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Smart approach for cost-effective genotyping of single nucleotide polymorphisms

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Abstract

Single Nucleotide Polymorphisms (SNPs) are in the prime focus of genomic studies for their probable roles in diagnostics and prognosis of diseases and forensic science. SNaPshot/minisequencing reaction-based genotyping of targeted SNPs is a method of choice due to its fast and reliable detection assay. Here we described smart modifications in minisequencing reaction to make it cost-effective to detect 15 SNPs in a single assay. The target SNPs were amplified in a multiplex PCR from genomic DNA, and these multiplex PCR amplicons were utilized as a template in modified SNaPshot reaction for SNPs identification. The modified protocol was assessed for reproducibility on more than 50 human DNA samples, and it was observed that this modified method is at least five times more productive than the original protocol recommended by the manufacturer. The current smart modifications in SNaPshot reaction were successfully optimized for susceptible asthma SNPs. However, these can be applied for cost-effective genotyping of any type of genomic Single Nucleotide Polymorphisms.

Keywords: Assay; genotyping; single nucleotide polymorphism; smart approach; SNaPshot.

1. Introduction:

Currently, there is substantial interest in the use of single nucleotide polymorphisms (SNPs) for understanding the genetic basis of complex human diseases. SNPs are the most common type of DNA sequence variations in the human genome (on average once every 200-300 base pairs of the human genome) that are responsible for functional changes (Tebbutt *et al*., 2007, Shahid *et al*., 2015). The use of SNapShot minisequencing has quite increased due to its fast and reliable detections of multiple SNPs in a single polymerase chain reaction (PCR). This method is a preferred choice for SNPs based diagnostics and forensic studies because of its robustness, multiplexing, detection sensitivity, and less dependence on quality and quantity of DNA (Gilbert *et al*., 2007, Babol-Pokora & Berent, 2008, Sabar *et al*., 2016).

Many disease association and genetic screening studies using SNaPshot minisequencing assay revealed that it is a very reliable method for the detection of SNPs. The assay was very successful in determining the significant association of GATA3 haplotypes with asthma in Finnish families (Pykalainen *et al*., 2005). Single nucleotide polymorphism of *BCL11A* and *HBS1L-MYB* genes is found to be strongly associated with HbF (fetal

hemoglobin) level using the same approach (Fanis *et al*., 2014). It is also an important tool for screening disease-related mutation for the purpose of the diagnostic (Sagong *et al*., 2013). Bouakaze and co-workers successfully performed the typing of Y-SNP from ancient DNA (5500 to 1800 years old) of southern Siberia origin using SNaPshot minisequencing, showing efficiency, robustness, and convenience of the assay in the field of forensic and evolutionary biology even with too ancient and degraded DNA samples (Bouakaze *et al*., 2007). *Fas* gene is a member of the TNF-receptor superfamily, encodes a protein that mediates apoptosis (programmed cell death). SNaPshot assay was employed to confirm the outcomes of a direct sequencing-based study that developed the novel DNA biomarkers present in the *Fas* gene associated with some economical traits in cattle (Kim *et al*., 2016).

This shows that SNaPshot minisequencing is a powerful tool that can serve the scientific community in forensic (Huang *et al*., 2010), medical genetics (Fanis *et al*., 2014), and diagnostics fields (Sagong *et al*., 2013). In most of the studies, the SNaPshot assay was performed according to the manufacturer's provided guidelines, and no doubt, it is a method of choice due to its accuracy, efficacy, reliability, and robustness. Cost-effective research is also one of the priorities of the scientific community, especially from developing or less developed countries.

This study presents a cost-effective 15-plex minisequencing protocol that was optimized to decrease the cost of qualitative detection of 15 "Single Nucleotide Polymorphisms" in a single multiplex PCR.

2. Materials and methods

2.1 Reference samples

The optimization and validation of cost-effective SNaPshot assay were performed on 50 DNA samples (25 normal healthy and 25 asthma patients) of children; The DNA samples were taken from the DNA bank of DNA Core facility-CAMB, University of Punjab. The samples were collected for research purposes after ethical approval from "The Children's Hospital & The Institute of Child Health" (Ref. No. 01/158/16), and written informed consents were obtained from the guardians/parents of children whose DNA was used in this study.

These DNA samples were extracted from the peripheral blood samples of study participants. DNA Core Facility-CAMB completed ethical approvals and legal formalities for the use of blood samples for research purposes as mentioned in our previous publications (Ghani *et al*., 2019b, Ghani *et al*., 2019a, Sabar *et al*., 2017).

2.2 SNPs selection and primers designing

Fifteen genomic SNP variants selected for multiplex PCR are described in Table I. These variants were already reported as potent asthma susceptible genomic variants in different populations; however, their detailed role in asthma development is still to be explored in Pakistani children; therefore, these SNPs were selected to optimize the modified SNP genotyping method which can be used to study the role of these SNPs in the pathogenesis of asthma disease in Pakistan. Twelve pairs of amplification primers were designed with Primer-3 software and technically analyzed for auto dimerization, self-complementarity, etc., by FastPCR software (http://primerdigital.com/tools/pcr.html). Primers were designed by keeping a minimum difference of 26bp between amplification PCR products. SBE (Single base extension) primers were designed with

Primer 3 Plus software (http://bioinfo.ut.ee/primer3-0.4.0/).

Poly-GACT tailing was added on the 5' position of primers to increase the length and keep at least 4bp difference between single base extension (SBE) primers.

2.3 Amplification in Multiplex PCR

Target SNPs were amplified with Phusion U multiplex PCR mix (ThermoFisher Scientific, Cat#F562S) in a 15µl reaction volume as described in Table II. 05µl of purified PCR amplicons were treated with 1.2µl ExoNuclease-I (01 IU/µl), and 1.6µl of 5X SAP (Shrimp Alkaline Phosphatase), and the mixture was incubated at 37°C for 60 minutes to remove primers and inactivate dNTPs followed by deactivation of these enzymes at 80°C for15 minutes

Table 1. SNPs optimized in 15-plex SNaPshot reaction.

Sr. No	Gene.	SNP ID
1	CHI3L1	rs4950928
2	CHI3L1	rs880633
3	CD14	rs2569190
4	IL4	rs2070874
5	IL3	rs40401
6	IL3	rs31480
7	CSF2	rs25882
8	IL5	rs2069812
9	RAD50	rs2244012
10	RAD50	rs6871536
11	IL13	rs1881457
12	IL13	rs1800925
13	IL13	rs1295686
14	IL13	rs20541
15	IL4	rs2243250

Table 2. Multiplex PCR protocol to amplify SNPs from genomic DNA.

Amplification PCR Reaction Mix Composition		**PCR Amplification Protocol**			
PCR Component	Volume	Step	Temperature	Time	Cycles
dH2O	6µl	Hot Start	95°C	5 Min.	1
PCR Ready Mix	7.5µl	Denaturation	95°C	0.45 Min.	30
DNA (25ng/µl)	01µl	Annealing	58°C	0.45 Min.	
Primers Mix (1µM)	0.5µl	Elongation	72°C	1.30 min.	
Total	15µl	Final elongation	72°C	10 min.	1

2.4 Modified SNaPshot Reaction

Mutiplex SNaPshot reaction was first optimized for genotyping of target SNPs in 10µl reaction as per the protocol of SNaPshot® Multiplex kit (ThermoFisher Scientific, cat# 4323159) just by varying concentration of SBE primers and amplified multiplex PCR product. The concentration of primers was optimized to the minimum possible concentration by decreasing from 0.2µM to 0.01µM to avoid junk or non-specific peaks in SNP genotyping results electropherogram. After optimization of standard reaction, modifications were performed in the SNaPshot reaction to make it cost-effective. For 5µl reaction volume, 5X sequencing buffer (BigDye ver 3.1, ThermoFisher Scientific, cat# 4337455) was used as a SNaPshot reaction buffer to balance the stoichiometric ratios of PCR reaction components by a stepwise decreasing concentration of "SNaPshot reaction ready mix" from 2.5µl to 0.5µl. For 15 SNPs-plex and 05 SNPs-plex, the SNaPshot reaction was optimized by taking 01µl and 0.5µl of ready mix, respectively (Table III). However, reproducibility of the modified method was only accessed for 15 SNPs-plex.

2.5 Purification of SNaPshot reaction products and Capillary Electrophoresis

0.5 µl 5X SAP (Shrimp Alkaline Phosphatase) added in SNaPshot reaction product and incubated at 37°C for one hour with subsequent inactivation of an enzyme at 75°C for 15 minutes. 01µl of the enzymatically cleaned product along with 0.05 µl of GeneScan-120LIZ (internal size standard-Life Technologies) was added in 8.95µl HiDi formamide and incubated the mixture at 95°C for 05 minutes followed by readily placement of mixture on ice for 03-5 minutes to denature and linearize the SNaPshot reaction product fragments. These reaction fragments were placed on 3130XL Genetic Analyzer for SNP genotyping based on the principle of CE (capillary electrophoresis). GeneMapper IDX analyzed

the electrophoresis results to interpret the alleles of target SNPs.

3. Results

The modified method was successfully optimized to detect 15 SNPs in a single reaction by changing stoichiometric ratios of PCR reaction components as describes in Table III. In optimization-1, the volume of PCR reaction components was reduced to half. Strategy to reduce the volume of reaction components was also optimized for our previous study to analyze the asthma susceptible SNP variants of the ADAM33 gene (Sabar *et al*., 2016). In optimization-2, we decreased the quantity of SNaPshot ready mix to half of optimization-1 and added 0.25µl sequencing buffer (5X) to adjusted stoichiometric ratios of reaction components. In optimization-3, we further decreased the quantity of SNaPshot ready mix to 1µl and added 0.5µl sequencing buffer (5X) to adjusted stoichiometric ratios of reaction components.

The optimization-3 is the best-optimized method, and the whole reaction process is demonstrated in Table III. The representative electropherogram of SNPs is given in figure I, and optimized capillary electrophoresis setting/parameters of ABI 3130XL are given in table IV.

Table 3. Optimization of cost-effective SNaPshot genotyping protocol.

PCR Components	Standard Reaction Volume (µl)	Optimization-1 Volume (µl)	Optimization-2 Volume (µl)	Optimization-3 Volume (µl)
SNaPshot kit	5	2.5	1.25	1
dH2O	2	1	2	2
PCR Amplicons	2	1	1	1
SBE Primer Mix (0.01µM)	1	0.5	0.5	0.5
Sequencing Buffer (5X)	0	0	0.25	0.5
Total Volume (µl)	10	5	5	5

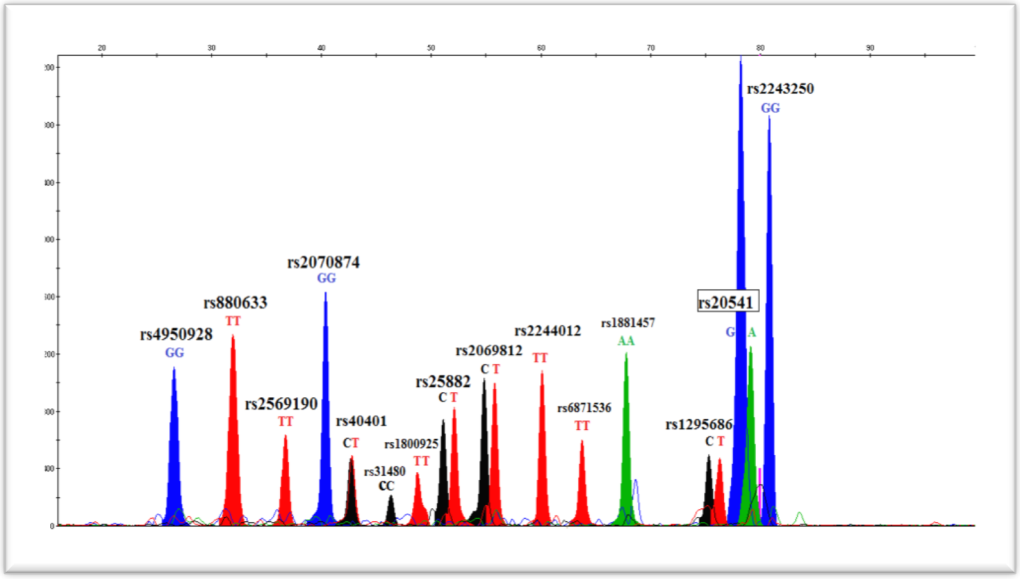

Fig. 1. Different colours of peaks differentiate among alleles. "G" allele is represented by blue, "C" allele by black, "T" by red, and "A" allele by green colour.

Table 4. Parameters of 3130xL Genetic Analyzer to perform electrophoresis of SNaPshot multiplex fragments.

Parameters	Control Module (E5 with POP-4 polymer)
Injection time	33 Sec
Electrophoresis voltage	15 kV
Injection Voltage	2 KV
Capillary Length	36 cm
Oven temperature	60 °C
Run Time	1000 Sec

Table 5. The whole optimized & modified protocol for genomic SNPs analysis.

Process	Step ID	Reagents and Protocols
Amplification PCR	A	PCR Conditions: Annealing Temperature 58°C Primers Mix: UP-01 to UP-12 PCR reaction Volume: 15µl
Post Amplification Multiplexing & Purification	B	PCR Product(05µl) + SAP (08 Unit) + Exo Nucl-1 (1.2 Unit) Incubation: 37°C for 1 hour & 80°C for 15 mint
SNaP-Shot Reaction	C	**Reaction Mix**: 01µl of purified amplicon + 0.5µl SBE Primer Mix + 01µl SNP-Shot Kit + 0.5 µl Sequencing Buffer (Big Dye Ter Ver 3.1) + 02µl dH_2O **Positive Control**: 01µl of Template + 0.5µl Primer Mix + 01µl SNP-Shot Kit + 0.5 µl Sequencing Buffer (Big Dye Ter Ver 3.1) + 02 µl dH_2O **Negative Control**: 0.5ul SBE Primers mixture + 01µl SNaP-Shot Kit + 0.5 µl Sequencing Buffer (Big Dye Ter Ver 3.1) + 03µl dH_2O **25cycle PCR:** 96°C for 10sec, 50°C for 5sec & 60°C for 30sec
Post-Extension Treatment	D	Add 2.5 Unit of SAP & Incubate at 37°C for 1 hour and then at 75°C for 15 mints
SNaP-Shot Genotyping	E	01µl of SNaP-Shot product + 0.05µl Liz120 + 8.95µl Formamide & Heat shock at 96°C for 5 mints.

4. Discussions

The minisequencing/single base extension assay used in this method utilizes the SNaPshot Multiplex Kit that comprises a ready reaction mixture of four fluorescently labeled dideoxy nucleotide triphosphates (ddNTPs), which allows the incorporation of only one base at the targeted mutation site. The incorporated ddNTPs are identified and distinguished based on the colors produced by fluorescently labeled dyes to call alleles on mutation/polymorphic site(Hurst *et al*., 2009).

SNaPshot minisequencing assay was first developed by Smith and his co-workers (Smith *et al*., 1998), and now it has been widely used in medical genetics (Bertoncini *et al*., 2011), population genetics (Drobnic *et al*., 2010), disease-related case-control studies (Kumar *et al*., 2012) and diagnostics (Al-Aama *et al*., 2019). This technique is equally important in the identification of an individual by determining the haplogroup of mtDNA followed by phylogenetic analysis (Coutinho *et al*., 2014).

Another important application of SNaPshot assay is the identification of closely related species by comparing the selected portions of the genome to develop biomarkers that lead to the discrimination between species (Maroso *et al*., 2019, Huang *et al*., 2011)

Currently, multiplex SNaPshot assay is successfully used to unravel the genetic basis of many hereditary diseases like ischemic stroke (Yang *et al*., 2016), asthma (Lyon *et al*., 2004), and large artery atherosclerotic stroke (Li *et al*., 2019).

It has an advantage over other genetic techniques that a single multiplex SNaPshot reaction can easily detect multiple SNPs in different genes.

Due to robustness and compatibility with capillary – based genetic analyzers , the manufacturer's standard SNaPshot assay has been adopted in several studies to genotype SNPs located at different positions on the human genome (Sabar *et al*., 2016, Shahid *et al*., 2019). So far, none of the studies have been designed to reduce the cost of SNaPshot minisequencing based methodology/research. To the best of our knowledge, the current study is the first effort of its kind that involved some successful modifications in the multiplex SNaPshot technique for cost-effective genotyping of SNPs without affecting the quality of research work. This modified protocol will help the scientific community from developing and very less developed countries where the research budget is very limited.

Originally SNaPshot Multiplex Kit is designed by the manufacturer to analyze 01 to 10 SNPs in a 10µl reaction volume. However, in this article, we present a more cost-effective and productive assay that can analyze up to 15 SNPs in a single tube reaction using 01µl SNaPshot multiplex kit ready mix instead of 05µl. With the suggested modifications, a kit of 100 samples with 10X multiplex (1000 SNPs) can be used to analyze about 500 samples with 15X SNPs multiplex (7500 SNPs) that make it cost-effective and more productive relative to standard protocol. It is observed that SBE primers may anneal at nonspecific genomic sites to produce nonspecific single base products; therefore, targeted alleles are needed to observe critically and be confirmed through DNA during the optimization step sequencing technique.

However, SNaPshot genotyping/misequencing protocol is still a method of choice to genotype SNPs due to robustness and cost-effectiveness. The optimized assay was assessed on 25 healthy and 25 asthma patients. The genotyping results were concordant with the results of the assay of the SNaPshot Multiplex Kit without modification.

5. Conclusion

The main focus of this study was the modification in SNaPshot minisequencing assay to make it cost-effective without compromising the quality of research data which has been achieved successfully by developing the 15X multiplex. The SNPs analyzed in the current study have been reported as potential asthma susceptible variants in different populations. We have reported the modified assay's efficiency, providing a low-cost and robust approach to analyze the SNPs efficiently. This approach may also provide sufficient information to understand the role of SNPs in the development of asthma and other diseases in different populations. The reaction success rate with the modified protocol was exactly the same as the reactions performed with the manufacturer’s provided standard protocol, suggesting that the quality of analysis work was not compromised over cost. In countries like Pakistan, where the budget for research activities is limited, such smart modifications in assays allow researchers to complete their targets within their limited financial resources without affecting the quality of research work.

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