Printed in the Republic of Korea
ANALYTICAL SCIENCE & TECHNOLOGY
Vol. 32 No. 4, 155-162, 2019
https://doi.org/10.5806/AST.2019.32.4.155

Application study of PCR additives to improve the split peaks in direct PCR

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(Received April 10, 2019; Revised August 2, 2019; Accepted August 2, 2019)

Abstract Analysis techniques using DNA profiling are widely used in various fields including forensic science and new technologies such as the Direct PCR amplification method are being developed continuously in order to acquire the DNA profiles efficiently. However, it has a limits such as non-specific amplification according to the quality of crime scene evidence samples. Especially, split peaks caused by excessive DNA samples are one of the important factors that could cause the debate to allow researchers to interpret the DNA profile results. In this study, we confirmed the occurrence rate of split peaks in each STR (short tandem repeats) locus of the GlobalFiler^{TM} kit and investigated the possibility of improving the split peaks using several PCR additives such as DMSO (dimethylsulfoxide), $MgCl_2$, Betaine and Tween-20. As a result, we could make three groups according to the occurrence rate of split peaks in Direct PCR and it was confirmed that the ratio of split peaks could be reduced by DMSO (87.4 %), $MgCl_2$ (84.5 %) and Betaine (86.1 %), respectively. These results indicate that PCR additives such as DMSO, $MgCl_2$ and Betaine can be improve the split peaks in Direct PCR and thereby facilitate subsequently a successful DNA profile results.

Key words: Direct PCR, PCR additive, split peaks, DNA profile, STR genotyping

1. Introduction

DNA fingerprinting, first invented by Alec Jeffreys in 1985, was rapidly developed since the 1990s to be used in a wide range of fields, such as crime investigations using DNA profile, individual identification, and paternity tests.[1-3] This was made possible due to the development of scientific investigation techniques that led to a higher success rate of DNA extraction from human samples including crime scene samples, and improvement of STR amplification kit that uses the extracted DNA, resulting in a more reliable DNA profiling result. In addition, direct PCR amplification techniques to obtain a DNA profile from samples more effectively have been recently developed using various methods.[4-7] The direct PCR method has been widely used since the early 1990s in the field of microbiology and is known as colony PCR. This

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method uses specific primers for the colony of bacteria or yeast for direct amplification and has been mainly used to detect successful gene cloning or transfection.

The mode of action includes 1) at the high temperature of the hot-start stage of PCR, the cell membrane is destroyed to release amplifiable genomic DNA and 2) in the PCR amplification step, the target gene is amplified using specific primers on the released genomic DNA.[8,9] The method employed in direct PCR directly amplifies DNA from the sample and has the advantage of minimizing DNA loss during the DNA extraction process. R.A.H. van Oorschot *et al.* reported that 20−70 % of sample DNA is lost during the DNA extraction process and there is a possibility of contamination with unnecessary DNA.[10] Further, direct PCR does not require intermediate stages, such as DNA extraction and purification, thereby reducing a large portion of the experimental time required for successful DNA profiling from the samples, leading to another advantage of reducing the required manpower and experimental cost. Such benefits led to attempts to apply the direct PCR method to the control samples in the forensic science field since the mid-2000s, leading to the development and improvement of many direct PCR reagents that are currently used to obtain evidence from crime scenes.[4,11-13] However, since direct PCR directly amplifies the target gene without DNA extraction from the sample, purification, or quantification, it can lead to abnormal amplification phenomena, such as stutters, split peaks, allele imbalance, and allele drop-in/out due to the state of the sample (low-quality or low- or high-copy number of DNA sample) and various PCR inhibitors.[14,15] For this reason, continuous improvement studies are required to obtain successful DNA profiles through direct PCR.

In DNA analysis, PCR is one of the key techniques to obtain the DNA profile. However, characteristics of the samples used in the amplification can lead to insufficient amplification of the target gene or abnormal amplification phenomena, and thus, many studies are focusing on these aspects for further improvement.[16] In particular, various organic additives, such as DMSO, $MgCl_2$, betaine, and Tween-20 are being reported to enhance PCR amplification yield and specificity,[16-18] and the chemicals are named PCR additives or PCR enhancers. In this study, Prep-*n*-Go™ reagent, which has been frequently used as direct PCR recently, was used to research the applicability of PCR additive in improving split peak phenomenon that occurs in sample amplification with a large amount of DNA. In this study, we suggest the developed experimental method for successful DNA profiling in various types of crime scene evidences.

2. Materials and Methods

2.1. DNA samples and reagents

Standard DNA was used in the experiment at 2800M (Promega Co., WI, USA) and a maximum of 20 ng according to the experimental condition. DMSO (1−5 %, v/v), betaine (0.2−1 M), and Tween-20 (0.1−1 %, v/v) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA), while $MgCl_2$ (1.25−4.5 mM) was purchased from Roche (Roche Diagnostics, Mannheim, Germany).

2.2. Direct PCR amplification

Standard DNA of 2800M was subjected to reaction using direct PCR reagent, Prep-*n*-Go™ (Applied Biosystems, Carlsbad, CA, USA) according to the user manual, followed by amplification using the GlobalFiler™ PCR amplification kit (Applied Biosystems) and GeneAmp PCR system 9700 (Applied Biosystems) machine. DNA amplification was performed according to the user manual with the final 25 μL of the PCR reaction solution. The amplified PCR product was subjected to electrophoresis on 2% agarose gel and visualized with Image Analyzer (UVP).

2.3. Capillary electrophoresis and result analysis

The PCR amplification product was mixed with 18.5 μL of Hi-Di formamide and 0.5 μL of standard per sample and heated to 95 °C for 3 min for denaturation. The denatured sample was stabilized at 4 °C and was subjected to capillary electrophoresis using 3500xl Genetic Analyzer (Applied Biosystems)

according to the user manual. The final results were analyzed with GeneMapper ID-X v.1.2 software (Applied Biosystems). Analysis of the split peak ratio according to the STR locus was performed using RFU (relative fluorescence units) calculated for each locus from the GeneMapper ID-X software.

3. Results and Discussion

3.1. Identification of the expression pattern of split peaks in direct PCR according to the increased amount of DNA sample

In the direct PCR of the standard DNA sample using the Prep-*n*-Go™ reagent, the expression pattern of the split peaks that occurred according to an increasing amount of 2800M DNA was analyzed. In general, the STR DNA analysis recommends the input DNA amount to be between 200 pg and 2−3 ng, whereas the commercial STR kit recommends 1 ng DNA as an appropriate amount for input.[19,20] However, a large amount of input DNA was reported to cause split peak phenomenon, one of the abnormal amplification phenomena, and thus, in this study, the amount of 2800M DNA was increased to the maximum of 20 ng to analyze the expression pattern of the split peaks. Using the TH01 locus that shows the most prominent split peak phenomenon at various STR locus in the GlobalFiler™ kit showed no special phenomenon when the amount of 2800M DNA was 1 ng. However, increased split peaks with an increased amount of DNA leading to a maximum of 61.4 % of split peaks were found using 20 ng of DNA (*Fig.* 1). Based on this result, the expression pattern of the split peaks in 22 STR locus excluding Y indel and DYS391 locus from the GlobalFiler™ kit were grouped by the occurrence rate, resulting in a high-level group showing a high split peak phenomenon including D3S1358, vWA, D2S441, TH01, D10S1248, D1S1656, and D12S391 locus with an average of 46.7−75.5 % split peak occurrence rate. Further, the moderate level group with an intermediate level of split peak phenomenon included TPOX, Amelogenin, FGA, D5S818, SE33, and D2S1338 locus with an average of 10.5−28.8 % split peak occurrence rate, while the low-level group without any split peak phenomenon included D16S539, CSF1PO, D8S1179,

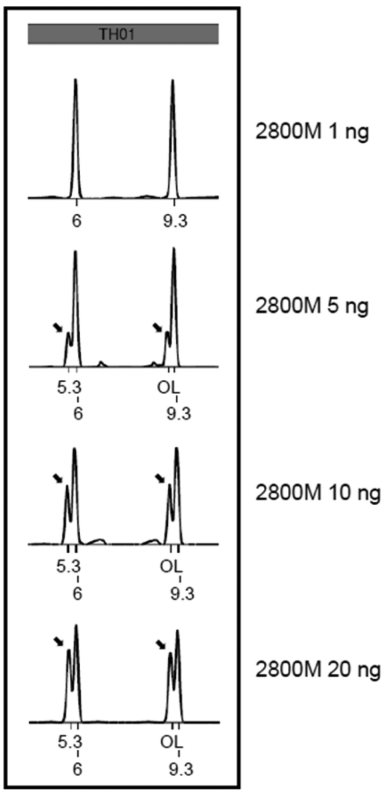

Fig. 1. Identification of the occurrence rate of split peaks with dose-dependent (up to 20 ng) of 2800M DNA in Direct PCR.

Table 1. Lists for split peaks occurrence group

Split peaks occurrence group	Locus	Split peaks occurrence ratios [%]
High level group	D3S1358, vWA, D2S441, TH01, D10S1248, D1S1656, D12S391	46.7~75.5
Moderate level group	TPOX, Amelogenin, FGA, D5S818, SE33, D2S1338	10.5~28.8
Low level group	D16S539, CSF1PO, D8S1179, D21S11, D18S51, D19S433, D22S1045, D13S317, D7S820	Not detected

D21S11, D18S51, D19S433, D22S1045, D13S317, and D7S820 locus (*Table* 1).

3.2. Reduction of split peaks in direct PCR according to the PCR additive treatment

In order to reduce the split peak phenomenon occurring in direct PCR when using a large amount of DNA sample, the PCR additives reported so far and are being widely used. DMSO, $MgCl_2$, betaine, and Tween-20 were used to evaluate their effects in reducing split peaks. split peaks produced in direct PCR using Prep-*n*-Go™ reagents when using 20 ng of 2800M DNA were evaluated at the TH01 locus with the most prominent split peak phenomenon from various STR locus in the GlobalFiler™ kit. The results showed a reduced proportion of split peaks to high concentration-dependent of DMSO, $MgCl_2$, and betaine, while even a high concentration of Tween-20 did not reduce the split peaks (*Fig.* 2). In addition, to identify the optimum concentrations of DMSO, $MgCl_2$, betaine, and Tween-20 to reduce split peaks, PCR amplification efficiency according to the concentration of each PCR additive was evaluated using the average RFU values of 22 STR locus in GlobalFiler™ kit. The results showed that a high concentration of DMSO, $MgCl_2$, betaine, and Tween-20 inhibited PCR amplification (*Fig.* 3), which is in agreement with the results of the previous studies using PCR additives.[17,21,22] Based on this result, the optimum concentrations of PCR additives used in this study were selected for the TH01 locus. In case of DMSO, 3.75 % concentration most effectively reduced split peaks by 9.3 %, whereas $MgCl_2$ reduced the split peaks by 9.5 % at 2.5 mM concentration and betaine reduced those by 9.1 % at 0.75 M concentration. However, Tween-20 did not show a

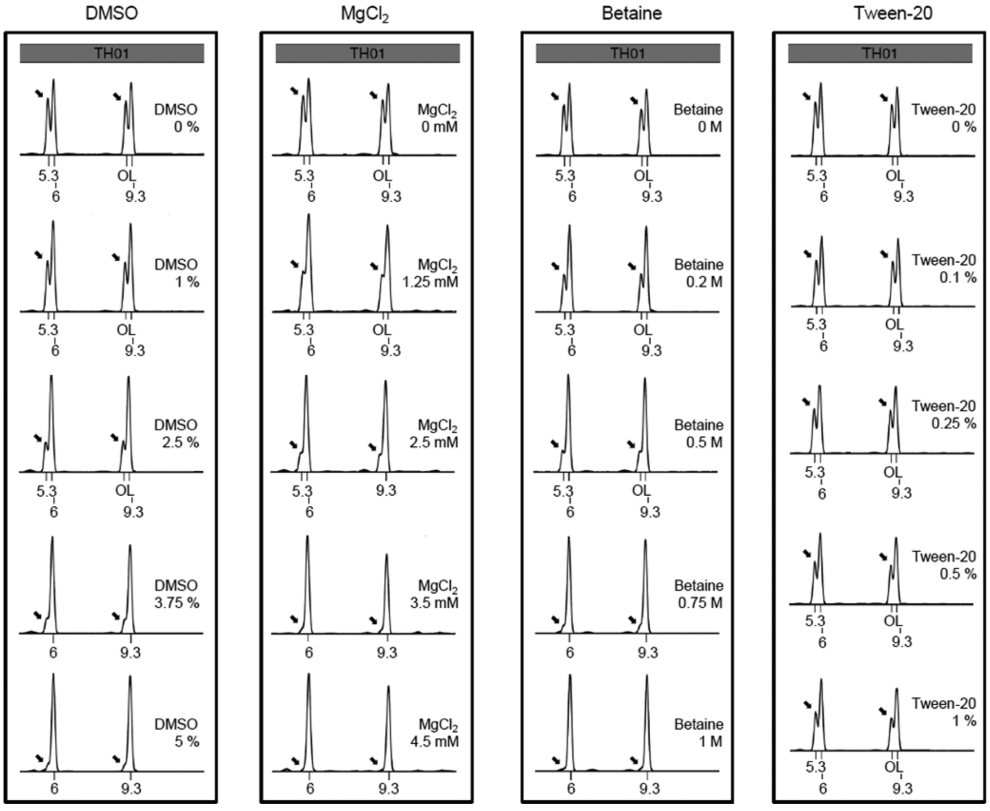

Fig. 2. Identification of improving the split peaks in Direct PCR with dose-dependent of PCR additives such as DMSO, $MgCl_2$ and Betaine, but not Tween-20.

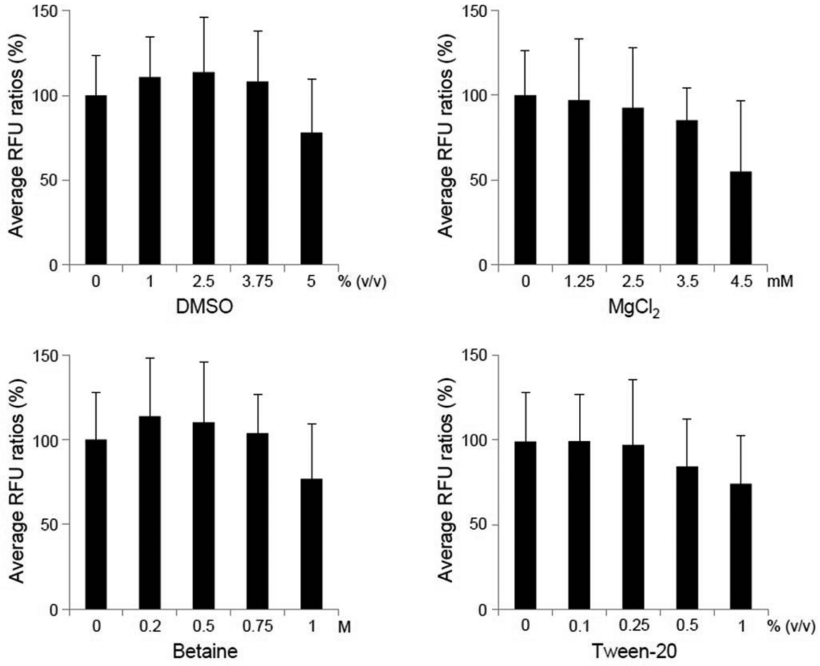

Fig. 3. Average RFU ratios of 22 STR locus of GlobalFiler™ amplification kit with dose-dependent of PCR additives such as DMSO, $MgCl_2$, Betaine and Tween-20 in Direct PCR.

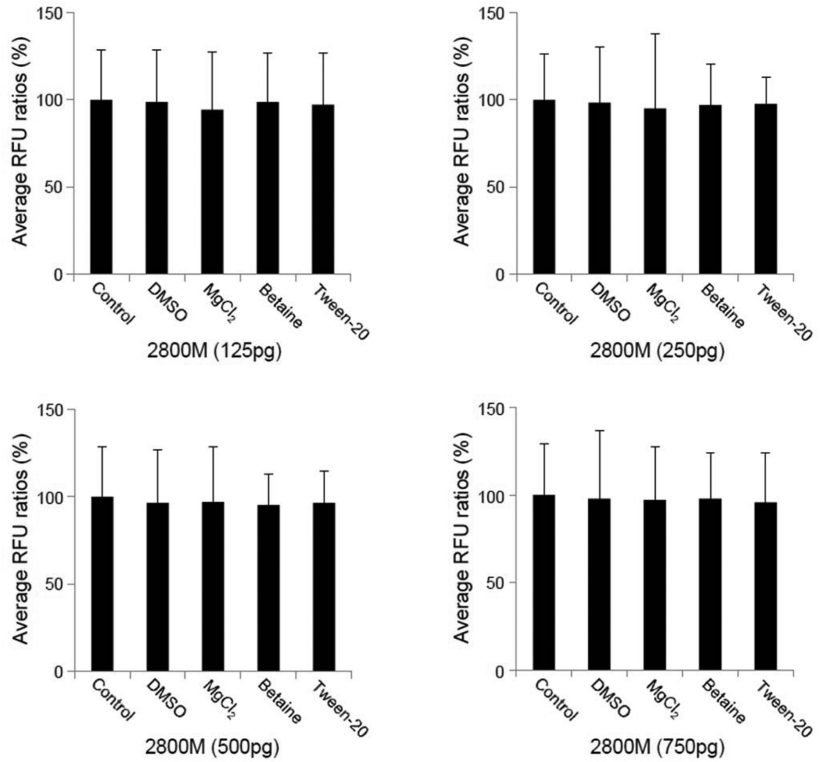

Fig. 4. Average RFU ratios of 22 STR locus of GlobalFiler™ amplification kit on various low-dose 2800M DNA by PCR additives such as DMSO (3.75 %), $MgCl_2$ (2.5 mM), Betaine (0.75 M) and Tween-20 (0.25 %) in Direct PCR.

Table 2. Split peaks occurrence ratios per STR locus according to PCR additives treatment

Locus	Split peaks occurrence ratios [%]				
	Control (No treatment)	DMSO (3.75 %)	$MgCl_2$ (2.5 mM)	Betaine (0.75 M)	Tween-20 (0.25 %)
D3S1358	47.3	8.9	14.6	9.9	34.3
vWA	48.5	8.2	12.6	8.4	32.8
D16S539	-	-	-	-	-
CSF1PO	-	-	-	-	-
TPOX	28.6	6.3	7.2	7.4	26.6
Amelogenin	12.8	4.1	3.5	5.0	16.3
D8S1179	-	-	-	-	-
D21S11	-	-	-	-	-
D18S51	-	-	-	-	-
D2S441	46.7	5.9	12.7	6.5	30.8
D19S433	-	-	-	-	-
TH01	61.4	9.3	9.5	9.1	40.3
FGA	13.3	4.9	7.5	6.2	11.6
D22S1045	-	-	-	-	-
D5S818	10.5	3.6	4.2	5.6	11.3
D13S317	-	-	-	-	-
D7S820	-	-	-	-	-
SE33	55.2	8.4	11.7	9.8	34.3
D10S1248	70.9	24.7	21.3	20.5	55.3
D1S1656	72.2	21.6	18.1	19.4	53.4
D12S391	75.5	18.6	29.5	19.0	59.1
D2S1338	28.8	4.8	13.0	6.9	27.0

good split peaks reduction, and thus, its effective concentration could not be selected.

Further, inhibition of PCR amplification by PCR additives using low concentration DNA (< 1 ng) was evaluated using the average RFU values of the 22 STR locus. However, the results did not show inhibition of PCR amplification at the selected optimum concentrations for DMSO (3.75 %), $MgCl_2$ (2.5 mM), and betaine (0.75 M). For Tween-20, for which the optimum concentration could not be selected, an intermediate concentration that did not inhibit PCR amplification (0.25 %) was used in the experiment. It showed a stable PCR amplification result even with a low concentration of DNA. This shows that PCR additive treatment in direct PCR does not affect PCR amplification efficiency in a low concentration DNA (*Fig.* 4).

Using optimum concentrations selected for each PCR additive based on the results above, the results for comprehensive evaluation on the 22 STR locus from GlobalFiler™ kit (*Table* 2) showed that the high-level group with a higher frequency of split peaks led to an average reduction of 5.9−24.7 % using DMSO (3.75 %), 9.5−29.5 % using $MgCl_2$ (2.5 mM), and 6.5−20.5 % using betaine (0.75 M), reflecting a large extent of reduction in the split peak phenomenon, However, an intermediate concentration of Tween-20 (0.25 %) led to a small reduction of 30.8−59.1 %. Such a phenomenon was similar in the moderate level group with an intermediate level of split peak occurrence. Effective PCR additives, such as DMSO, $MgCl_2$, and betaine could be used in the future to improve split peak phenomenon that occurs by using a large amount of DNA sample in direct PCR.

4. Conclusions

In this study, the occurrence rate of split peaks, one of the abnormal amplification phenomena that occur in direct PCR using Prep-*n*-Go™ reagent was

evaluated for the STR locus in the GlobalFiler™ kit. The results showed that the split peak occurrence rate can categorize the STR locus into a high-level group (D3S1358, vWA, D2S441, TH01, D10S1248, D1S1656, and D12S391), moderate level group (TPOX, Amelogenin, FGA, D5S818, SE33, and D2S1338), and low level group (D16S539, CSF1PO, D8S1179, D21S11, D18S51, D19S433, D22S1045, D13S317, and D7S820). PCR additives were used to evaluate its potential in reducing the split peak phenomenon. In regard to the PCR additives that showed effectiveness in this study, DMSO and betaine have been reported to reduce the secondary structure of DNA enriched in GC nucleic acid for stabilization and increasing DNA amplification. In particular, betaine is widely used as a main component in the commercialized reagents, such as Q-Solution (QIAGEN), GC-melt (Clontech), GC-RICH solution enhancer (Roche), TaqMaster enhancer (Eppendorf), and FailSafe enhancer (Epicentre) due to such characteristics.[23,24] In addition, magnesium ions in $MgCl_2$ act as an accessory factor of PCR polymerase to augment the enzyme activity, and thus, a higher DNA amplification has been reported.[25] Due to such activities, DMSO, $MgCl_2$, and betaine significantly reduce split peak phenomenon that occurs in direct PCR using a large amount of DNA sample. The ratios of reduction in the high-level group compared to the control sample were 65.2−87.4 %, 60.9−84.5 %, and 71.1−86.1, respectively. However, in contrast, the non-ionic detergent Tween-20 could not significantly reduce the occurrence of split peaks. These results suggest that various reported PCR additives act differently to improve the abnormal amplification phenomenon that occurs due to the sample characteristics. Therefore, in regards to the split peak phenomenon occurring during DNA amplification by direct PCR while using a large amount of DNA sample, PCR additives, such as DMSO, $MgCl_2$, and betaine can be applied, as suggested in this study, to more effectively obtain DNA from various types of samples collected in the crime scenes as well as the control samples.

Acknowledgements

This research was funded by the Forensic Technique Development Project from the National Forensic Service (NFS2019-DNA-03) in 2019 by the Korean government, for which we are grateful.

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