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A streamlined gDNA Sequencing Protocol for Low Sample Consumption and Reduced Reagent Costs Using the Mastercycler® pro

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Abstract

This application note provides a streamlined protocol for DNA sequencing using small reaction volumes on the Mastercycler® pro. We performed PCR in reaction volumes of 10 µL, 20 µL and 50 µL and compared the obtained PCR product concentration. Furthermore, sequencing reactions were carried out in 5 µL, 10 µL and 20 µL and the quality of the obtained sequencing data was evaluated. We show that PCR and sequencing reactions with small reaction volumes can be performed successfully on the Mastercycler pro: The obtained concentration of the PCR products and the quality of the obtained sequences, including the lowest volume reactions, were comparable. Robustness of the method was proven by sequencing the entire coding sequence of the human IRS1 gene in 95 bladder tumor samples using the smallest tested volumes. Using this protocol, sample and reagent consumption was considerably reduced.

Introduction

Classical DNA sequencing consists of template amplification by PCR, purification, sequencing reaction and sequence analysis [1,2]. Functional requirements for an optimized sequencing protocol are low sample and reagent consumption, robustness and fast and easy handling. Low sample consumption is essential when using precious samples that are difficult to obtain, as tumor tissue or forensic material [3]. Small reagent consumption is in particular important for higher throughput as reagent costs quickly become a limiting factor. Lack of robustness impairs the quality of the results and is time consuming, as failed PCR or sequencing reactions have to be repeated individually.
Minimizing reaction volumes is one possibility to reduce sample and reagent consumption. However, PCR and sequencing reactions need stable conditions for reliable results and good quality. For example, small reaction volumes increase the sensitivity of the reaction to evaporation. Heat sealing of PCR vessels provides a high level of protection against evaporation, but special consumables and a heat sealing machine are needed. With the new Mastercycler pro, we performed 760 low volume PCR reactions and 1520 sequencing reactions on 95 bladder tumor gDNA samples with standard PCR consumables and adhesive foils.
To assess the quantity and quality of PCR products obtained in the different PCR reaction volumes, we performed agarose gel electrophoresis. The obtained sequencing data issued from different reaction volumes was compared for signal intensity and background level.

Material and Methods

First, PCR product concentration and quality of sequencing data issued from different reaction volumes have been compared. Therefore, using PCR, two different regions of the IRS1 gene were amplified:

PCR1
A 699 bp fragment of IRS1 was amplified using the forward primer 5'-TCCACCTCGGATTGTCTCTTC-3' and the reverse primer 5'-GGGCATATAGTCTCCACTGCC-3'.

PCR2
A 674 bp fragment of IRS1 was amplified using the forward primer 5'-CACAGAGATGATGCCTGCCTA-3' and the reverse primer 5'-GCTGGGTGTGCTTAAAGGATC-3'.

PCR was carried out using HotStarTaq® PCR kit (Qiagen®, Germany) referring to the manufacturer's instructions in 50 µL, 20 µL and 10 µLl total volume using a concentration of 2 ng/µL gDNA. The PCR conditions are shown in Table 1.

Table 1: PCR program

Time	Temperature	Number of cycles
12 min.	94 °C	1 x
15 s. 30 s. 2 min.	94 °C 64 °C 72 °C	2 x
15 s. 30 s. 2 min.	94 °C 63 °C 72 °C	2 x
15 s. 30 s. 2 min.	94 °C 62 °C 72 °C	2 x
15 s. 30 s. 2 min.	94 °C 61 °C 72 °C	3 x
15 s. 30 s. 2 min.	94 °C 60 °C 72 °C	3 x
15 s. 30 s. 2 min.	94 °C 59 °C 72 °C	4 x
15 s. 30 s. 2 min.	94 °C 58 °C 72 °C	4 x
15 s. 30 s. 2 min.	94 °C 57 °C 72 °C	5 x
15 s. 30 s. 2 min.	94 °C 56 °C 72 °C	5 x
10 min.	72 °C	1 x

PCR product purification:
5 µl of PCR product were purified by adding 1 µl of ExoSAP-It® (GE Healthcare®, USA) and incubated for 15 min at 37 °C followed by 15 min at 80 °C.

Sequencing reaction:
0.1 µL of PCR product per 1 µL of total reaction volume was subjected to sequencing analysis using BigDye® Terminator v3.1 (Applied Biosystems®, USA) according to the manufacturer's instructions in 5 µL, 10 µL and 20 µL. After thermal cycling (Table 2), the total volume of the reactions was adjusted to 20 µl using Milli-Q® water (Millipore®, USA).

Table 2: Sequencing program

Time	Temperature	Number of cycles
1 min.	96 °C	1 x
30 s. 15 s. 4 min.	96 °C 50 °C 60 °C	29 x

After these initial tests, we performed the amplification and following sequencing of 8 different loci in the IRS1 gene on 95 bladder cancer samples to test the robustness of the reactions performed in the smallest tested volumes. 8.4 µL of PCR Mastermix containing reaction buffer, dNTP, primers and polymerase were pipetted with an automatic pipette into each well of an Eppendorf twin.tec® PCR Plate 96 (skirted). Bladder cancer gDNA samples were organized in a 96 well plate. One well of the plate contained only Milli-Q water, serving as a negative control.
With an 8 channel pipette, 1.6 µl of gDNA at 12.5 ng/µL were added into the corresponding well. Plates were sealed with Eppendorf PCR Film (self adhesive) and centrifuged at 500 g for 1 min.
After thermal cycling, the sealing PCR film was removed and 5 µl of the PCR product were subjected to analysis on a 1.5% agarose gel.
To the remaining 5 µl of PCR product, 1 µL of ExoSAP-It was added with an automatic pipette to each well of the plate. The plate was then sealed with a PCR film and centrifuged at 500 g for 1 min. After thermal incubation referring to the manufacturer's instructions, the purified PCR products were subjected to sequence analysis.
A sequencing Mastermix containing the reaction buffer, BigDye Terminator v3.1 and the sequencing primer was prepared and 4.5 µL were pipetted in each well of a twin.tec PCR Plate 96 (skirted). With an 8 channel pipette, 0.5 µL of purified PCR product were transferred into each corresponding well of the sequencing plate. The sequencing plate was then sealed with a PCR film and centrifuged at 500 g for 1 min. After thermal cycling, 15 µL of Milli-Q water were added to each well with an 8 channel pipette.
The sequencing reactions were analyzed on an ABI Prism® 3130XL (Applied Biosystems, USA).
All thermal incubations were performed on a Mastercycler pro (Eppendorf AG, Germany)

Results and Discussion

Agarose gel analysis of PCR products showed a single band at approximately 700 bp. The concentration of the PCR product was comparable in 10 µl, 20 µl and 50 µl total reaction volume. In 50 µl, a weak decrease of PCR product concentration was observed.

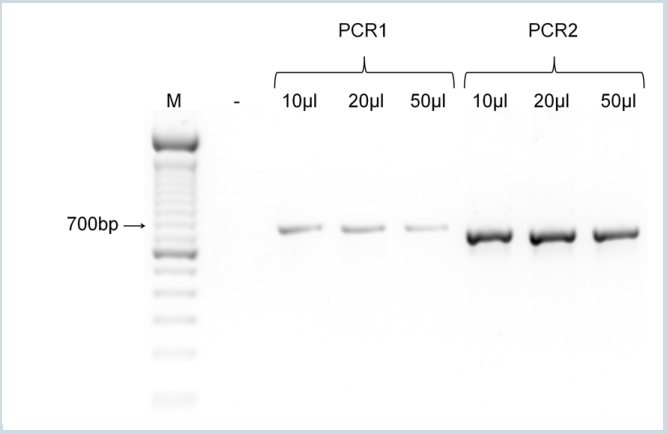

Figure 1: Comparison of PCR-product concentration on an agarose gel. 5 µL of PCR product were loaded on a 1.5% agarose gel. M = 100bp ladder, - = negative control.

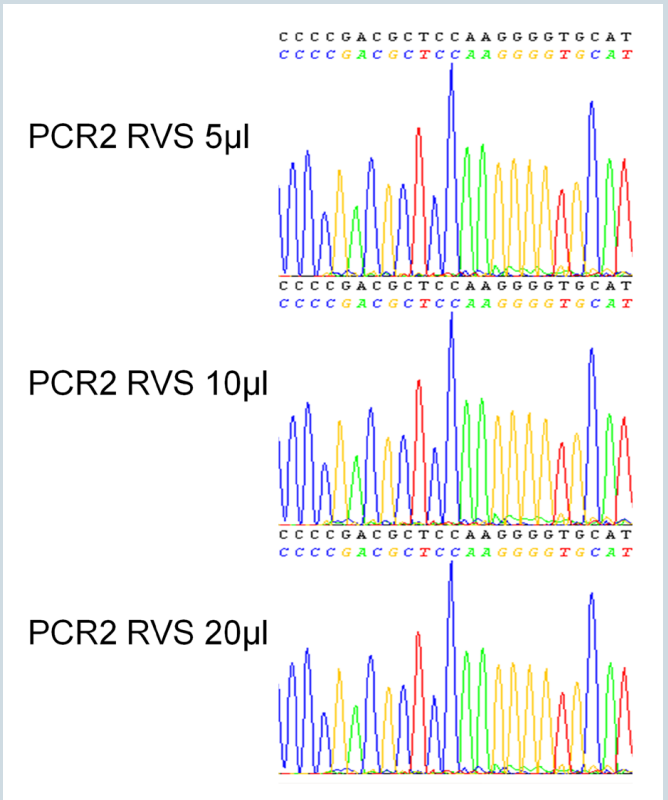

Figure 2: Representative section of the electropherogram obtained from 5 µl, 10 µl and 20 µl sequencing reactions.

In Figure 2, a representative section of three electropherograms is shown. The electropherograms obtained from 5 µL, 10 µL and 20 µL sequencing reaction volumes were comparable (Figure 2). No differences in background level were observed. With decreasing total reaction volumes, a diminution of the absolute signal intensity was observed in the raw data of the sequences, without affecting the quality of the analyzed sequences (Figure 2). To test the robustness of low volume PCR and sequencing reactions, we sequenced the entire coding of the IRS1 gene (3720 bp) in 95 bladder cancer samples. We obtained in total 1520 sequences. All the sequences could be determined in all 95 bladder cancer samples without ambiguity (data not shown).

The selected PCR reactions indicate that thermal cycling can be performed in volumes of 10 µL, 20 µL and 50 µL on the Mastercycler pro without compromising PCR product concentration. A weak decrease in concentration was observed in 50 µL, maybe due to slower temperature equilibration in the wells with the highest volume.
The comparison of sequences obtained from 5 µL, 10 µL and 20 µl revealed a loss in absolute signal intensity in the lower volumes. However, the electropherogram of the analyzed data from different volumes was almost not distinguishable (Fig. 2). Sealing the Eppendorf twin.tec PCR Plates 96 (skirted) with an adhesive film was sufficient to systematically avoid evaporation. We did not need to use heat sealing on the Mastercycler pro. Our sequencing project on the human IRS1 gene performed in low sample volumes provided 760 PCR products and 1520 sequences. As all of the sequences were of good quality and none of the reactions had to be repeated, we are confident in the robustness of low volume PCR and sequencing reactions on the Mastercycler pro.
With this streamlined protocol, PCR reagent costs and sample consumption can be cut to half compared with 20 µL reactions. Sequencing reaction reagent costs can be reduced by 75% compared with 20 µL reactions. Our results demonstrate that PCR and sequencing reactions can successfully be performed in low reaction volumes with high robustness and without compromising quality.

Acknowledgement

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References

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Ordering information

Description	Order no. International	Order no. North America
Mastercycler® pro (96 well aluminum block) 230 V / 50 - 60 Hz	6321 000.019	–
Mastercycler® pro (96 well aluminum block) 120 V / 50 Hz, with US-plug	6321 000.027	950030010
Mastercycler® pro S (96 well silver block) 230 V / 50 - 60 Hz	6325 000.013	–
Mastercycler® pro S (96 well silver block) 120 V / 50 Hz, with US-plug	6325 000.021	950030020
Mastercycler® pro 384 (384 well aluminum block) 230 V / 50 - 60 Hz	6324 000.010	–
Mastercycler® pro 384 (384 well aluminum block) 120 V / 50 Hz, with US-plug	6324 000.028	950030030
Control Panel incl. connecting cable	6320 000.007	950030050
Eppendorf twin.tec® PCR Plate 96, skirted wells colorless, 25 pcs	0030 128.648	951020401
PCR Film self-adhesive, 100 pcs	0030 127.480	951023019

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