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APPLICATION NOTE No. 428

PCR Optimization for Single-Molecule Experiments using the Mastercycler® X50 2D-gradient

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Abstract

In this work, we illustrate the powerful capabilities of the Mastercycler X50 2D-gradient in producing suitable DNA filaments for single-molecule applications. Our results indicate that its characteristic of simultaneously modulate both the annealing temperature (T_A) and the denaturation temperature (T_D) results in a very potent tool for GC-rich template applications. This innovation in gradient technology allows the setting-up of the best conditions to obtain an efficient amplification of a long DNA sequence with a high GC-content in a fast and convenient way.

Introduction

Single-molecule techniques are the methods of choice to study the nanomechanical properties of nucleic acids *in vitro*, among them the most commonly used are Magnetic Tweezers (MT), Optical Tweezers (OT) and Atomic Force Microscopy (AFM) [1][2][3]. The investigation on DNA properties, especially based on OT and MT, requires DNA molecules with stringent and peculiar characteristics: they have to be sufficiently long – ranging from two thousand to more than twenty thousand base pairs – and nicked-free. The simplest way to obtain such long constructs is a gel-purification after proper digestion of a specific sequence, coming from commercial or synthetic DNA preparations.
However, for the study of DNA denaturation and supercoiling in a single molecule, the imposition of a torsional constraints over a DNA filament is mandatory, and the presence of nicks in the sequence, as a result of the gel extraction procedure, would per se impede the torsion. Indeed, the nicked double helix would be free to rotate around the single bond present in the backbone and would not denature or supercoil. Hence, as a matter of principle, the only way to obtain an intact double helix is to take advantage of the Polymerase Chain Reaction (PCR). However, with this approach, the problem is only shifted through the complications arising from the PCR amplification of long sequences. It is well known that the optimization of a PCR for long amplicons needs fine-tuning. Indeed, it is necessary to obtain the conditions that are able to gain sufficiently abundant amplification products, and the presence of shorter and non-specific amplification products be extremely reduced, if not completely abolished. To reach these optimal conditions, the annealing temperature (T_A) and time have to be conveniently modulated.

Moreover, the local characteristics of DNA such as GC-content and distribution frequently attract the attention of molecular biologists, since they seem to be involved in the principal aspects of DNA metabolism and DNA associated pathologies [4][5]. In this scenario, the amplification of a long DNA sequence, which also has a high GC-content (>70%) becomes even more complicated. Indeed, GC-rich DNA sequences have a melting temperature higher than DNA sequences with a normal CG-content (around 50%). Thus, working with this kind of sequences imply the need for simultaneous modulation of both the annealing temperature (T_A) and the denaturation temperature (T_D), to find the best conditions for amplification. Using a classical thermal cycler, an approach that could only independently vary the two parameters (T_A and T_D) in an analytical screening would require a large expenditure of both time and resources.
Here in this work, we optimize the amplification of a 6,098 bp-long DNA sequence with 78% CG-content by taking advantage of the Mastercycler X50 2D-gradient technology. Our results clearly indicate that the exclusive feature of the Mastercycler X50 2D-gradient, which can modulate both the T_A and T_D simultaneously, is crucial to turning a difficult and time-consuming procedure into a rapid and easy process.

Material and Methods

To obtain a 6,098 bp amplicon with a 78% GC-content, the pSC-77%GC_ΔAsc vector was used as a template for the PCR. The said vector is a modified version of the pSC-77%GC vector [5], in which the 4,017 bp sequence between the two AscI restriction sites have been removed. The PCR was performed using the Mastercycler X50 with the 2D-gradient function in a final volume of 15 µL, with the following primers:

Forward primer: 5'- TAATACGACTCACTATAGGG -3'
Reverse primer: 5'- GCAATTAACCCTCACTAAAGG -3'

The PCR was performed using 1 ng of DNA template, 0.5 mM primers, 2 U/ml of Q5 DNA polymerase (NEB®, Cat. no. M0491) and 0.2 mM dNTPs in 1X Q5 reaction buffer. The cycling conditions are as specified in Table 1. Following amplification, the PCR products were subjected to an agarose gel electrophoresis (with 0.5 g/mL ethidium bromide) and visualized using the Amersham® Imager 600 (GE Healthcare-Life Sciences).

Table 1: PCR cycling condition using 2D gradient settings, both at the denaturation and annealing step.

Header (Eppendorf settings)	Lid	105 °C
	TSP/ESP	ON
	Lid auto-off	ON
	Temperature mode	Fast
Initial Denaturation		98 °C/1 min
Cycles: 35x	Denaturation	Gradient at 95-99 °C/20 s
	Annealing	Gradient at 52-62 °C/15 s
	Elongation	72 °C/2 min
Post-Cycle Elongation		72 °C/2 min
Storage	Hold	10 °C

Results and Discussion

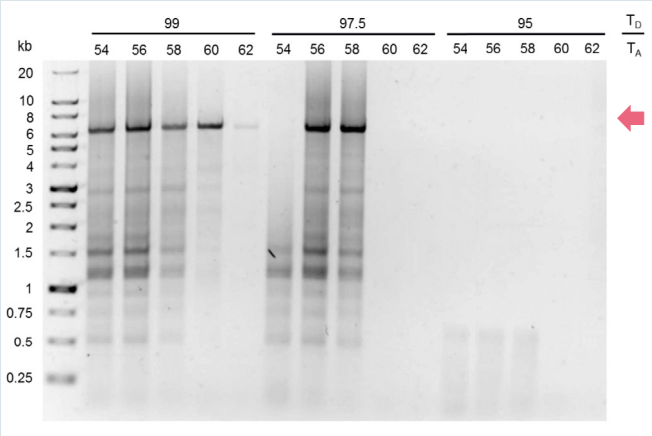

Figure 1: PCR optimization of a 6kb-long amplicon with a 78% GC-content, using the 2D-gradient technique. One representative run out of three independent experiments. Arrow indicates 6 kb target amplicon.

The opportunity to simultaneously operate contemporary T_A and T_D variations is a specific feature of the Mastercycler X50 equipped with an innovative 2D-gradient function. Indeed, it has been shown that this instrument allows the optimization of difficult amplifications, by applying a 2D-gradient that permits a simple and simultaneous modulation of these two critical temperature parameters [6]. For this reason, we decided to optimize the PCR conditions for the amplification of a 6 kb DNA sequence with a high GC-content (78%), for use in single-molecule experiments, by taking advantage of this powerful instrument. With a theoretical optimal T_A of 56 °C, four different T_A were tested (54, 56, 58, 60 and 62 °C) while at the same time, three T_D were scanned (95-97.5-99 °C).

Figure 1 shows the representative result obtained from a single PCR run using the 2D-gradient function. It was immediately clear that the lower T_D was not sufficient to allow the amplification of the desired sequence, which should be visible at around 6,000 bp. Only non-specific bands, which showed up mainly as smears, can be detected at a lower molecular weight of around 500 bp, and only in the case of the three more permissive T_A (54, 56, 58 °C). This finding indicates that with this DNA template that has a high GC-content, a higher T_D is necessary to start and carry on the amplification of this 6 kb-long sequence.

We can instead, observe some specific amplicons at both the higher T_D (97.5 and 99 °C). In the case of 97.5 °C, an abundant and specific band is present at the theoretical T_A (56 °C) and a more stringent T_A of 58 °C. However, in both cases, undesirable non-specific bands ranging from 500 bp to 3kb are also present in abundance. On the other hand, at T_D of 99 °C, we observe a general improvement in PCR specificity: a specific band is present at all the T_A tested. This result confirms the hypothesis that a higher T_D is required to allow the amplification of long amplicons with a high GC-content. Moreover, it is worthwhile to note that at this higher T_D, the amplicons obtained with the most stringent T_A tested (60 °C) produces the most specific amplification, whereby the pattern of non-specific bands at the lower molecular weights is shown to be critically reduced. Thus, we can conclude that this temperature combination (99 °C T_D + 60 °C T_A) can be considered as the best amplification conditions. This fulfil the demand of highest specificity being mandatory for obtaining the most suitable construct for single-molecule experiments.

The results show that the Mastercycler X50 2D-gradient is an extremely powerful tool. Only one PCR experiment was sufficient to obtain the desired conditions, which took approximately 2 hours. If this experiment was to be carried out separately using conventional one-dimensional gradient technology, three PCRs would be required, which would have been a full day's work. Thus, the 2D-gradient technology helped in significantly reducing the operational time, making a complex and time-consuming screening more easier and faster. Moreover, the possibility to modulate both T_D and T_A at the same time allows the optimization to be carried out using directly comparable conditions. Thus, run-to-run variations between three different PCR experiments because of running the PCRs on conventional thermal cycler without 2D-gradient can be avoided. In conclusion, our results showed that the Mastercycler X50 2D-gradient can be considered the best choice for the optimization of PCR conditions for long amplicons, in particular those with extremely high GC-content. The possibilities and benefits offered by this tool would surely help in simplifying the production of complex DNA constructs necessary for single-molecule experiments.

References

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Ordering information

Description	Order no. international	Order no. North America
Mastercycler® X50s (96-well silver block, with touchscreen interface)	6311 000.010	6311000010
Mastercycler® X50a (96-well alu block, with touchscreen interface)	6313 000.018	6313000018
Mastercycler® X50p (96-well alu block, high pressure, with touchscreen interface)	6315 000.015	6315000015
Mastercycler® X50h (384-well alu block, with touchscreen interface)	6316 000.019	6316000019
Mastercycler® X50i (96-well silver block, eco module)	6301 000.012	6301000012
Mastercycler® X50l (96-well alu block, eco module)	6303 000.010	6303000010
Mastercycler® X50r (96-well alu block, high pressure, eco module)	6305 000.017	6305000017
Mastercycler® X50t (384-well alu block, eco module)	6306 000.010	6306000010
Eppendorf twin.tec® PCR Plate 96 semi-skirted	0030 128.575	951020303
Eppendorf twin.tec® PCR plate 96 skirted	0030 128.648	951020401
Eppendorf Fast PCR Tube Strips, 0.1 mL, with flat cap strips	0030 124.910	0030124910
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