Comprehensive Gene Sequence Analysis from Bloodspot and Saliva DNA

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Abstract

Ambry Genetics' proprietary full gene sequence detection methods have been validated for DNA isolated from blood spots and saliva, in addition to DNA from whole blood and cultured amniocytes used to date. Blood can be collected on Schleicher & Schuell (S&S) 903 specimen collection paper commonly used in newborn screening programs. Alternatively, saliva can be collected into an Oragene™ DNA Self-Collection container (DNA Genotek Inc.). High quality DNA can be obtained from either starting material, with a half of a blood spot (0.5 inch diameter) or 250 ul saliva giving yields of approximately 1.5-4.5 ug and 8-12 ug DNA, respectively. The isolated DNA has been successfully analyzed for sequence variations in the entire Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) coding sequence using the Ambry Test™: CF, utilizing modified temporal temperature gradient electrophoresis (mTTGE) and dye terminator sequencing methods. In addition, DNA from bloodspots has been successfully analyzed for sequence variations in the coding and flanking regions of the Beta Globin (HBB) gene using a dye terminator sequencing method. We observed a PCR amplification template size limit of approximately 800 bp for DNA isolated from bloodspots. Analytical sensitivities and specificities between the specimen types for these two genes were shown to be similar. Furthermore, the specimens are intact for years in their collected media and the isolated DNA are as stable as whole blood DNA when stored at –20ºC or –80ºC.

By making blood spots an acceptable specimen type, Ambry Genetics can now offer CFTR and HBB full gene sequence analysis to newborn screening programs, eliminating the need for additional blood collection and allowing for more rapid processing of samples. In addition, clinics or individuals not having customary access to phlebotomists can utilize the saliva kits to submit their DNA for comprehensive genetic analysis.

Introduction

Comprehensive gene sequence detection methods typically rely on ample amounts of DNA isolated from whole blood for analysis. Our assays, in particular for cystic fibrosis (CF) and beta globin (HBB) are often requested for newborns, babies and small children. In addition, specimens are sent from locations worldwide. We investigated if our methods could be optimized for use with bloodspot DNA and saliva DNA in order to accommodate a broader range of sample collection and shipping conditions. Advantages of providing these options are time and cost savings, and expedited diagnoses.

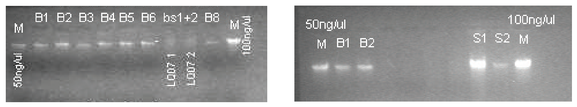
Methods

DNA extraction from blood spots: Blood was collected on FDA approved Schleicher & Schuell (S&S) 903 specimen collection paper (1), and DNA was extracted based on a method published by Walsh et al. (2). Briefly, half of a dried blood spot was incubated and rinsed three times in deionized water prior to cell centrifugation. A small volume of freshly made 5% Chelex 100 resin was then added. The suspension was incubated at 56ºC for 2 hours, then boiled for 8 minutes, and centrifuged at high speed. The DNA supernatant was removed from the resin pellet and used for PCR.

DNA extraction from saliva: Two milliliters (4-5 spits) of saliva were collected into a DNA Genotek's Oragene™ DNA Self-Collection container, which contains ~2 ml of cell lysis solution, and kept at room temperature. Saliva samples were processed according to manufacturer specifications (3). Briefly, Oragene Purifier solution (proprietary proteinase and precipitation solution) was added to the saliva mixture, and incubated at 50ºC for at least 1 hour prior to precipitation with ethanol. The DNA pellet was air dried and dissolved in 1X TE. The DNA was then used for PCR.

DNA quality and quantity assessments: The extracted DNA was analyzed on agarose gels with known amount of high molecular weight DNA to assess DNA qualities and quantities. DNA was tested with PCR amplification procedures and primers used for CFTR TTGE and sequencing analysis (4). TTGE: For bloodspots, 10-30 ng input DNA (2 ul) was used with HotStarTaq Master Mix (Qiagen) per 10 ul reaction. Typical PCR conditions were 1 cycle: 95ºC/ 15 min, 39 cycles: 94ºC/ 30 sec, 49-62ºC/ 30 sec, 72ºC/ 30 sec, 1 cycle: 72ºC/ 10 min. PCR products were then processed for TTGE on DCode gels (BioRad) in adherence with the Ambry Test technology. For saliva DNA, ~40-60 ng input DNA (0.5 ul) was used per 10 ul reaction, identical to the standard method for whole blood, and PCR amplification was performed for 35 cycles. Sequencing: For bloodspots, 10-30 ng input DNA and for saliva, 40-60 ng DNA were used per 10 ul PCR reactions to amplify template for subsequent dye terminator sequencing. Exons were amplified with unique primer sets using Taq PCR Master Mix (Qiagen, Valencia CA). Typical PCR conditions were 1 cycle: 95ºC/ 5 min, 35 cycles: 94ºC/ 30 sec, 50-55ºC/ 30 sec, 72ºC/ 30-45 sec, 1 cycle: 72ºC/ 10min. For HBB sequencing, the template length was kept under 800 bp. Dye terminator sequencing was set up using Beckman DTCS reagents and templates were analyzed on a CEQ8000.

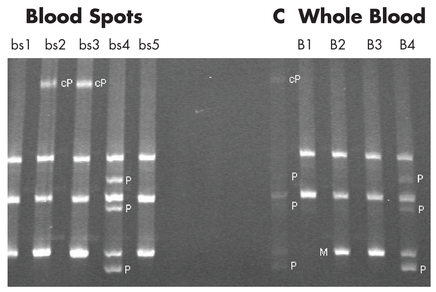
Figure 1: Extracted Genomic DNA



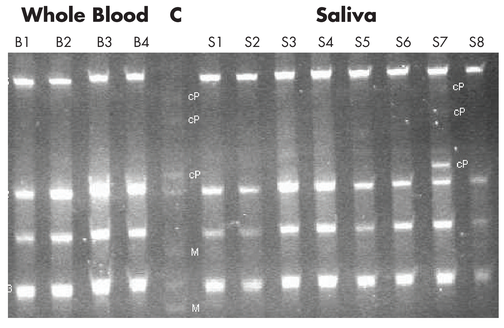
A. 4 ul of genomic DNA extracted from two halves of a blood spot (bs1+2) were analyzed on agarose gel along with 2 ul of DNA extracted from 1 ml of whole blood (B1-B6 and B8).

B.2 ul of genomic DNA extracted from 250 ul of saliva (S1 and S2) were analyzed on agarose gel along with 2 ul of DNA extracted from 1 ml of whole bloods (B1 and B2). To assess the DNA quantity and quality, 2 ul of 50 ng/ul and 100 ng/ul high molecular weight DNA (M) were also included in the gels. The saliva samples S1 and S2 represent the maximum (~200 ng/ul) and minimum yields (~80 ng/ul) obtained from several saliva samples. Most samples gave yields between ~80-120 ng/ul.

Figure 2: TTGE Comparison

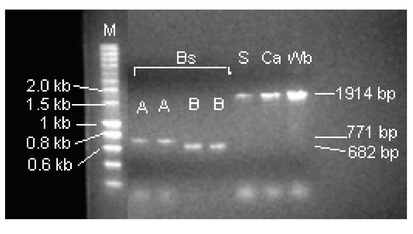


A.TTGE gel showing 3 different PCR products amplified from CFTR gene using DNA extracted from 5 blood spot (bs1-bs5) and 4 whole blood (B1-B4) samples. A common polymorphism (cP) is detected in samples bs2 and bs3 and a polymorphic sequence (P) is detected in samples bs4 and B4. Both of the sequence variants are included in the control (C) sample. Potential mutation (M) is observed in sample B1. The absent band could result from a sequence variation where the PCR primers anneal or a homozygous deletion of the genomic sequence. Any sequence variations are verified by direct sequencing.



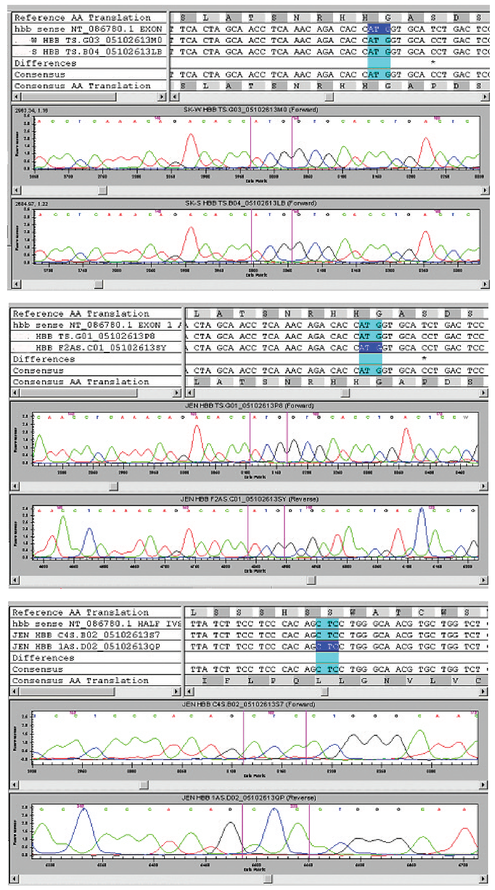
B.TTGE gel showing 4 different PCR products amplified from CFTR gene using DNA extracted from 4 whole blood (B1-B4) and 8 saliva (S1-S8) samples. A common polymorphism (cP) is detected in saliva S7 and control (C) samples. The control sample also contains a mutation (M) located in the bottom PCR fragment.

Figure 3: Sequence Templates



A.The agarose gel shows amplification product for three different HBB templates synthesized for sequencing. The DNA was amplified with PCR to yield a 1914 bp fragment for the Beta-Hemoglobin gene (HBB) containing exons 1, 2 and 3. Larger templates were more difficult to generate from bloodspot DNA. Thus, the HBB sequencing templates were generated in 2 separate reactions giving rise to two smaller PCR templates of 771 bp (A, covering exons 1 and 2), and a 682 bp template (B, covering exon 3). We have observed a consistent drop in yields for templates over 800 bp for bloodspot DNA and design template lengths accordingly. (Bs) refers to DNA extracted from a blood spot sample; (S) to DNA extracted from a saliva sample; (Ca) to DNA extracted from a cultured amniocyte sample; and (Wb) to DNA extracted from a whole blood sample.

Figure 4: Sequence Analysis



A.The chromatograph shows a sample sequencing result from whole blood (W) versus saliva (S) DNA template from the same individual at the beginning of exon 1 of HBB. Both templates were 1914 bp long.

B.The same region in HBB exon 1 is shown in sense and antisense direction when synthesized from bloodspot DNA, template A.

C.The beginning of HBB exon 3 is shown in sense and antisense direction when synthesized from bloodspot DNA, template B.

Results

- DNA extraction methods for half of a blood spot (0.5 inch diameter) and ~250 ul of saliva typically gave yields of ~300 ul of 5-15 ng/ul (1.5-4.5 ug total) and ~100 ul of 80-120 ng/ul (8-12 ug total) of DNA, respectively.
- Bloodspot DNA was of high quality, although PCR products generated from blood spots generally need to be less than 800 bp to amplify reliably.
- In order to obtain sufficient PCR product from bloodspot DNA for TTGE analysis, PCR amplification cycles were increased by 4 cycles. Sequencing analysis did not necessitate amplification cycle changes compared to whole blood DNA. The assays' analytical sensitivities and specificities were unchanged.
- DNA yield from ~250 ul of saliva is similar to yield from 1 ml of whole blood, and similar performance on TTGE and sequencing protocols were observed.

Conclusion

- Ambry Genetics' proprietary full gene sequence detection method utilizing a combination of TTGE scanning and sequencing has been validated for DNA extracted from blood spots and saliva specimens.
- Analytical sensitivities and specificities for blood spot and saliva DNA are similar to those of whole blood DNA in The Ambry Test: CF and in The Ambry Test: HBB.
- Blood spots collected as part of newborn screening programs now can be used in Ambry Genetics' CFTR and HBB full gene sequence analysis when follow-up molecular studies are needed, thus avoiding multiple blood collection and office visits, while expediting diagnosis. Potentially, DNA from stored bloodspots of deceased patients could be evaluated to help establish diagnoses and complete pedigrees.
- Clinics or individuals without customary access to phlebotomists can utilize the Oragene saliva kits to submit their DNA to Ambry Genetics for CFTR and HBB full gene sequence analysis.

References

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2. Walsh, PS, Metzger, DA, and Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10(4): 506-513, 1991.
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