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Thesis

Application of DNA Containing Forensic Blood Substitute in the Bloodstain Pattern Analysis

Sang-Yoon Lee[1,3]*, Jun-Soo Park[2], Hye-Hyun Lee[3,4], Young-il Seo[3], Si-Keun Lim[5] and Se-Hyun Shin[1]

[1]Department of Mechanical Engineering, Korea University, Seoul 02841, Republic of Korea

[2]Department of Micro/Nano System, Korea University, Seoul 02841, Republic of Korea

[3]National Forensic Service, Wonju 26460, Republic of Korea

[4]Department of Microbiology, Pusan National University, Busan 46241, Republic of Korea

[5]Department of Forensic Sciences, Sungkyunkwan University, Suwon 16419, Republic of Korea

Abstract

Bloodstain pattern analysis, one of the fields of forensic science, can reconstruct a bloodshed crime scene by analyzing the physical characteristics of blood, such as the size, shape, and spatter pattern, and can contribute to the resolution of a case by determining the authenticity of a suspect's statement and estimating the suspect for some cases. Following the BPA at the crime scene, DNA profiling can be of great help in solving cases based on more accurate crime scene reconstruction where the bloodstains of multiple people are mixed. Through recognition of the importance of DNA labelling systems that can accurately identify individuals, numerous studies focusing on the diversity and stability of DNA that can improve existing use in various forensic research have been conducted. Thus, we used a developed blood substitute and synthetic DNA to test the combination of different DNA sequences or size to increase the availability of the BPA by diversifying the analysis in conjunction with already established methods. In this study, we produced synthetic DNA applied this DNA to developed blood substitute, identified the size through PCR, thereby identified characteristics of developed blood substitute. Accordingly, we established conditions for amplifying synthetic DNA in developed blood substitute and conducted verification through a stability test of synthetic DNA. We were able to diversify blood substitutes that emphasized the existing physical characteristics by adding the synthetic DNA, which was designed and produced in this study, to developed blood substitute. By doing so, we could improve the availability of developed blood substitute to the point where it can be identified similarly as in DNA analysis from the human blood.

***Corresponding author:** Sang-Yoon Lee, Department of Mechanical Engineering, Korea University, Republic of Korea, Tel: +82-10-9774-6119; E-mail: sylee1221@korea.ac.kr

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Introduction

Bloodstain pattern analysis (BPA), one of the fields of forensic science, can reconstruct a bloodshed crime scene by analyzing the physical characteristics of blood, such as the size, shape, and spatter pattern, and can contribute to the resolution of a case by determining the authenticity of a suspect's statement and estimating the suspect for some cases [1,2]. To understand the generation mechanisms of bloodstains in the crime scene, to eventually identify characteristics of the bloodstains and reconstruct the scene, scientific reliability must be secured by validating the hypothesis that can explain the site most clearly of the many explanations obtained through experiments. This is important because the BPA result can be selected as proof of evidence in court [3]. Human blood collected from subjects, animal blood (bovine or porcine) supplied from butcheries, and blood substitutes developed in other countries (Arrowhead, Sirchie, Tri-Tech Forensics in the USA) are currently used for the BPA experiment. However, blood substitutes are not suitable for experimentations due to various problems and are inconvenient to use [3,4]. Numerous studies on suitable alternatives for blood to solve these problems have been conducted by multiple scientists such as [3, 5-8]. Following the BPA at the crime scene, it is critical to identify individuals by DNA sampling of each type of BPA [9, 10]. However, DNA analysis is underutilized and practically impossible to test all bloodstains. Therefore, if there are not enough details of a case, bloodstains observed in the field are generally assumed to belong to victims and analyzed accordingly [11]. However, suspects can do multiple things to conceal the bloodstains and bloodstains from a third party cannot be excluded, DNA profiling can be of great help in solving cases based on more accurate crime scene reconstruction where the bloodstains of multiple people are mixed [12].

Through recognition of the importance of DNA labelling systems that can accurately identify individuals, numerous studies focusing on the diversity and stability of DNA that can improve existing use in various forensic research have been conducted [13,14]. Thus, in this study, we used a developed blood substitute (NFBS) of [3] and synthetic DNA to test the combination of different DNA sequences or size to increase the availability of the BPA by diversifying the analysis in conjunction with already established methods. In this study, we produced synthetic DNA as shown in [14], applied this DNA to NFBS, identified the size through PCR, thereby identified characteristics of NFBS. Accordingly, we established conditions for amplifying synthetic DNA in NFBS and conducted verification through a stability test of synthetic DNA. Also, to recreate a situation where we need to identify bloodstains from multiple people, we used the synthetic DNA (72bp) to NFBS, which was used to the fluorescent paste in the previous study, and, at the same time, used two different synthetic DNA of different sizes (90bp and 140bp), and produced three different NFBS (Figure 1). Three identifiable types of NFBS could be used for the reconstruction of three subjects and, we could include three or

more subjects for the education and training of the BPA by adding different synthetic DNA depending on the situation. NFBS with 3 different sizes of synthetic DNA can be quickly and easily identified by the size of amplified products through electrophoresis. Among them, two types of NFBS were mixed in various ratios to assume the situation of mixed-blood condition, and real-time PCR was used to quantify the proportion of each synthetic DNA of different sizes within the NFBS to estimate the ratio of the blood mixed. Among them, two types of NFBS were mixed in various ratios to assume the situation of mixed-blood condition, and real-time PCR was used to quantify the proportion of each synthetic DNA of different size within the NFBS to estimate the ratio of the blood mixed. Based on this, we aim to diversify and increase the practicality of NFBS to increase the availability of BPA research and test, and further help solve bloodshed crimes.

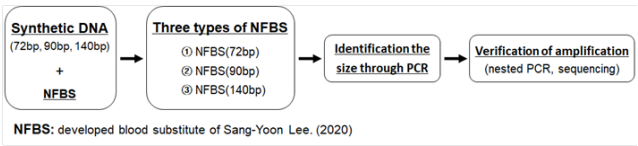

Figure 1: Mimic diagram of the experiment.

Materials and Methods

Design and synthesis of synthetic DNA

Synthetic DNA consists of a random combination of A, G, C, and T with lengths of 90bp and 140bp. To eliminate the possibility of randomly designed DNA being contaminated and amplified, we validated the sequences of the DNA by using the "BLAST" search (https://blast.ncbi.nlm.nih.gov/Blast.cgi)" provided by the National Center for Biotechnology Information (NCBI) and confirmed there is no sequence that completely matches. The primers used in DNA synthesis and polymerase chain reaction (PCR) were produced by Bioneer (Bioneer, Daejeon, Korea) (Table 1).

Synthetic DNA was amplified by PCR for economical use. For the PCR, 2μl of the synthetic DNA diluted to 250pg/μl, 10pmol of forward and reverse primers, and 10μl of AccuPower® PCR master mix were used, and the rest was filled with distilled water to have a final volume of 20μl. Using a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, California, USA), the mixture was pre-denatured at 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final elongation step at 72°C for 5 min. The amplicons were subjected to electrophoresis in a 3% agarose gel with 50bp DNA Ladder (Dyne Bio, Gyeonggi-do, Korea) and were confirmed and photographed using a Gel Doc XR+Imaging System (Bio-Rad Laboratories, California, USA).

Amplification and verification of NFBS with synthetic DNA

To select the concentration of synthetic DNA, When synthetic DNA was mixed with NFBS, we performed PCR to verify whether the synthetic DNA can successfully be amplified. The 140bp long synthetic DNA was used for the verification process. After establishing the optimal condition, results for 90bp long DNA were shown. When NFBS is used in forensic science, a large amount of NFBS is needed, thus to save resources, we compared the result of below two conditions and selected the optimal concentration; ① 1ml of NFBS with 1μl of synthetic DNA stock solution (estimated concentration after dilution: 50pg/μl) and ② 1ml of NFBS with 10μl of synthetic DNA stock solution (estimated concentration after dilution: 500pg/μl). When NFBS was not diluted as shown in the above section, PCR

DNA/Primer		Sequences	Expected Size (bp)
Synthesized DNA		TACAGAGTAGGAGCTGCTAGTCAGCGATCTGCTTCCAAGC ACCTAGACTGAATCCTGTAGGCATCTGATCTGGCTAGTCG AGCTAAGCTA	90bp
Original primer	Forward	TACAGAGTAGGAGCTGCT	90bp
	Reverse	TAGCTTAGCTCGACTAGC	
Nested primer	Reverse	CAGATCAGATGCCTACAG	72bp
Synthesized DNA		GTCATCAGATGACTAGGCGATGAACCATGCATTGACTACT GCGTACCTATAAGGTACGGTACGGCATAACTTTCAAGCTC GTACCTACGCTAGCACGTCGAGATGCATGAACGTGCATAC GGCGCATTGTAGCCGCTGCA	140bp
Original primer	Forward	GTCATCAGATGACTAGGC	140bp
	Reverse	TGCAGCGGCTACAATGCG	
Nested primer	Reverse	CCGTATGCACGTTCATGC	122bp

Table 1: The sequence information of synthesized DNA and primers.

was successful; leading us to assume that there is a PCR inhibitor in NFBS. Therefore, we decided to establish an optimal protocol for the PCR through dilution. The experiments were conducted by diluting the mixture of NFBS and synthetic DNA in a ratio of 1:1, 1:10, 1:100, and 1:1000.

Next, an experiment was conducted to confirm the PCR reaction of synthetic DNA by diluting the mixture of 1 ml of NFBS with 1μl of synthetic DNA stock solution at a ratio of 1:1000 as selected from the above section. For verification of PCR amplification products, a mixed solution obtained by 1000-fold dilution of 1ml of NFBS with 1μl of the synthetic DNA stock solution was amplified using the original primer. The amplicons were subjected to electrophoresis in an agarose gel and then to nested PCR with nested primer for further verification. After amplifying the positive control group (PC) and DNA with NFBS (S, Specific), purified the DNA from the agarose gel using AccuPrep® PCR/Gel DNA Purification Kit (Bioneer, Daejeon, Korea). Then, nested PCR was performed with purified DNA diluted at a ratio of 1:1000, the same forward primer as before, and the nested primer as in table 1. For the synthetic DNA of 90bp, 15pmol of nested and forward primer, 10μl of AccuPower® PCR master mix (Bioneer, Daejeon, Korea) were used, and the rest was filled with distilled water to have a final volume of 20μl. For 140bp long synthetic DNA, 10pmol of the nested and forward primer and 10μl of AccuPower® PCR master mix were used, and the rest was filled with distilled water to have a final volume of 20μl.

Stability Test of NFBS with Synthetic DNA

To verify the stability of synthetic DNA mixed with NFBS, PCR was performed after storing 1000-fold diluted solution at 4°C and room temperature for 1 to 4 weeks. To be specific, we first diluted the NFBS containing synthetic DNA at a ratio of 1:1000, then put the mixture at either 4°C or room temperature for 1 to 4 weeks, then performed PCR with the solution which was stored for one, two, three, and four weeks, and finally confirmed the stability of the synthetic DNA mixed with NFBS through the PCR.

Application of identifiable NFBS

First, to confirm whether the DNA with different sequences and lengths can be identified, we mixed 72bp long synthetic DNA

constructed in [14] and synthetic DNA used in this study. As mentioned above, we diluted the mixture of NFBS with synthetic DNA at a ratio of 1:1000 then confirmed the result of the PCR. Next, to verify whether the 90bp and 140bp long synthetic DNA constructed in this study and 72bp long DNA from the work of in the previous study are identifiable, we mixed these three synthetic DNAs with NFBS, then dilute them 1/1000 as above, and observed the result of the PCR. Finally, When NFBS Ⓐ and NFBS Ⓑ are mixed, we want to check whether the mixed ratio can be estimated through the ratio of synthetic DNA in each NFBS. NFBS Ⓐ and NFBS Ⓑ were prepared, and two NFBS were mixed in a ratio of 1:1, 2:1, 1:5, 5:1, 2:1 to make a mixed sample, and then the mixed samples were respectively 90bp and 140bp primers. The concentration was measured using.

Results

Design and synthesis of synthetic DNA

Experiments have shown that the synthetic DNAs are successfully amplified, and the amplified products were confirmed through the nested PCR. It was confirmed through sequencing analysis that went through the TA cloning process (Figure 2). Furthermore, amplification using 90bp, 140bp long synthetic DNAs and primer set designed in this study showed a single band of expected sizes (90bp, 72bp, 140bp, and 122bp). This result confirms that the synthetic DNA can be accurately amplified and that it can be used economically when used in large quantities.

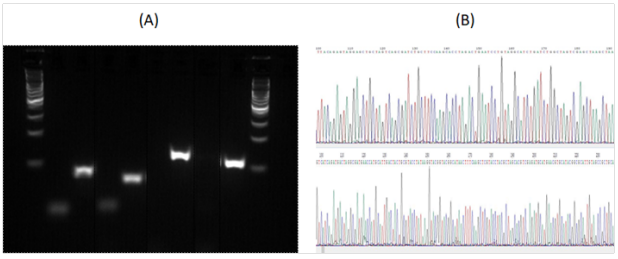

Figure 2: Amplification of synthetic DNA and verification of Amplified products with nested PCR and sequencing. **A:** (a) 90bp, (b) 90bp - nested (72bp), (c) 140bp, (d) 140bp - nested (122bp), B: Sequencing results of (a) and (c) of A (Top: 90bp, Under: 140bp)

Amplification and verification of NFBS with synthetic DNA

Concentration selection experiment results, the results have shown that PCR was not successful in 1ml NFBS + synthetic DNA mixture, through which we assumed the presence of PCR inhibitors in the NFBS. To avoid the PCR inhibition, the PCR was conducted with a 1000-fold dilution of (a) and (c). Both diluted solutions (b) and (d) were amplified, from which we assumed that the diluted solutions are not affected by the inhibitors in the NFBS. Since the amplified products of (b) and (d) are similar and to save the resources, we chose (b) with a lower concentration of synthetic DNA for further experiments (Figure 3-A).

In selecting the optimal test method, we could confirm that without diluting the mixture of the NFBS and synthetic DNA, amplification is not possible and that the PCR inhibition can be avoided with at least 1:1 dilution. Furthermore, we could show that the dilution factor can be increased (~ 1:1000) for convenience, so further experiments were conducted with a 1000-fold dilution (Figure 3-B).

Based on the results of (Figure 3-A and 3-B), we verified the amplified products of the mixed solution of NFBS + synthetic DNA (90bp) and NFBS + synthetic DNA (140bp). As a result, we showed that PCR was successful in both mixtures. Also, when compared with the positive control, we could identify a single band of the same size from the mixtures (Figure 3-C). This result shows that both amplification and size identification is possible. We could further confirm that the synthetic DNA was stably detected in NFBS regardless of the size of the DNA used in this study even at low concentrations.

After extracting the amplicons from the gel of (Figure 3-C), we validated the amplicon through the nested PCR. The results have shown that amplification was not successful when nested primers, which are not complementary to the synthetic DNA, were used. On the other hand, amplification was successful when the primer complementary to the synthetic DNA was used (Figure 3-D). From this, we could show the identification of two different synthetic DNA is based on the characteristics of the primer that specifically binds to the DNA whose sequence is complementary (Figure 3).

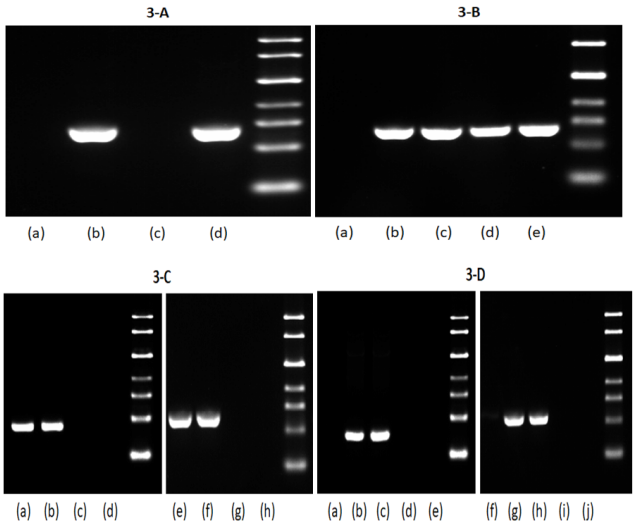

Figure 3-A: Determination of synthetic DNA concentration added to NFBS. (a) NFBS 1㎖+ DNA undiluted solution 1㎕ (b) Dilute 1㎖of NFBS + 1 ㎕of DNA undiluted solution to 1:1000 (c) NFBS 1㎖+ DNA undiluted solution 10㎕(d) Dilute 1㎖of NFBS + 10㎕of DNA undiluted solution to 1:1000.

Figure 3-B: Selection of the optimal test method for NFBS PCR containing synthetic DNA (a) NFBS 1㎖+ DNA undiluted solution 1 ㎕(b) Dilute 1㎖of NFBS + 1㎕of DNA undiluted solution to 1:1 (c) Dilute 1㎖of NFBS + 1㎕of DNA undiluted solution to 1:10 (d) Dilute 1㎖of NFBS + 1㎕of DNA undiluted solution to 1:100 (e) Dilute 1㎖of NFBS + 1㎕of DNA undiluted solution to 1:1000.

Figure 3-C: Identification of NFBS PCR amplification products with synthetic DNA (A) NFBS with 90bp synthetic DNA (B) NFBS with 140bp synthetic DNA (a),(e) 1/1000 dilution of NFBS including synthetic DNA (b),(f) Positive control (c),(g) 1/1000 NFBS without DNA (d),(h) PCR blank.

Figure 3-D: Verification of Amplified Products with Nested PCR. (A) NFBS with 90bp synthetic DNA (B) NFBS with 140bp synthetic DNA (a), (f) nonspecific nested PCR, (a) Use 140bp nested primers for 90bp (f) Use 90bp nested primers for 140bp (b),(g) Specific nested PCR (c),(h) positive control (d),(i) 1/1000 NFBS without DNA (e),(j) PCR blank.

Figure 3: Amplification and verification of NFBS with synthetic DNA.

Stability test of NFBS with Synthetic DNA

The results have shown that the synthetic DNA contained in the NFBS was stable for 1 to 4 weeks at 4°C and room temperature

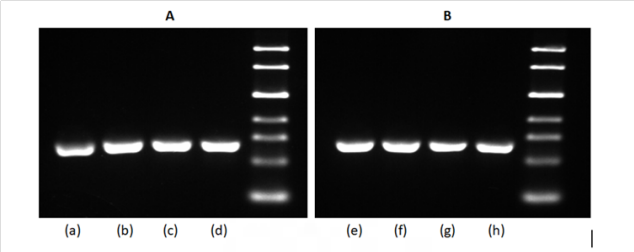

Figure 4: Stability test for 1 to 4 weeks at refrigerated storage (4°C) and room temperature. (A) Refrigerated (B) room temperature (a),(e) PCR results after 1 week (b),(f) PCR results after 2 week (c),(g) PCR results after 3 week (d), (h) PCR results after 4 week.

(Figure 4). This means that the synthetic DNA is stable in the NFBS even if the mixture is stored at 4°C or room temperature for up to 4 weeks.

Application of identifiable NFBS

Experimental results of NFBS containing synthetic DNA (72bp) developed in previous research, it has been shown that the mixture containing 72bp long synthetic DNA and NFBS can be successfully amplified with a 1000-fold dilution and with a set of primer complementary to the DNA (Figure 5). This means that only primers specific to the sequence of the synthetic DNA can participate in the PCR and that it is possible to distinguish the synthetic DNA from others (Figure 5-A).

Next, in the NFBS identification experiment containing synthetic DNA (72bp, 90bp, 140bp), 72bp, 90bp and 140bp long synthetic DNAs are all identified for each size in a 1:1000 diluted solution including NFBS (Figure 5-B).

Finally, in the experiment for measuring the mixing ratio of NFBS Ⓐ and NFBS Ⓑ, which mixed 90bp and 120bp of synthetic DNA, it was possible to estimate the mixed ratio at 1:1, 1:2, 1:5, 5:1, and 2:1 through the ratio of synthetic DNA in the mixed NFBS (Table 2) (Figure 6).

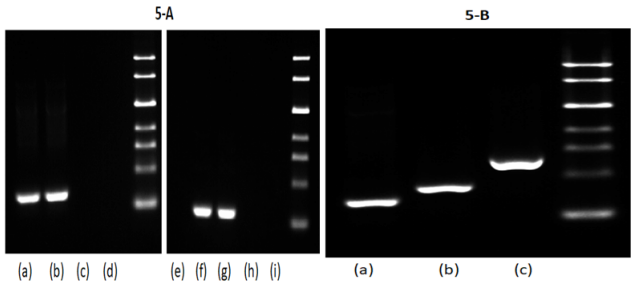

Figure 5-A: NFBS application of 72bp of synthetic DNA developed in prior research (A) Identification of NFBS PCR amplification products with synthetic DNA (B) Verification of Amplified Products with Nested PCR (a) 1/1000 dilution of NFBS including synthetic DNA (b) Positive control (c) 1/1000 NFBS without DNA (d) PCR blank (e) nonspecific nested PCR, use 140bp nested primers for 72bp (f) Specific nested PCR (g) positive control (h) 1/1000 NFBS without DNA (i) PCR blank.

Figure 5-B: Identify three NFBS including synthetic DNA developed in this study (a) 72bp (b) 90bp (c) 140bp.

Figure 5: Application of identifiable NFBS.

	NFBS and DNA mixing sample				
	S1	S2	S3	S4	S5
Mixing ratio (90bp:140bp)	1.00 : 1.00	1.00 : 2.00	1.00 : 5.00	5.00 : 1.00	2.00 : 1.00
Measured mixing ratio (90bp:140bp)	1.00 : 0.90	1.00 : 2.02	1.00 : 4.48	4.82 : 1.00	1.54 : 1.00
Measured DNA concentration (90bp)	53pg ±2.1	28pg ±1.7	12pg ±0.4	53pg ±2.1	54pg ±0.4
Measured DNA concentration (140bp)	47pg ±1.2	56pg ±2.4	54pg ±0.4	11pg	35pg ±0.9

Table 2: Two NFBS mix applications.

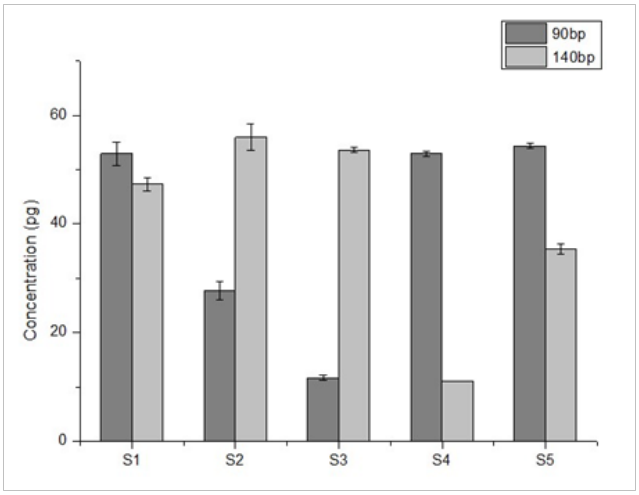

Figure 6: Two NFBS measured mixing ratio.

Conclusion

In this study, when synthetic DNA was mixed with NFBS, it was not amplified due to the influence of the PCR inhibitors in NFBS, but we could confirm that synthetic DNA can be amplified smoothly without being affected by the inhibitors through at least 1:1 dilution. Since the amplified products were verified by the nested PCR and sequencing, we could confirm that the synthetic DNA mixed with NFBS diversifies NFBS and enables identification. Also, these synthetic DNA was stably amplified for up to 4 weeks at 4°C and room temperature, suggesting the possibility of envisaging wider use in terms of storage temperature or shelf life when it comes to actual BPA.

Three types of NFBS were produced with the synthetic DNA (90bp, 140bp) as well as the one (72bp) suggested in the previous research. In addition, by estimating the mixing ratio through quantitative analysis of the ratio of the amount of synthetic DNA after mixing two types of NFBS including synthetic DNA (90bp, 140bp), Through these, we reconstructed a bleeding site where the bloodstains of numerous people are mixed, confirming the possible application of BPA. However, when synthetic DNA of different sizes are mixed, it is necessary to confirm whether they can be used normally and to validate a wider range of temperature and humidity conditions in the stability test. Besides, when synthetic DNA is mixed with NFBS which is not diluted, PCR cannot be performed due to inhibitors in NFBS, so we suggest that an additional experiment is needed for accurate validation.

In this study, we were able to diversify blood substitutes that emphasized the existing physical characteristics by adding the synthetic DNA, which was designed and produced in this study, to NFBS. By doing so, we could improve the availability of NFBS to the point

where it can be identified similarly as in DNA analysis from the human blood. Furthermore, we believe that BPA will be available by estimating the proportion of blood through quantification of synthetic DNA in the mixed blood, and through this, it will greatly contribute to solving the case through experimentation, research and education, and further reconstruction of the bloodshed crime scene.

Highlights

Through the development of appropriate blood substitute that can replace human blood, it can greatly help in the development of forensic science and bloodstain pattern analysis. Following the BPA at the crime scene, DNA profiling can be of great help in solving cases based on more accurate crime scene reconstruction where the bloodstains of multiple people are mixed. A developed blood substitute of [3] and synthetic DNA to test the combination of different DNA sequences or size to increase the availability of the BPA by diversifying the analysis in conjunction with already established methods aim to diversify and increase the practicality of blood substitute to increase the availability of BPA research and test, and further help solve bloodshed crimes.

Acknowledgment

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Conflict of Interest

To the best of our know ledge, the named authors have no conflict of interest, financial or otherwise.

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