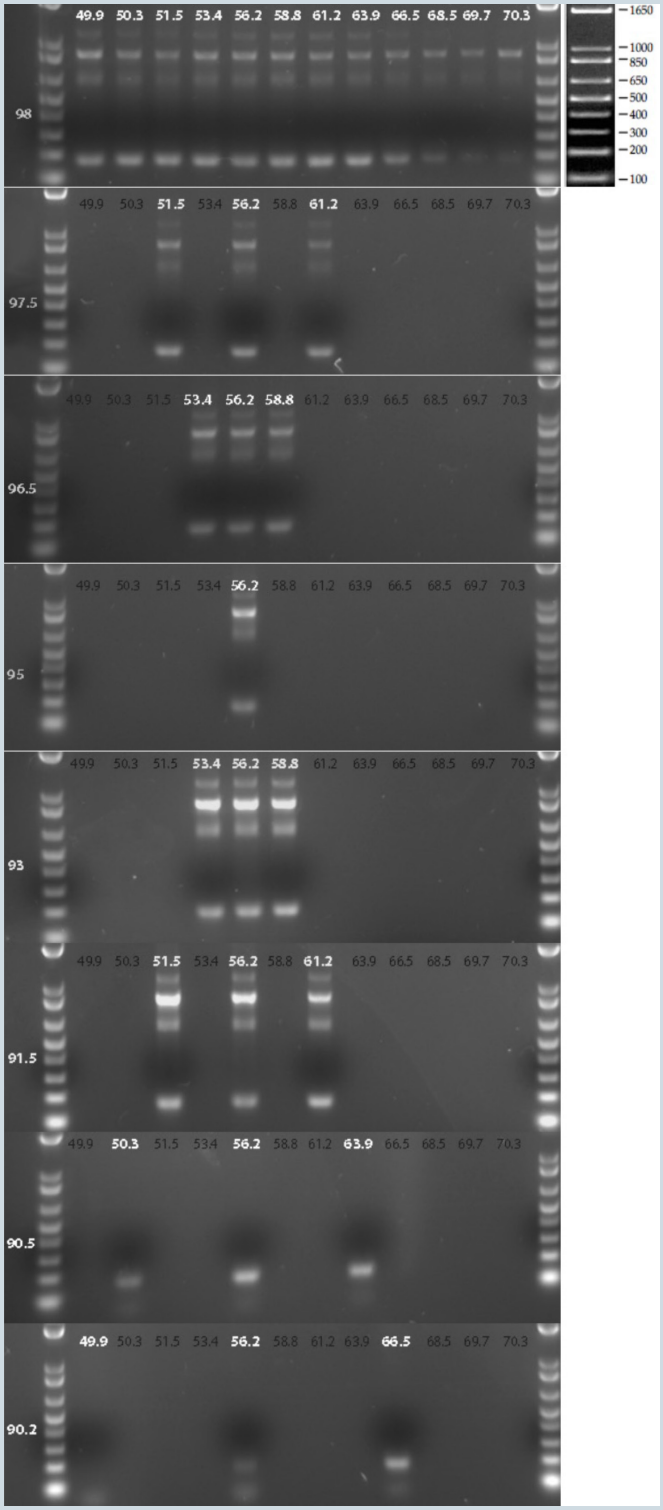

Figure 3: Agarose-Gel analysis of the plasmid located gene A. It is clearly visible that the unspecific signals with a molecular weight size larger than 200 bp disappear if the denaturation temperature is decreased to 90.5 °C or less. This indicates a clear improvement by the change in denaturation temperature.

Mouse located gene B

The target amplicon here has a size of 600 bp and is present in all samples except for one. There are no unspecific products visible, hence an optimization on this side is not necessary. However, running a 2D-Gradient shows variation in yield if the denaturation temperature is modulated. The product yield is acceptable in a denaturation temperature range of 96.5 to 90.2 °C. The highest yield is achieved in a denaturation temperature range of 91.5 to 90 °C and an annealing temperature of 56.2 °C (compare figure 4). The initially used denaturation temperature was 94 °C thus delivering product in an acceptable range but not being in the optimal denaturation temperature. This leads to a decreased product yield that can be easily optimized using a 2D-Gradient.

This shows that not only the annealing temperature needs optimization but also the denaturation temperature. Both factors have an impact on product yield. Thus, optimizing both temperature conditions could lead to better yield without increasing the number of PCR cycles in one PCR run and thus drastically saving time and resources. Optimization is easily achieved in one PCR run if the 2D-Gradient is used. If not, at least 8 PCR runs must be performed to achieve the displayed results.

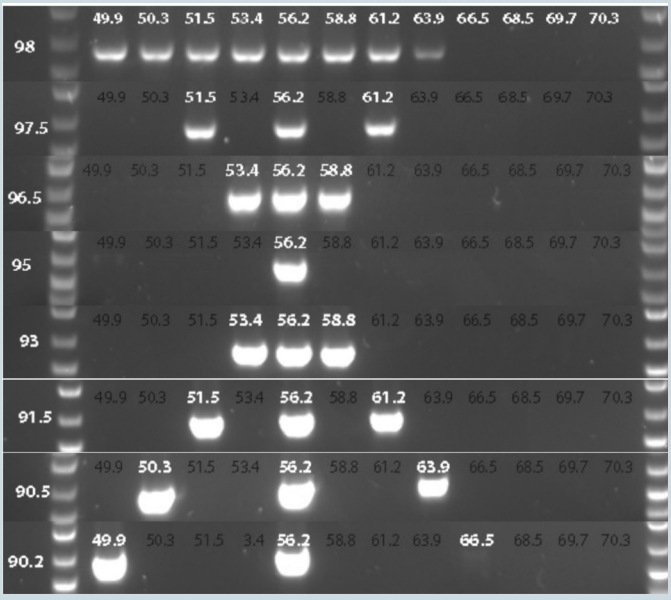

Figure 4: Agarose-Gel analysis of the mouse located gene B. This experiment shows an increase in yield if the denaturation temperature is decreased. This data shows that the most optimal yield can be achieved at a denaturation temperature of approximately 91 °C.

Zebrafish located Gene C

This gene has two target amplicons with a size of 502 bp for the wild type and 453 bp for the deletion mutant. This analysis does not show any nonspecific products. PCR results are visible in all but two PCR conditions. However, this experiment exemplifies the importance of the right denaturation temperature since a denaturation temperature that is too low led to the amplification of the mutant band only, leaving out the wildtype band. Thus, having the wrong denaturation temperature might lead to false negative results. In this case denaturation temperatures below 91.5 °C led to the loss of the wild type signal at 502 bp (see figure 5), again clearly indicating that the modulation of denaturation temperature is indeed of great importance. The denaturation temperature originally used was 98 °C thus already ensuring the presence of both specific products. However, it would have been possible to miss one of the specific bands due to a non-optimal denaturation temperature, reinforcing the importance of an optimized denaturation temperature.

Conclusion

We could show for three cases with different organisms of origin and different target genes that the denaturation temperature has a crucial effect on the PCR results, be it specificity, yield or false negative results. Optimizing the annealing and the denaturation temperature however proofs to be a very time consuming and tedious process using conventional thermocyclers with 1D-Gradients. This setup would require at least 8 different PCR runs to obtain the wanted results.

Using the Mastercycler X50 with its 2D-Gradient cuts down this optimization time to one single PCR since all gradients can be run in one go on one thermoblock.

Acknowledgement

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Literature

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[4] US patent US603236B2, 1990
[5] US patent US7504241B2, 1990
[6] European patent EP 1 426 110 A3, 1997

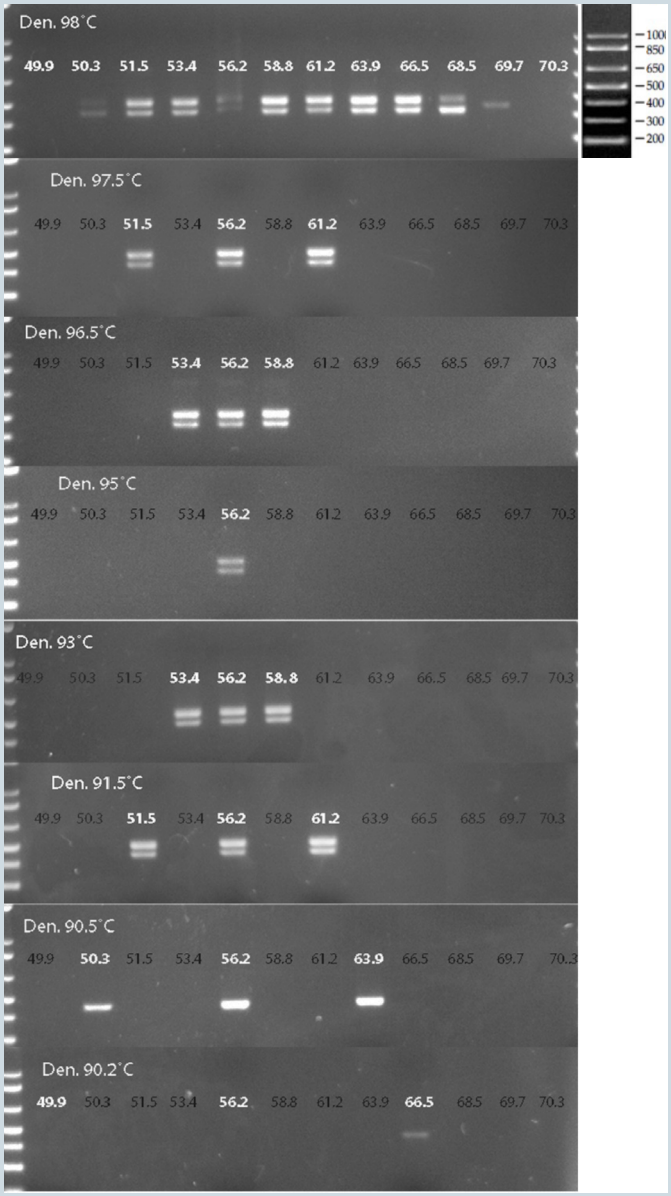

Figure 5: Agarose-Gel analysis of the zebrafish located gene C. Both of the visible signals are specific in this assay, thus showing that decreasing the denaturation temperature below 91.5°C results in the loss of one of the specific bands and by that giving a false negative analysis.

Ordering information

Description	Order no. international	Order no. North America
Mastercycler® X50s	6311 000.010	6311000010
Eppendorf twin.tec® PCR Plate 96 semi-skirted	0030128575	951020303
Eppendorf twin.tec® PCR plate 96 skirted	0030128648	951020401
PCR Film self-adhesive	0030127781	0030127781
Heat Sealing Film	0030127838	0030127838

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