Separation and detection of SYBR Green II labeled-ssDNA by Automated Capillary Electrophoresis

Sirinan Sangkara[1], Nathinee Panvisavas[1&2], Punchapat Sojikul[3], Wanasphon Bunakkharasawat[1]

[1] Forensic Science Graduate Programme, [2] Department of Plant Science, [3] Department of Biotechnology, Faculty of Science, Mahidol University, Rama VI Road,Bangkok, Thailand.

Abstract

In this study, we developed a protocol for separation and detection of SYBR Green II labeled-ssDNA utilizing the automated CE instrument (ABI Prism®310 Genetic Analyzer). Firstly, the separation condition of the instrument was optimized based on the STR typing condition. The result demonstrated that SG-II/ssDNA complex can be separated and detected, though SYBR Green II dye was not present in the commercial dye set of the instrument. In addition, it was also demonstrated that reproducible peaks were obtained when LIZ500 internal size standard was co-injected with SG-II/ssDNA complex. The labeled-ssDNA fragment can be subsequently analyzed and sized. Next, the amount of dye used for ssDNA labelling was then determined by using different combination of dye, varying from 10,000X to 1X, and ssDNA fragments, varying from 300ng to 18.75ng. Good quality peaks were presented in the electropherogram and correctly sized when ssDNA was labeled with 1,000X SYBR Green II. It was also observed that imbalance labeling ratio between dye and ssDNA concentration affected on dye labeled-ssDNA uptake *via* electrokinetic injection resulting in no peak in the electropherogram. In conclusion, besides the routine forensic DNA typing work, the automated CE can also separate and detect SYBR Green II labeled-ssDNA products generated from technique such as rolling circle amplification (RCA). Co-injection of ssDNA product with LIZ500 internal size standard allows sizing by GeneMapper®Software.

Key words: Automated capillary electrophoresis, SYBR Green II, ssDNA

Introduction

SYBR Green II (SG-II) is a cyanine dye, which the core structure is a monomeric asymmetrical cyanine, similar to other cyanine dyes such as Thiazole orange (TO) , TO-PRO-1, PicoGreen (PG), and SYBR Green I (SG-I) (1). This core structure compose of an N-alkylated benzothiazolium or benzoxazolium ring system, which is linked by a monomethine bridge to a pyridinium or quinolinium ring system that carries a substituent with a heteroatom (see Figure 1) (2). SG-II has been developed for the analysis of nucleic acids, especially ssDNA/RNA. Although it is not specific to ssDNA/RNA, the SG-II dye exhibits a superior fluorescence quantum yield when bound to ssDNA/RNA (~0.54) than dsDNA (~0.36). The SG-II/RNA complex shows the fluorescence quantum yield that is seven times greater than ethidium bromide/RNA complex. Binding affinity of SG-II to ssDNA/RNA is also higher than ethidium bromide. SG-II has a very low intrinsic fluorescence property, and SG-II/DNA complex does not quench in the presence of urea or formaldehyde. Therefore, there is no need to destain gels neither to remove excess dye after electrophoresis nor to wash these denaturants prior to staining (3-5). Due to these advantageous properties of SG-II, it has been utilized in nucleic acids analysis in a wide variety of gel – based separation technique, such as slab gel electrophoresis (6) and capillary electrophoresis (7).

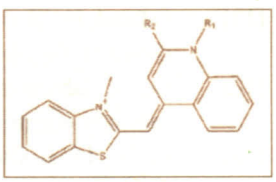

Figure 1 The core structures of monomeric cyanine dyes (1)

Automated Capillary Electrophoresis (CE) has been become a routine separation technique for DNA fragment analysis in forensic DNA laboratories. By capillary electrophoresis, minute amount of sample can be separated in the gel or polymer media, based on size of molecule, under electric field (8-9). Comparing to slab gel electrophoresis, automated CE provides faster separation with better resolution, higher sensitivity, and being automated (10). With this capability, it has been applied for DNA fragment sizing and sequencing in a variety of applications including diagnosis of diseases, genotyping for personal medicine (11), and also short tandem repeat (STR) typing in forensic DNA laboratories (12). CE coupled with laser-induced fluorescence (LIF) detection system provides higher sensitivity than UV detection, and is used for nucleic acid analysis (13). The LIF detection involves the use of fluorescent intercalating dyes to visualize the DNA fragments. LIF detection provides fine resolution that DNA fragments which are 1 base differ can be discriminated. There are two methods widely used to generate fluorescent DNA molecules. The first method is the covalent coupling of DNA with fluorophores at the 5'–end of primers or probes. The second method is post-labeling of single or double strand DNA

with intercalating dye (14). In routine forensic DNA analysis, DNA fragments were PCR amplified by fluorescent-labeled primers at the 5'-end, and then were separated by automated CE coupled with LIF detection. However, there are assays such as Rolling Circle Amplification (RCA) technique, which ssDNA molecules would be generated using unlabeled primers, thus needs post-labeling of DNA strands for separation and detection by automated CE coupled with LIF detection. Considering forensic DNA analysis, small amount of amplified-DNA molecules from forensic samples with low quality nature would be available. Therefore, the florescent dye selected for forensic application must then have high binding affinity with the type of DNA molecule being analyzed as to enhance sensitivity of the detection method.

In this study, we developed a protocol for separation and detection of SYBR Green II labeled ssDNA utilizing the automated CE instrument (ABI Prism®310 Genetic Analyzer).

Materials and Methods

Preparation of SYBR Green II/ssDNA complex

A 79-nt synthetic ssDNA (Bio Basic Inc.) was mixed with different volumes of 10,000X SYBR®green II RNA gel stain in DMSO (Lonza Rockland, USA), and incubated for 30 min. Then, the labeled product was mixed with a 10-µL reaction mixture including Hidi-formamide (Applied Biosystems, UK) and the internal size standard LIZ 500 (Applied Biosystems, UK) and heated at 95°C for 5 min, rapidly chilled on ice until loaded into ABI Prism®310 Genetic Analyzer.

Separation and detection of DNA molecule

The instrument, ABI Prism®310 Genetic Analyzer equipped with LIF detector (Applied Biosystems, USA) was used to perform separation and detection of the labeled DNA molecule. Separation was carried out in a 47-cm capillary with diameter of 50 µm (Applied Biosystems, USA) filled with optimized polymer 4 (POP-4; Applied Biosystems, USA). The instrument was equipped with argon ion laser (10 mW, ABI PRISM®), excitation wavelength of 488 nm, and connected to a CCD cmera detector that monitors fluorescent wavelengths from 520 to 660 nm. Because SYBR Green II has $\lambda_{ex\ max}$ = 497 nm and $\lambda_{em\ max}$ = 520 nm, virtual filter sets optimized for ABI PRISM® dye sets were utilized for detection.

Electrophoresis was carried out at 60°C using injection voltage of 15kV for 5 sec, separation voltage of 15kV, and separation was carried out for 28 min, unless otherwise stated. DNA fragments were analyzed and sized by using GeneMapper™ *ID* Software, version 3.2 (Applied Biosystems, USA).

Results and discussion

Optimization of SYBR Green II-ssDNA complex separation and detection using ABI Prism®310 genetic analyzer

The SYBR Green II-ssDNA complex was separated and detected under the default STR typing run condition, i.e. injection voltage of 15kV for 5 sec, separation voltage of 15kV for 28 min, and electrophoresis temperature at 60°C. The resulting electropherogram showed a peak sized at 73 with peak height approximately 700 rfu (figure 2a). In order to efficiently separate and detect SYBR Green II-ssDNA complex by the genetic analyzer instrument, modifications were made to the default STR typing run condition.

Firstly, the injection time was varied to 5, 30, 60, and 90 sec. Electropherograms showed that heights of the peak increased as injection time was increased from 5 to 30, 60, and 90 sec, respectively (Figure 2a-d). When injection time at 30 sec was applied, a sharp apex peak with peak height approximately 2,000 rfu (Figure 2b) was present. However, longer injection times, i.e., 60 and 90 sec, caused broad and off scale peaks (Figure 2c and 2d). This resulted in a substantial loss of resolution (15), and the DNA fragment cannot be analyzed by the installed software.

The next factor that affected uptake of DNA sample into the capillary was injection voltage. Injection voltage was then varied to 5, 10, and 15kV, while a 30-sec of injection time was applied. The default injection voltage at 15kV for 30 sec produced the highest peak of SG-II/ssDNA complex among the three different injection voltages (Figure 3). Peaks of lower heights were obtained when injection voltage were 5 and 10 kV (Figure 3a and b). According to Butler, J. M (12), higher injection voltage promotes the process of sample stacking that is caused by large amount of DNA molecule from the sample reservior migrating rapidly into the capillary.

Control of temperature is also important for the efficiency of labeled-ssDNA sample separation under the denaturing condition by CE. The separation of SG-II/ssDNA complex was observed under the three different temperatures; 30°, 40°, and 60°C. Electropherograms in figure 4 demonstrated that the best peak resolution and signals was obtained when electrophoresis was carried out at 60°C (figure 4c). Moreover, shorter period of time was required to obtain a complete set of the internal size standard fragments (LIZ 500), when compared to lower temperatures at 30°and 40°C (figure 4a and b, respectively). In addition, noise background was observed in low temperature separation (figure 4a). These may cause less precision of DNA sizing because of the DNA strands may not be fully denatured, thus forming secondary structures in the capillary (12). However, increasing the capillary temperature can have both positive and negative effects on separation, which depends on particular sample (16). Temperature of the chamber can be increased to as high as 60°C in most commercial CE units. Performing the electrophoresis separation at a high temperature at 60°C can minimize the formation of DNA secondary structure or intra-strand hybrid structure that impacts the DNA separation (17).

Experimental results suggested that the optimal condition for separation using ABI prism® 310 for SG-II/ssDNA complex analysis was as following; injection voltage of 15kV for 30 sec, separation voltage of 15kV for 28 min, and electrophoresis temperature at 60°C.

Electropherogram shown in figure 5 demonstrated peaks resulting from co-injection of SYBR Green II labeled-ssDNA complex with LIZ 500, a commercial internal size standard. This enabled the SG-II/79-nt ssDNA complex to be sized as 73.85 nt. According to Butler, J. M (12), the size of a labeled-DNA fragment may differ from its original size due to the change of the new molecule's electrophoretic mobility. This is because the physical size and shape of the dye changes the overall size of the dye-DNA conjugate. The ionic charge, which is present on the dye, also alters the charge to size ratio of the nucleic acid conjugate.

Determination of labeling ratio (Dye: ssDNA)

The amount of dye used for ssDNA labelling was determined by using different combination of dye from 10,000X to 1X (10-fold dilution) and ssDNA fragments from 300ng to 18.75ng (2-fold dilution). Then each combination was separated and detected under the optimal CE condition. The bar chart in figure 6 demonstrated the effect of different labeling ratio and peak heights obtained from CE separation. The set of SG-II/ssDNA complex peaks were highest when 10,000X SYBR Green II was used for labeling. However, peaks were present in only 4 electropherograms; there was no peak when using 10,000X SYBR Green II to label 18.75 ng of ssDNA. When using 1,000X SYBR Green II dye to label 18.75 – 300 ng DNA, peaks of SG-II/ssDNA complex were present in all electropherograms. All peak heights were higher than 100 rfu, which was more than the 50-rfu cut-off limit. In addition, the peak heights of samples containing larger amount of DNA were higher than the ones with lower amount of DNA, respectively. Peaks of lower peak heights were present when lower concentrations of SYBR Green II dye was used for labeling. Labeling ssDNA with 100X SYBR Green II dye resulted in peak heights less than 100 rfu. No product was present in the electropherogram when the working concentrations of dyes were 10X and 1X, suggesting that if insufficient dye was added for labeling, it would then result in smaller peaks; and if small amount of DNA was present for labeling, then no peaks was called (because peak height was less than 50 rfu).

These result also suggested that using imbalance labeling ratio between dye and ssDNA concentrations might have an impact on the sample up take *via* electrokinetic injection. Excess dye produced highly positive charge, which decreased the amount of labeled-ssDNA fragments being introduced into the capillary. On the other hand, using too low amount of dye resulted in faster drawing of unlabeled-ssDNA fragments into the capillary because of its higher negatively charge, competing the labeled-ssDNA complex. Thus, resulting in no peak in the electropherogram. Because cyanine dyes interact with dsDNA *via* 2 binding modes, i.e., intercalation and binding to the minor groove, and the change of binding mode can occur if there are subtle variations in dye structure or DNA sequences (18). In this experiment, SG-II, which is a cyanine dye, was used for ssDNA labeling; therefore, minor groove binding is the only interaction that holds the two molecules together by insertion of dye into the minor groove of ssDNA. Because of these, DNA fragments which a fluorophore was coupled at the 5'-end would then be more stable than SGII/ssDNA complex under denaturing condition.

After the optimal condition for separation and detection SG-II/ssDNA complex and labeling ratio was obtained, reproducibility of the procedure was conducted. Results suggested that the procedure was reproducible. As shown in figure 7, peaks of SG-II/79-nt ssDNA complex were sized as 72.57, 72.55, and 72.56-nt (S.D.±0.01).

Separation and Detection of ssDNA from rolling circle amplification (RCA)

The ssDNA obtained from RCA and the corresponding ssDNA marker were taken from Thaichareon, P.'s work to challenge the optimized separation and detection procedure. As shown in figure 8, the RCA product was sized at the same position as the corresponding ssDNA marker (figure 8a and 8b). No peak was present in the negative control (figure 8c).

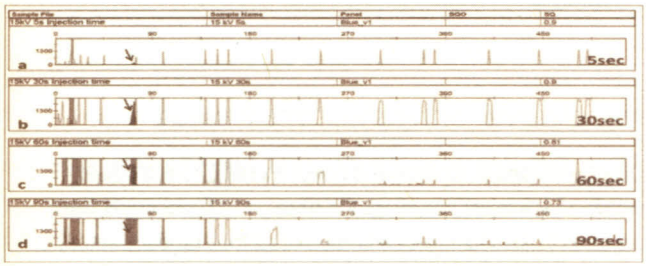

Figure 2 Optimization of injection time. Electropherograms shows peaks resulted from different injection time; (a) 5, (b) 30, (c) 60, and (d) 90sec. The arrow indicates the position of SG-II/ssDNA complex peaks. Injection time at (a) 5 sec gave low peak, (b) 30 sec produced the best peak morphology, (c) and (d) 60 and 90 sec caused broad and off scale peaks.

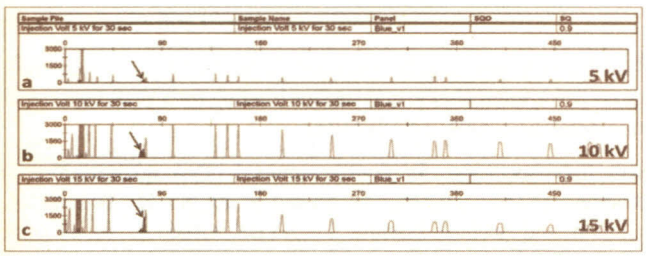

Figure 3 Optimization of injection voltage. Electropherograms demonstrate peaks of SG-II/ssDNA complex from various injection voltages at (a) 5, (b) 10, and (d) 15 kV, while a 30-sec of injection time was applied. Injection voltage at (c) 15 kV for 30 sec produced the highest peak, while the two lower voltages at (a) 5 and (b) 10 kV resulted in lower peaks.

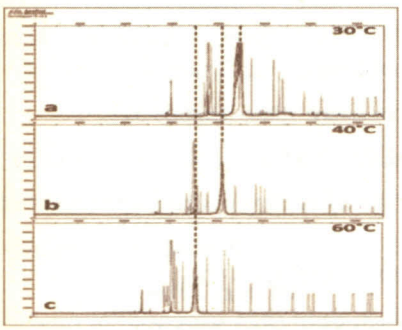

Figure 4 Optimization of separation temperature. Electropherograms of the SG-II/ssDNA complex separated under different temperatures of (a) 30, (b) 40, and (c) 60°C. Each dotted line indicates the position of SG-II/ssDNA complex peak. The best peak resolution and a complete set of an internal size standard were obtained at (c) 60°C, while the lower temperature separation of (a) 30°C and (b) 40°C caused uncompleted an internal size standard. The noise background presented at (a) 30°C.

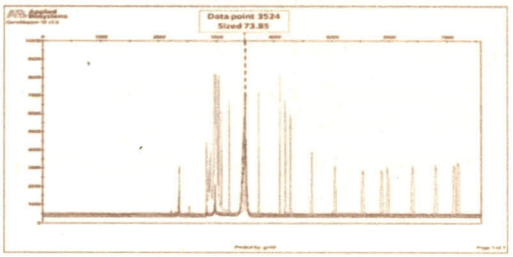

Figure 5 Separation and detection of SG-II/ssDNA complex under the optimal condition. The SG-II/ssDNA complex was sized at 73.85-nt when co-injection with LIZ500.

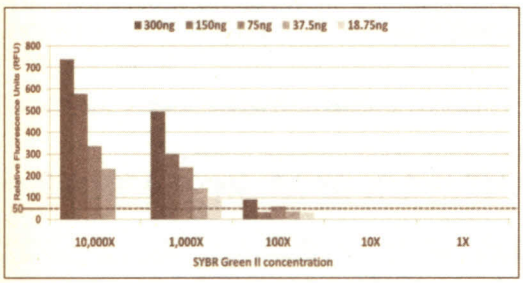

Figure 6 The bar chart presents heights of peaks generated from different labeling ratio between dye and ssDNA. The dotted line represented the cut-off value of 50 RFU. Using 1,000X SG-II for labeling ssDNA 18.75-300ng, peaks of SG-II/ssDNA complex were presented in all electropherograms.

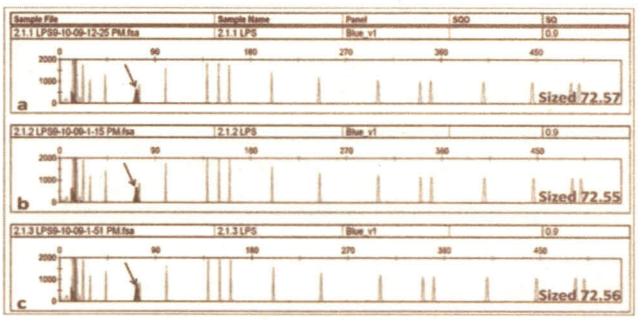

Figure 7 Reproducibility test. SG-II/ssDNA complex was injected 3 times. The arrow indicates peaks of the SG-II/ssDNA complex. Electropherogram showed peaks sized at (a) 72.57, (b) 72.55 and (c) 72.56. S.D. was ± 0.01.

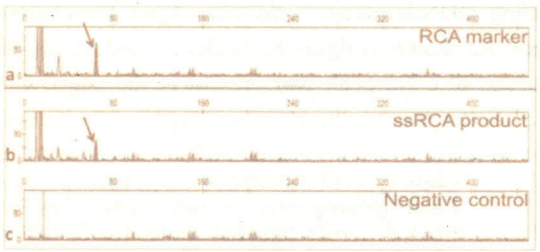

Figure 8 Separation and detection of RCA product under the optimal procedure. Electropherograms shows (b) peaks of the RCA product was sized at the same position as (a) the corresponding ssDNA marker. (c) There was no peak in the negative control.

Conclusion

The automated CE, ABI prism® 310 Genetic Analyzer, can be utilized to separate and detect SYBR Green II labeled-ssDNA products by using the optimized CE condition, which is as following; 15 kV injection voltage for 30 sec, 15 kV electrophoresis voltage, and separation was carried out for 28 min, at constant temperature of 60°C. In addition, co-injection of ssDNA product with LIZ500 internal size standard allows sizing by GeneMapper®Software.

References

1. Zipper H., Brunner H., Bernhagen J., Vitzthum F. Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Res.* 2004; 32(12):e103.
2. Vitzthum F., & Bernhagen J. SYBR Green I: an ultrasensitive fluorescent dye for double-stranded DNA quantification in solution and other applications. Recent Res. Devel. Anal Biochem. 2002; 2, 65-93. As quoted in Zipper H., Brunner H., Bernhagen J., Vitzthum F. Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological impliçations. *Nucleic Acids Res.* 2004; 32(12):e103.
3. A Guide to Fluorescent Probes and Labeling Technologies. Nov 11, 2009. Available from: http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The Handbook.html
4. Lee L.G., Chen C.H., Chiu L.A. Thiazole orange: a new dye for reticulocyte analysis. *Cytometry.* 1986; 7(6):508-517.
5. Lonza. Instructions for using SYBR® Green II Nucleic Acid Gel Stain. Lonza Rockland, Inc. 2007.
6. Emanuel J.R., Damico C., Ahn S., Bautista D., Costa J. Highly sensitive nonradioactive single-strand conformational polymorphism. Detection of Ki-ras mutations. *Diagn. Mol. Pathol.* 1996; 5(4):260-264.
7. Deyl Z., Miksík I., Tagliaro F. Advances in capillary electrophoresis. *Forensic Sci. Int.* 1998; 92(2-3):89-124.
8. Luckey J.A., Drossman H., Kostichka A.J., Mead D.A., D'Cunha J., Norris T.B., et al. High speed DNA sequencing by capillary electrophoresis. *Nucleic Acids Res.* 1990; 18(15):4417-4421.
9. Watts D. & MacBeath J.R. Automated fluorescent DNA sequencing on the ABI PRISM 310 Genetic Analyzer. *Methods Mol. Biol.* 2001; 167:153-170.
10. McCord B., Hartzell-Baguley B., King S. Separation of DNA by Capillary Electrophoresis. In: Walker JM, Schmitt-Kopplin P, editors. Capillary Electrophoresis. Humana Press; 2008. p. 415-429. Available from: http://dx.doi.org/10.1007/978-1-59745-376-9_15
11. Le H., Fung D., Trent RJ. Applications of capillary electrophoresis in DNA mutation analysis of genetic disorders. *Mol Pathol.* 1997; 50(5):261-265.
12. Butler J.M. *Forensic DNA Typing Biology, Technology, and Genetics of STR Markers.* 2ed. UK; Elsevier Academic Press; 2005.
13. Ulfelder J.K. Capillary Electrophoresis of dsDNA Fragments with UV and Laser-Induced Fluorescence Detection. *Beckman Instrument, Inc* 1993; A-1748.
14. Ulfelder K.J. Application Information Bulletin. Beckman Instrument, Inc 1994; A-1774.
As quoted in Schwartz H., & Guttman A. Separation of DNA by Capillary Electrophoresis. California: Beckman Coulter.
15. Weinberger R. *Practical Capillary Electrophoresis.* 2 ed.Chappaqua,New York: Academic Press; 2000.
16. Guttman A., Horvath J., Cooke N. Influence of temperature on the sieving effect of different polymer matrixes in capillary SDS gel electrophoresis of proteins. *Analytical Chemistry.* 1993; 65(3):199-203.