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(Citation)

The Kobe journal of the medical sciences, 65(2):54-58

(Issue Date)

2019

(Resource Type)

departmental bulletin paper

(Version)

Version of Record

(JaLCD0I)

<https://doi.org/10.24546/81011839>

(URL)

<https://hdl.handle.net/20.500.14094/81011839>



Nested PCR Amplification Secures DNA Template Quality and Quantity in Real-time mCOP-PCR Screening for SMA

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Received 9 January 2018 / Accepted 27 May, 2019

Keywords: spinal muscular atrophy, *SMN1*, *SMN2*, mCOP-PCR, targeted pre-amplification, PCR-RFLP

BACKGROUND: Spinal Muscular Atrophy (SMA) is a common autosomal recessive disorder caused by *SMN1* gene deletion. SMA has been considered an incurable disease. However, a newly-developed antisense oligonucleotide drug, nusinersen, brings about a good outcome to SMA patients in the clinical trials. Now, a screening for SMA is required for early diagnosis and early treatment so as to give a better clinical outcome to the patients. We have invented a new technology, mCOP-PCR, for SMA screening using dried blood spot (DBS) on the filter paper. One of the problems encountered in SMA screening is poor quality and quantity of DNA extracted from DBS. **METHODS:** DNA was extracted from DBS of six individuals. Fresh blood DNA of each individual had already been genotyped using PCR/RFLP. The fragments including the sequence of *SMN1/SMN2* exon 7 were pre-amplified with conventional PCR. To determine which pre-amplified product is a better template for the real-time mCOP-PCR, we did pre-amplification with a single PCR or pre-amplification with a nested PCR. **RESULTS:** The real-time mCOP-PCR using pre-amplified products with a single PCR brought about ambiguous results in some *SMN1*-carrying individuals. However, the results of real-time mCOP-PCR following pre-amplification with a nested PCR were completely matched with those of PCR-RFLP. **CONCLUSION:** In our study on the real-time mCOP-PCR screening system for SMA, a nested PCR secured the DNA template quality and quantity, leading to unambiguous results of SMA screening.

INTRODUCTION

Spinal Muscular Atrophy (SMA) is a common autosomal recessive neuromuscular disease characterized by defects of lower motor neurons. SMA has been recognized as an incurable disease. In 1995, the *SMN* genes *SMN1* and *SMN2* were identified as SMA-related genes in chromosome 5q [8]. *SMN1*, which produces the SMN protein, is present in all healthy individuals. However, more than 95% of SMA patients show homozygous *SMN1* deletion, while the remaining patients harbor some deleterious mutations in *SMN1* [8]. *SMN2*, a gene highly homologous to *SMN1*, also produces small amount of SMN and modifies the SMA phenotype [4,14].

Improved understanding of the molecular mechanisms of *SMN2* expression has facilitated development of therapeutic compounds. In 2016, clinical trial results of intrathecal administration of an antisense-oligo drug, nusinersen, demonstrated encouraging clinical efficacy of the drug [5]. Food and Drug Administration (FDA) of US gave their approval to the drug in December, 2016, followed by the approval of the Ministry of Health, Labor and Welfare (MHLW) of Japan in July, 2017. We are now about to enter an era of the possibility of SMA treatment.

Identifying infants with *SMN1*-deletion will become more important. In order to address this demand, we have developed a new screening system with real-time “modified competitive oligonucleotide priming”-polymerase chain reaction (mCOP-PCR) using dried blood spot (DBS) [3, 7,15] or fresh blood [13], and combined it with PCR restriction fragment length polymorphism (PCR-RFLP) analysis for the purpose of confirming the results of real-time mCOP-PCR [12].

Although the real-time mCOP-PCR in our combination system can differentiate *SMN1* and *SMN2*, its amplification efficiency is not so good because the gene-specific reverse primers carry a nucleotide mismatched with the sequence of the pre-amplified product [12]. The mismatch has consequently been generated in the process of introducing a restriction enzyme site in the pre-amplified products for PCR-RFLP. We developed a more rapid screening system with newly-designed reverse primers used in the real-time mCOP-PCR in the combination system [11].

However, we still worry about another problem which will be encountered in the real-time mCOP-PCR screening for SMA, that is poor quality and quantity of DNA extracted from DBS. In this study, to overcome this problem, we did a nested PCR in the pre-amplification step instead of a single PCR.

MATERIALS AND METHODS

Patient and control samples

Six dried blood spot (DBS) samples collected from three SMA patients and three healthy controls were analyzed in this study. The DBS samples were stored at room temperature in dark room for 1-3 months. Genotypes of the patients and healthy controls had been determined using PCR-RFLP. All patients were negative for *SMN1* and positive for *SMN2*, while all controls were positive for *SMN1* and *SMN2*. Informed consent had been obtained from participants prior to this study. This study was approved by the Ethics Committee of Kobe University Graduate School of Medicine.

DNA extraction from DBS

Outline of this study is shown in Figure 1. DNA from DBS (DBS-DNA) on FTA Elute card (Thermo Fisher Scientific, Waltham, MA, USA) was extracted as follows: (1) one circle (3 mm diameter) was punched out of the filter paper using sterile paper plunger and placed into 1.5 mL tube, (2) the punched circle was washed in 125 μ L distilled water, (3) after removal of the washing water, 50 μ L 1 x Tris-EDTA buffer, pH 8.0 (TE buffer) was added into the tube and heated at 95°C for 30 minutes, (3) the paper was discarded and the remaining solution in the tube was used as a DNA source. The concentration and absorbance ratio at 260/280 of the extracted DNA solution was determined with NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific).

Pre-amplification with a single PCR and pre-amplification with a nested PCR

To secure quality and quantity of DNA template for real time mCOP-PCR, pre-amplification with a single PCR or pre-amplification with a nested PCR were performed. The primer sets for pre-amplification with a single PCR were RIII forward primer (5'-AGA CTA TCA ACT TAA TTT CTG ATC A-3') and X7Dra reverse primer (5'-CTT CCT TCT TTT TGA TTT TGT TT-3'). The primer sets for a nested PCR were (1) the 1st round: RIII forward primer and 541C770 reverse primer (5'-TAA GGA ATG TGA GCA CCT TCC TTC- 3'), and (2) the 2nd round: RIII forward primer and X7Dra reverse primer. Figure 1 illustrates locations of the primer sets.

For pre-amplification with a single PCR, 2 μ L of DNA solution was added to 28 μ L of PCR mixture containing 1x PCR buffer, 2 mM MgCl₂, 0.1 mM of each dNTP, 0.3 μ M of each primer (R111, X7-Dra), and 1.0 U FastStart Taq DNA polymerase (Roche Applied Science, Mannheim, Germany). The PCR condition for each step was: (1) initial denaturation at 94°C for 7 min, (2) 35 cycles of denaturation at 94°C, annealing at 56°C for 1 min, and extension at 72°C for 1 min, (3) additional extension at 72°C for 7 min, and (4) hold at 10°C (GeneAmp PCR system 9700 Applied Bio-system, Foster city, CA, USA). Gel electrophoresis was performed in each pre-amplification step on 4% agarose gel and visualized by Midori-Green staining (Nippon Genetics, Tokyo, Japan).

For pre-amplification with a nested PCR, the 1st round was performed in the same manner as above, except the reverse primer. Here, 541C770 reverse primer was used instead of X7Dra reverse primer. A hundred times diluted solution of the 1st round PCR product was used as a template in the second round PCR. The other PCR conditions were completely same as the pre-amplification with a single PCR.

Real time mCOP-PCR amplification

The real-time mCOP-PCR was performed using the LightCycler® 96 Real-time PCR system (Roche Molecular Systems, Inc) (11). Two microliter of 100 times diluted solution of pre-amplification products were added into 28 μ L of PCR mixture containing 1x PCR buffer, 2 mM MgCl₂, 0.1 mM of each dNTP, 50pmol/ μ L of common forward primer (R111), 50pmol/ μ L of gene-specific reverse primer *SMN1*-COP-Dra (5'-TTG TCT GAA ACC-3') or *SMN2*-COP-Dra (5'-TTG TCT AAA ACC-3'), and 1.0 U FastStart Taq DNA polymerase. The PCR conditions were: (1) initial denaturation at 94°C for 7 min; (2) 30 cycles of denaturation at 94°C for 1

min, annealing at 35°C for 1 min, and extension at 72°C for 1 min; (3) additional extension at 72°C for 7 min; and (4) hold at 10°C. Fluorescence signals were detected at the end of each extension procedure. Melting curve analysis was performed after PCR amplification, with 10 sec of denaturation at 95°C, 1 min of renaturation at 60°C, and then melting, which consisted of a continuous fluorescence reading from 65°C to 97°C at the rate of five data acquisitions per °C.

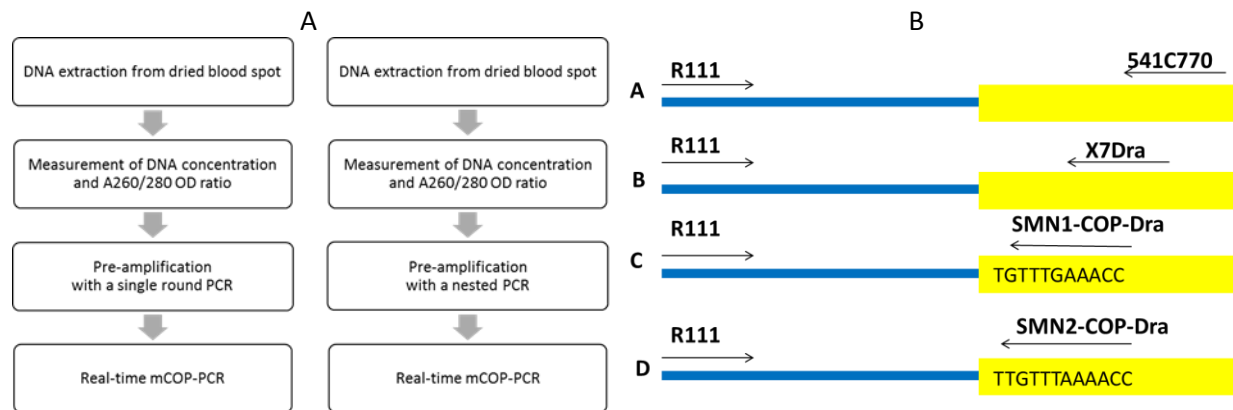


Figure 1. Outline of our study. (A) Flow chart of the procedures in this study. (B) Primer sets and their locations. Primer set A refers to the 1st round of nested PCR step, primer set B refers to pre-amplification and primer sets C and D refer to real time mCOP-PCR. The blue and yellow bars represent intron 6 and exon 7 respectively.

RESULTS

DBS-DNA concentration

The concentration of DBS-DNA extracted from a single circle punched from the filter paper was presented in table 1. DNA concentration ranged between 89.0 and 198.2 ng/μL, and A260/280 OD ratio ranged between 1.50 and 2.31 (Table I).

Table I. DNA Concentration of DBS samples

Sample	1	2	3	4	5	6
DNA concentration (ng/μL)	119.4	132.8	89.0	197.3	137.9	198.2
A260/280 OD ratio	1.65	1.90	1.50	2.31	1.78	1.80

Pre-amplification with a single PCR and pre-amplification with a nested PCR

In this study, only two clear bands were observed (sample number 1 and 3) in gel electrophoresis of pre-amplification products with a single PCR (Figure 2). We encountered similar tendency in the 1st round PCR results. Only two samples (sample number 1 and 3) exhibited clear bands on gel electrophoresis. However, the 2nd round PCR products presented promising results. Gel electrophoresis revealed clear bands in all six samples even in the samples showing faint bands in the 1st round PCR (sample number 2, 4, 5 and 6).

Real time mCOP-PCR

In the real time mCOP-PCR screening system, the presence and/or absence of *SMN1* and *SMN2* was determined based on the Cq values. In this study, as for *SMN1*, the Cq value <15 was considered as positive, >17 was considered as negative, and between 15-17 was considered as indecisive result. As for *SMN2*, the Cq value <20 was considered as positive, >23 was considered as negative, and between 20-23 was considered as indecisive result.

In this study, the real time mCOP-PCR following a pre-amplification with a single PCR showed weak amplification peaks of *SMN1* in all six samples. All the Cq values of *SMN1* in all six samples were high (18.7, 18.6, 18.4, 16.8, 16.4, and 15.4 respectively). We failed to determine *SMN1*-positive even in healthy controls (sample number 4, 5, and 6). In addition, similar results were obtained in the *SMN2* determination. The Cq values of *SMN2* gene were also too high (20.8, 28.8, 20.9, 29.1, 28.9, and 29.4 respectively). Thus, we failed to determine *SMN2*-positive in all samples. In contrast, the real time mCOP-PCR following a pre-amplification with a nested PCR showed clear results; *SMN1*-positive in samples number 4, 5, and 6 (Cq value were 12.8, 11.8, and 11.0 respectively), and *SMN1*-negative in samples number 1, 2, and 3 (Cq value were 20.8, 17.3, and 19.2 respectively). Furthermore, all samples had Cq value less than 20 in *SMN2* quantitation, indicating that they were

SMN2-positive. These data were completely compatible with those of PCR-RFLP (data not shown). In addition, melting curve analysis demonstrated no unexpected peaks, suggesting no non-specific amplification in the real-time mCOP-PCR (data not shown).

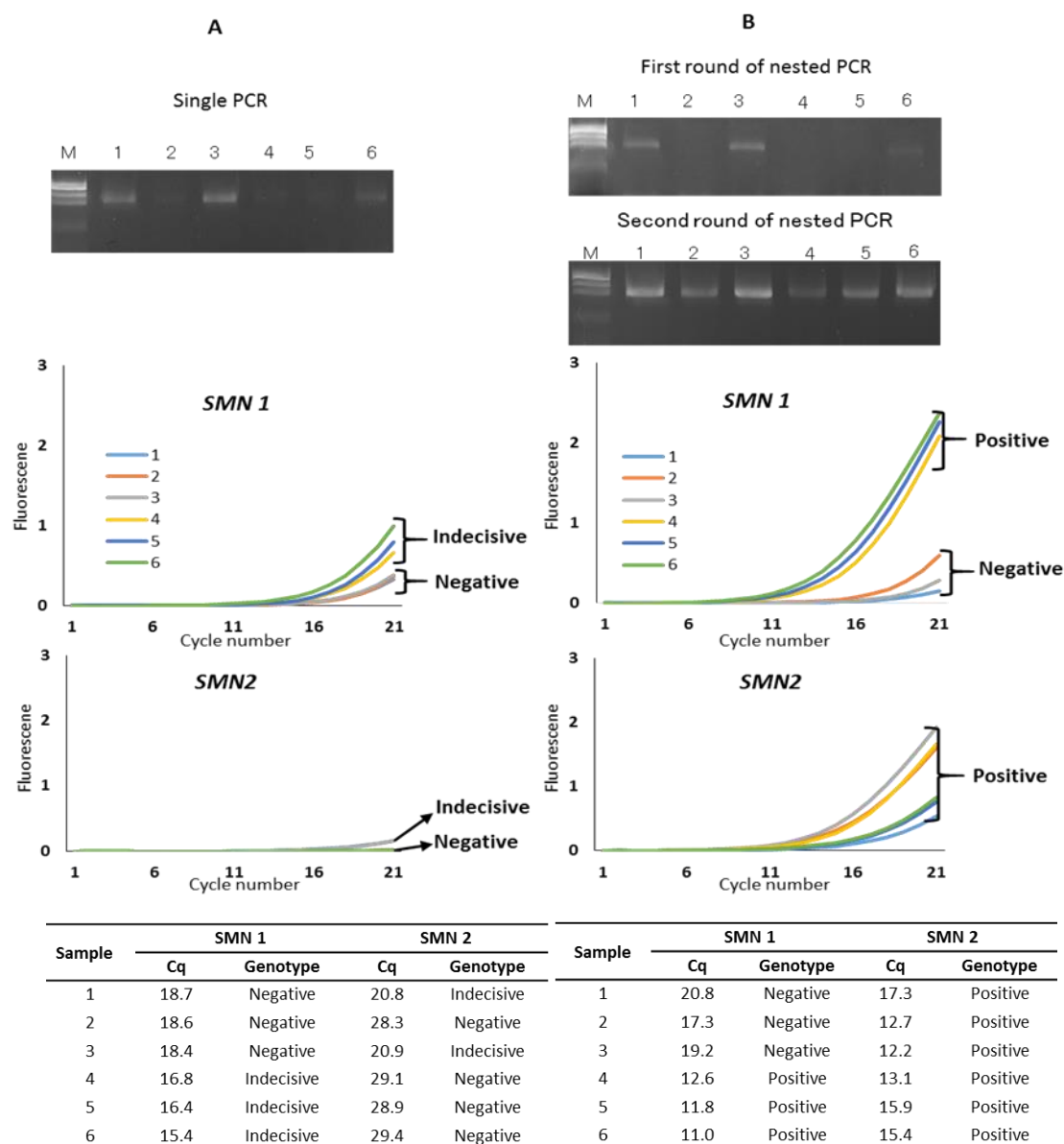


Figure 2. Gel electrophoresis and real-time mCOP PCR results. (A) Pre-amplification with a single PCR. (B) Pre-amplification with a nested PCR. For *SMN1*, the genotype is determined as positive (Cq value <15), indecisive (Cq value 15-17) and negative (Cq value >17). For *SMN2*, the genotype is determined as positive (Cq value <20), indecisive (Cq value 20-23) and negative (Cq value >23).

DISCUSSION

SMA has been considered as incurable disease. However, a newly-developed antisense oligonucleotide drug, nusinersen, brings about a good outcome to SMA patients in the clinical trials [5, 6]. Such data strongly suggested that early, accurate and efficient screening system for SMA will bring a better outcome for the patients.

For SMA, screening system with DBS may be preferable because nation-wide newborn screening of other diseases has already been performed with this. And when the DBS-DNA would become available for SMA screening, it would expand the newborn screening field in no time because any genetic disorder can be screened in the same manner.

The quality or quantity of DBS DNA is usually poorer compared to genomic DNA extracted from freshly collected blood. Moreover, DBS DNA might contain some PCR inhibitors either natural blood components

(hemoglobin, carbonic anhydrase, lactoferrin and human immunoglobulin G) or added anticoagulant like heparin that could not be completely eliminated during extraction procedure [1, 2, 11, 16]. These inhibitors were increasingly become fixed in the filter paper with time [9]. To overcome such problems, pre-amplification step has been recognized as essential in the real time mCOP-PCR screening system [3]. If quality or quantity of DNA extracted from DBS were too poor, pre-amplification with a single PCR could not provide enough amount of template for real-time mCOP-PCR, leading to misdiagnosis. In such situation, pre-amplification with nested-PCR should be tried.

Regarding a blood collection tool, we used an FTA Elute card in this study. FTA Elute cards are well designed so as not to contaminate the DNA solution with PCR-inhibitors. Thus, we are planning to use FTA Elute cards for the pilot study of newborn screening for SMA. However, one FTA Elute card costs ~5 dollars. For the purpose of mass screening, less expensive cards are preferable. We need to develop special blood collection cards for mass screening for SMA.

In conclusion, in the real-time mCOP-PCR screening system for SMA, our study demonstrated that a nested PCR was able to secure the DNA template quality and quantity, leading to unambiguous results of SMA screening.

ACKNOWLEDGMENTS

This research was supported by AMED under Grant Number JP16ek0109086h0002.

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