Newborn Screening Quality Assurance Program for CFTR Mutation Detection and Gene Sequencing to Identify Cystic Fibrosis

Miyono M. Hendrix, MS¹, Stephanie L. Foster, BS¹, and Suzanne K. Cordovado, PhD¹

Abstract
All newborn screening laboratories in the United States and many worldwide screen for cystic fibrosis. Most laboratories use a second-tier genotyping assay to identify a panel of mutations in the CF transmembrane regulator (CFTR) gene. Centers for Disease Control and Prevention’s Newborn Screening Quality Assurance Program houses a dried blood spot repository of samples containing CFTR mutations to assist newborn screening laboratories and ensure high-quality mutation detection in a high-throughput environment. Recently, CFTR mutation detection has increased in complexity with expanded genotyping panels and gene sequencing. To accommodate the growing quality assurance needs, the repository samples were characterized with several multiplex genotyping methods, Sanger sequencing, and 3 next-generation sequencing assays using a high-throughput, low-concentration DNA extraction method. The samples performed well in all of the assays, providing newborn screening laboratories with a resource for complex CFTR mutation detection and next-generation sequencing as they transition to new methods.

Keywords
cystic fibrosis, CFTR, mutation, newborn screening, next-generation sequencing

Introduction
Cystic fibrosis (CF) is one of the most common autosomal recessive disorders that affects approximately 1:4000 people of Western European, North American, and Australasian descent. When CF is identified and treated early, patients avoid many of the devastating clinical consequences, allowing for improved growth, reduced hospitalizations, and longer life span, which resulted in the US Centers for Disease Control and Prevention (CDC) recommending that CF be included in newborn screening panels in the United States.¹² Newborn screening for CF begins with an immunoassay that measures the pancreatic enzyme immunoreactive trypsinogen (IRT), which is elevated in newborns affected with CF.³⁴ Since IRT can be elevated for reasons other than CF, this test alone does not have the specificity required for newborn screening. In 1989, scientists discovered the CF transmembrane regulator (CFTR) gene on chromosome 7.⁵ Defects in the CFTR gene that alter structure, function, or expression of this protein can lead to malfunctions or disease processes in the lungs, upper respiratory tract, gastrointestinal tract, pancreas, liver, sweat glands, and genitourinary tract.⁶

All US states and many international programs have implemented routine newborn screening for CF. Most US programs use an algorithm that involves at least 1 initial measurement of IRT from a dried blood specimen (DBS) taken from all newborns and then testing for a panel of CFTR mutations on a subset of babies with elevated IRT.⁷⁸ The panel of CFTR mutations can be variable between programs but typically includes the American College of Medical Genetics (ACMG) recommended 23 mutations and often additional mutations.⁷-¹⁰ Newborns with either 1 or 2 CFTR mutations are considered screen positive by most programs and are sent to CF care centers for diagnostic

¹Centers for Disease Control and Prevention, Atlanta, GA, USA

Received April 12, 2016, and in revised form May 27, 2016. Accepted for publication May 27, 2016.

Corresponding Author:
Suzanne K. Cordovado, PhD, Centers for Disease Control and Prevention, 4770 Buford Highway, MS F24, Atlanta, GA, USA.
Email: scordovado@cdc.gov

This article is distributed under the terms of the Creative Commons Attribution 3.0 License (http://creativecommons.org/licenses/by/3.0/) which permits any use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (https://us.sagepub.com/en-us/nam/open-access-at-sage).
workup. Although this algorithm has a relatively low false-negative rate, it has quite a high false-positive rate with most babies being carriers of 1 CFTR mutation and do not display any symptoms associated with CF. As an example, the state of Wisconsin reported a false-positive rate as high as a 90% and the New York state found as high as a 94% false-positive rate (excludes screen positives with no mutations identified). The false-positive rates vary between programs most often because of the differences in the IRT cutoff but sometimes because of the mutation panels used. Currently, the only US program that identifies a screen positive as 2 identified CFTR mutations is the state of California, which initially tests for a panel of CFTR mutations in babies with elevated IRT. If a newborn has only 1 mutation from the California panel, the CFTR gene is sequenced and only those babies with 2 mutations are sent for clinical evaluation. Using this algorithm, California reported that 34% of their screen-positive newborns were CF, 53% had a milder form of CFTR-related metabolic syndrome (CRMS), and 13% were carriers with complex mutations.

False CF-positive results, while unavoidable in newborn screening, cause parental anxiety, unnecessary clinical testing, and downstream genetic counseling. Thus, there are ongoing efforts to redefine a positive newborn screening test such that it requires the identification of 2 CF-causing CFTR mutations similar to what is being done in California. Since there are >2000 mutations or variants in the CFTR gene with more still being discovered, the requirement of identifying 2 CF-causing mutations would likely necessitate either gene sequencing or a greatly expanded genotyping panel of CFTR mutations. As the complexity of CFTR mutation detection in newborn screening expands, there is a need for more extensively characterized dried blood spot quality assurance materials to ensure that high levels of accuracy are maintained in these analytical measurements.

The CDC’s Newborn Screening Quality Assurance Program (NSQAP) provides DBS proficiency testing to United States and international laboratories for both IRT (N = 215 laboratories, quarter 1 of 2016) and CFTR mutation detection (N = 68 laboratories, quarter 1 of 2016). The NSQAP’s CF DNA DBS repository, made from CF patient and family blood samples, contains a wide variety of CFTR mutations including the 23 recommended by ACMG as well as 47 additional mutations. Each repository sample is characterized extensively by CDC’s Molecular Quality Improvement Program Laboratories using Sanger sequencing and commonly used genotyping methods to ensure robust performance in newborn screening laboratories. As the complexity of CF molecular methods continues to evolve, CDC has performed a comprehensive evaluation and characterization of the CF DNA DBS repository samples using a diverse array of genotyping and next-generation sequencing methods that would be amenable in the newborn screening laboratory environment.

Materials and Methods

Samples

Samples from 198 patients and family members affected by CF were collected from CF care centers located in Maryland, Ohio, and Wisconsin and more recently in California in collaboration with the Sequoia Foundation and the California Department of Public Health. All blood was collected in EDTA blood collection tubes from adult donors with at least 1 CFTR mutation (Becton Dickinson, Franklin Lakes, New Jersey) and shipped to CDC, where blood was spotted on to Whatman 903 filter paper (Piscataway, New Jersey) to create dried blood spots (75 μL) for quality assurance. This project was approved by the institutional review boards of all participating CF care centers, and the CDC’s Office of Science at the National Center for Environmental Health determined that CDC was not involved with human subjects under 45 CFR 66.012(d,b) because all specimens are deidentified and cannot be traced back to the donor.

Newborn Screening Quality Assurance Program CF DNA Proficiency Testing (PT) Program

Each participating laboratory received 5 blind-coded proficiency testing specimens 4 times a year, and laboratories reported both the genotyping results and clinical assessments to CDC. These results were evaluated based on the program’s stated mutation panel and molecular algorithm. Programs were informed to assume all samples have an elevated IRT that would trigger their algorithm to test for CFTR mutations. To ensure accurate grading, each programs provided descriptive information including CFTR genotyping/sequencing method, mutation detected or exons sequenced if not using a commercial method, secondary/confirmatory method, description of when and how a secondary/confirmatory method is used, and DNA extraction method.

DNA Extraction and Quantitation

Genomic DNA was extracted from 250-μL whole blood (EDTA anticoagulant) using the Qiagen QIAcube Micro spin columns and resuspended in 100 μL of Tris-buffered EDTA (Valencia, California). The DNA was quantified using the NanoDrop spectrophotometer (Wilmington, Delaware) and diluted to 10 ng/μL for direct use only in Sanger sequencing. Genomic DNA was also extracted from one 3.2-mm DBS punch using the Qiagen Generation DNA Purification and Elution Solutions. The punch was washed 2 times for 15 minutes in 150 μL of DNA Purification Solution followed by one 15-minute wash with 150 μL of DNA Elution Solution. All washes were performed at room temperature with slight agitation. Genomic DNA was eluted from the punch in 50 μL of DNA Elution Solution after incubating at 99°C for 15 minutes. All assays other than Sanger sequencing used DNA extracted from DBS. When quantification of DNA extracted from DBS...
punches was required, a real-time polymerase chain reaction (PCR) of the RNase P gene using the TaqMan RNaseP Control Reagents was used (Thermo Fisher Scientific, Waltham, Massachusetts). The standard curve was made from human genomic DNA (Roche Applied Science, Penzberg, Germany).

**CFTR Genotyping**

Following the manufacturer’s instructions, the NSQAP samples were genotyped and analyzed using 3 commercially available products: InPlex CF Molecular Test 40+4 (Hologic, Marlborough, Massachusetts), xTAG CF39v2 and CF60v2 kits (Luminex, Austin, Texas), and the MiSeqDx CF 139-variant assay (Illumina, San Diego, California). The InPlex CF Molecular test 40+4 followed the manufacturer’s instruction for the in vitro diagnostic (IVD) InPlex CF Molecular Test IVD with 1 modification—the second cycling step during the amplification process was increased from 12 to 14 cycles. The DNA volume used for the Hologic InPlex CF Molecular Test 40+4, both the Luminex xTAG 39 and 60 kits, and the MiSeqDx CF 139-variant assay kit was 5 μL of the Generation extraction’s 50 μL total volume (5-10 ng DNA). Samples were also analyzed using 45 unique TaqMan CFTR single-nucleotide polymorphism (SNP) assays that include all but the I148T (c.443T>C) and I507V (c.1516A>G) variants. Each 10 μL reaction consisted of 1× DurAmp v2 Master Mix (Thermo Fisher Scientific), 1× of SNP genotyping probe mix, and 1 μL of the Generation extraction’s 50 μL total volume (1-2 ng DNA). All run data were loaded into Thermo Fisher Scientific’s GenoType software and analyzed with Hardy-Weinberg analysis with noted exceptions. Hardy-Weinberg analysis was disabled for the F508del (c.1521_1523delCTT), I507del (c.1519_1521delATC), I506V (c.1516A>G), I507V (c.1519A>G), F508C (c.1523T>G), 5T (c.1210-12[5]), and 9T (c.1210-12[9]) assays. (Note: since the writing of this manuscript, the Hologic Inplex CF assays have been recalled and discontinued.)

**Next-Generation Sequencing of CFTR**

**Ion AmpliSeq CFTR Panel on the Ion Torrent PGM.** Target regions of the CFTR gene were amplified in 2 amplicon pools that covered all exons, untranslated regions (UTRs), and regions of interest in introns 12 and 22 known to contain mutations 1811+1.6kbA>G (c.1679+1.6kbA>G) and 3849+10kbC>T (c.3717+12191C>T) using the Accel-Ampliplex CFTR Panel for the Illumina MiSeq Platform (Swift Biosciences, Ann Arbor, Michigan). The genomic DNA input was between 10 and 30 ng, and the libraries were prepared according to the manufacturer’s instructions. The libraries were diluted by 1:100 000 and quantified using KAPA Biosciences Library Quantification kit KK4835 (Wilmington, Massachusetts) according to the manufacturer’s recommendation on the QuantStudio 12K Flex (Thermo Fisher Scientific). Libraries were diluted to either 2 or 4 nmol/L and pooled together for denaturing and subsequent loading on to the MiSeq flow cell at a final concentration of 12 to 16 pmol/L. The samples were run on the MiSeqDx instrument research mode using either the MiSeq Reagent Kit v2 Standard (300 cycles) or MiSeq Reagent kit v2 Micro (300 cycles; Illumina).

As an open system, data from the Accel-Ampliplex CFTR Panel were processed using several freeware bioinformatic tools to create a custom analytical pipeline. The first step was to trim the 5’- and 3’-anchored primers using Cutadapt. The data were then aligned to the human genome reference sequence (hg19, build GRCh37) using Burrows-Wheeler Aligner BWA-MEM version 0.7.5a-r405, and variants were extracted using FreeBayes version v1.0.2 and GATK version 3.5 (3.5.0-g3628249) using a Browser Extensible Data (BED) file supplied by the manufacturer to limit the sequence area of interest. The data were also visually inspected using IGV. All analyzed data were then compared for concordance against the Sanger sequence data.

**Swift Biosciences Accel-Ampliplex CFTR Panel for Illumina Platforms.** Target regions of the CFTR gene were amplified in a single amplicon pool that covered all exons, UTRs, and regions of interest in introns 12 and 22 known to contain mutations 1811+1.6kbA>G (c.1679+1.6kbA>G) and 3849+10kbC>T (c.3717+12191C>T) using the Accel-Ampliplex CFTR Panel for the Illumina MiSeq Platform (Swift Biosciences, Ann Arbor, Michigan). The genomic DNA input was between 10 and 30 ng, and the libraries were prepared according to the manufacturer’s instructions. The libraries were diluted by 1:100 000 and quantified using KAPA Biosciences Library Quantification kit KK4835 (Wilmington, Massachusetts) according to the manufacturer’s recommendation on the QuantStudio 12K Flex (Thermo Fisher Scientific). Libraries were diluted to either 2 or 4 nmol/L and pooled together for denaturing and subsequent loading on to the MiSeq flow cell at a final concentration of 12 to 16 pmol/L. The samples were run on the MiSeqDx instrument research mode using either the MiSeq Reagent Kit v2 Standard (300 cycles) or MiSeq Reagent kit v2 Micro (300 cycles; Illumina).

As an open system, data from the Accel-Ampliplex CFTR Panel were processed using several freeware bioinformatic tools to create a custom analytical pipeline. The first step was to trim the 5’- and 3’-anchored primers using Cutadapt. The data were then aligned to the human genome reference sequence (hg19, build GRCh37) using Burrows-Wheeler Aligner BWA-MEM version 0.7.5a-r405, and variants were extracted using FreeBayes version v1.0.2 and GATK version 3.5 (3.5.0-g3628249) using a Browser Extensible Data (BED) file supplied by the manufacturer to limit the sequence area of interest. The data were also visually inspected using IGV. All analyzed data were then compared for concordance against the Sanger sequence data.

**Sanger Sequencing of CFTR**

Sanger sequencing was performed for all exons, intron/exon borders, and a region of interest in intron 22 known to contain mutation 3849+10kbC>T (c.3717+12191C>T). The CFTR gene was amplified using primer sets (RSS000010013) described in the National Center for Biotechnology Information’s Probe database. Each region was amplified from 5 to 10 ng of genomic DNA in a 10 μL reaction, using 10 pmol each of forward and reverse primers in the RSS000010013 primer sets, and 1× HotStarTaq Master Mix (Qiagen, Valencia, California). Cycling conditions were as follows: 10-minute denaturing step at 95°C; 40 cycles at 95°C for
Table 1. DNA Extraction Methods Used by the 2016 Quarter 1 CF DNA PT Participants.

| DNA Extraction Methods Used by CF DNA PT Participants | No. of Laboratories |
|-------------------------------------------------------|---------------------|
| Qiagen QIAamp spin columns (manual or robotic)         | 5                   |
| Qiagen magnetic bead kit (EZ1 or BioSprint 96)         | 2                   |
| Qiagen Generation DNA purification and DNA elution solutions | 22                  |
| Sigma Aldrich Extract-N-Amp                           | 3                   |
| In-house alkaline lysis prep                          | 7                   |
| In-house boiling prep                                 | 6                   |
| In-house lysis boiling prep                           | 1                   |
| Other                                                 | 11                  |
| No response                                           | 6                   |

Abbreviation: CF, cystic fibrosis.

Results

The CDC’s NSQAP sends DBS samples to participating laboratories engaged in CF newborn screening 4 times a year. Based on the information collected from the 63 laboratories that reported data for quarter 1 of 2016, the most commonly used method of DNA extractions was the Qiagen Generation DNA Purification and Elution Solution method (Table 1). This method is a relatively crude extraction that often results in a lower concentration of DNA. Thus, the Generation DNA extraction method was used in this study to validate the various genotyping and sequencing methods. The primary and secondary genotyping/sequencing methods reported by the laboratories for this quarter included 27 different methods that were either commercially available or laboratory developed. The number of mutations that each method detects is reported in Table 2 and ranged from 1 to 139 detected mutations for genotyping assays and 2 to an unlimited number of detected mutations for Sanger and next-generation sequencing methods.

DNA was extracted from a 3.2-mm punch taken from a DBS that contains approximately 3 μL of blood. Since newborn screening laboratories do not typically quantify DNA, a prescribed volume of extracted DNA was used in most of the assays rather than a set concentration or quantity. In order to better define the working range of DNA concentrations for the assays not commonly used in newborn screening laboratories, DNA extracts were quantified using real-time PCR, and the average quantity and range of concentrations are presented in Table 3.

All genotyping method results were compared with Sanger sequence data and found to have 100% concordance with the mutations included in their panels (Table 4 includes ACMG recommended mutations and variants, and Table 5 includes expanded panel mutations beyond the ACMG recommended). The CFTRdele2,3 (c.54-5940_273+10250del21kb) mutation detected by the xTAG CF60v2 kit was confirmed using the CF 139-variant assay kit because this mutation is not detectable using Sanger sequencing. The xTAG CF kits conditionally report the intron 9 poly T status (c.1210-12[5], c.1210-12[7], and c.1210-12[9]) when an R117H (c.350G>A) mutation is present, whereas these data can be seen for all samples using the InPlex 40+4 assay. Similarly, the F508C (c.1523T>G), 1506V (c.1516A>G), and 1507V (c.1519A>G) variants are assayed, but results are only displayed when the software designates a “Mut” call (indicating no normal sequence detected) for F508del (c.1521_1523delCTT) and/or I507del (c.1519_1521delATC) for the xTAG kits, but the F508C (c.1523T>G) is shown for all InPlex 40+4 samples. These variants when analyzed by the genotyping assays were 100% concordant with Sanger sequence data (Tables 4 and 5). For this study, only TaqMan Genotyping assays corresponding to the InPlex CF Molecular test 40+4 kit were assayed, however, additional CFTR mutation probe sets are available and can be used to create a more customized panel.

Three next-generation sequencing methods and 2 instrument platforms (MiSeqDx and Ion Torrent PGM) were used to characterize the CF DNA DBS repository. Both the MiSeqDx CFTR 139-variant genotyping assay and the AmpliSeq CFTR gene sequencing panel have a developed bioinformatics pipeline for analysis on their respective instruments, whereas the Accel-Amplicon CFTR panel, which is still in development, was analyzed using freeware bioinformatics tools.3-26 The results from all 3 next-generation sequencing methods were 100% concordant with Sanger sequence. Since Sanger sequencing cannot detect the CFTR dele2,3 mutation in the MiSeqDx CFTR 139-variant assay, this sample was compared with the xTAG CF60v2 and also found to be 100% concordant (Tables 4 and 5).

The MiSeqDx CFTR 139-variant next-generation sequencing assay which can accommodate up to 48 samples per flow cell had a density range between 473 ± 11 and 983 ± 9 K/mm², with an average quality score ≥Q30 of 88.6% for read 1 and 79.9% for read 2. The Ion AmpliSeq CFTR libraries were pooled and run on six 318 chips; five 318 chips were loaded with 20 samples, and one 318 chip was loaded with 32 samples. The average mapped reads for the 5 chips with 20 samples was 276 939, and 97.2% of reads were on target with a read depth of 2 476 and a uniformity of 88.1%. The 318 chip with 32 samples had 102 862 mapped reads and 94.8% reads on target with a read depth of 705 and uniformity of 88.3%. The Ion AmpliSeq CFTR Panel was designed to distinguish the intron 9 PolyT 5
| Genotyping/Sequencing Methods | Labs using Genotyping/Sequencing Method | Mutations/Variants Detected by Genotyping/Sequencing Methods | varients | Intron 9 (5/7/9T) |
|--------------------------------|----------------------------------------|-------------------------------------------------------------|--------|------------------|
|                                | # labs using as primary method | # labs using as secondary method | U.S. labs | non-U.S. labs | # mutations detected | # ACMG mutations | # expanded mutations | (F508C, IS06V, IS07V) |
| Hologic CF InPlex® Molecular Test—ACMG | 3 | 1 | 4 | 0 | 23 | 23 | 1<sup>a</sup> | F508C | 5/7/9T |
| Hologic CF InPlex® Molecular Test 40<sup>+</sup> | 20 | 6 | 19 | 1 | 40<sup>b</sup> | 23 | 19 | F508C | 5/7/9T |
| Lumine Molecular Diagnostics xTAG® CF—ACMG only | 0 | 0 | 0 | 0 | 23 | 23 | 0 | Yes | 5/7/9T |
| Lumine Molecular Diagnostics IVD xTAG® CF39 v2 | 7 | 4 | 5 | 2 | 39 | 23 | 16 | Yes | 5/7/9T |
| Lumine Molecular Diagnostics xTAG® CF60 v2 | 1 | 0 | 1 | 0 | 60 | 23 | 37 | Yes | 5/7/9T |
| Lumine Molecular Diagnostics xTAG® CF71 v2 | 0 | 0 | 0 | 0 | 71 | 23 | 48 | Yes | 5/7/9T |
| Lumine Platform and Laboratory Developed Test | 1 | 0 | 1 | 0 | 40 | 15 | 25 | No | No |
| Elucigen Diagnostics Elucigen® CF4v2 | 1 | 0 | 0 | 1 | 4 | 4 | 0 | No | No |
| Elucigen Diagnostics Elucigen® CF30v2 | 3 | 0 | 0 | 3 | 29 | 19 | 10 | No | No |
| Elucigen Diagnostics Elucigen® CF6U2v1 | 1 | 1 | 0 | 1 | 51 | 23 | 27 | No | 5/7/9T |
| Abbott Molecular CF Genotyping Assay v3 | 2 | 1 | 0 | 4 | 32 | 23 | 9 | Yes | 5/7/9T |
| Fujirebio INNO-LiPA® Strip 19 | 0 | 1 | 0 | 1 | 19 | 12 | 7 | No | No |
| Fujirebio INNO-LiPA® Strips 17-19 | 3 | 2 | 0 | 3 | 36 | 23 | 13 | No | 5/7/9T |
| Sequenom<sup>®</sup> assays other than HerediT™ CF | 1 | 1 | 0 | 3 | 12-42 | 11-21 | 1-21 | No | No |
| (MALDI-TOF Mass Spectrometry) | | | | | | | | | |
| ViennaLab Diagnostics GmbH CF StripAssay<sup>®</sup> | 1 | 0 | 0 | 1 | 34 | 23 | 11 | No | 5/7/9T |
| Allele-specific Oligonucleotide PCR | 2 | 0 | 1 | 1 | 1-9 | 1-9 | 0 | No | No |
| High Resolution Melt Technology | | 2 | 0 | 2 | 3-11 | 3-8 | 0-3 | Unknown | No |
| Real-time PCR Allelic Discrimination Assay (ie TaqMan<sup>®</sup>) | 2 | 0 | 2 | 0 | 1 | 1 | 0 | No | No |
| In-house Amplification Refractory Mutation System | I | 1 | 0 | 1 | 1 | 1 | 0 | No | No |
| In-house single nucleotide primer extension assay | | 1 | 0 | 0 | 1 | 12 | 10 | 1 | No | 5/7/9T |
| PCR/Heteroduplex Analysis/Gel Electrophoresis | 0 | 2 | 0 | 2 | 1 | 0 | 1 | No | No |
| Capillary Electrophoresis | 0 | 1 | 0 | 1 | 3 | 3 | 0 | No | No |
| Amplification and Restriction Fragment Length Polymorphism Analysis (PCR-RFLP) | 2 | 1 | 0 | 2 | 5-9 | 4-8 | 1-1 | No | No |
| Amplification and Polyacrylamide Gel Electrophoresis (PCR-PAGE) | | 2 | 0 | 0 | 1 | 1 | 1 | 0 | excludes variants | No |
| Next-generation sequencing—Ilumina MiSeq<sup>®</sup> Cystic Fibrosis 139 Variant Assay | 1 | 0 | 0 | 1 | 139 | 23 | 113 | Yes | 5/7/9T |
| Next-generation sequencing—Multiplicom Molecular Diagnostics CFTR MASTR<sup>®</sup> v2 | 2 | 0 | 0 | 1 | varies<sup>c</sup> | 20-23<sup>c</sup> | varies<sup>c</sup> | varies<sup>c</sup> | varies<sup>c</sup> | |
| Next-generation sequencing—Ion AmpliSeq<sup>®</sup> CFTR Community Panel | 0 | 1 | 0 | 1 | varies<sup>c</sup> | 23 | varies<sup>c</sup> | Yes | 5T |
| All other gene sequencing protocols including Sanger and Next Gen | 5 | 5 | 1 | 8 | varies<sup>c</sup> | 2-23 | varies<sup>c</sup> | Yes<sup>c</sup> | 5/7/9T<sup>d</sup> |
| Other—Hydrolysis probe | 1 | 0 | 0 | 1 | 4 | 4 | 0 | No | No |
| Other—LiGHT SNP | 0 | 1 | 0 | 1 | 7 | 7 | 0 | No | No |

<sup>a</sup> The 2183AA>G mutation is used for the interpretation of the 2184delA mutation and is not reported.

<sup>b</sup> Note that the InPlex 40<sup>+</sup> contains two non CF causing variants, I148T (c.443T>C) and D1270N (c.3808G>A) are not counted in these numbers.

<sup>c</sup> Varies by the sequencing technology used and/or whether filters are applied to mask certain results.

<sup>d</sup> Intron 9 - 5/7/9T detectable by Sanger if included in assay.
Expanding the CFTR2 panel of mutations to 276 gene CF was used to make variant calls because they for read 1 and 84.3% of reads on target with a Q30 for mutations, with read quality of 10 K/mm. With an increasingly diverse ethnic population occurring in the region when these mutations are present in the same sample. Examination of the sequence data did detect the presence of this mutation, and the correct genotype was called manually. In addition, an I507del/F508del (c.1519_1521delATC/c.1521_1523delGTT) sample required visual inspection for the final call for similar reasons. Since the results presented here are based on chemistry and algorithms from 2013, it is predicted that newer chemistries and algorithms may improve these calls.

The Accel-Amplicon CFTR panel libraries were run on the MiSeqDx in Research Run mode using 2 flow cells. A library of 94 samples was loaded on a micro flow cell and produced a density of 1299 ± 10 K/mm² with a read quality of ≥Q30 of 88.8% for read 1 and 84.3% for read 2. A second library of 74 samples was loaded on a standard flow cell and produced a density of 698 ± 10 K/mm² with read quality of ≥Q30 for 90.2% of read 1 and 80.4% for read 2. There was an average of 115 611 mapped reads with 87.2% of reads on target with a read depth of 1142 and 74.9% uniformity. Both FreeBayes and GATK was used to make variant calls because they produced different call frequencies. The output from both programs was used along with visualization of the data for analysis. As with the AmpliSeq assay, a sample containing I507del/F508del (c.1519_1521delATC/c.1521_1523delGTT) required visual inspection for the final call again due to the complexity in the region when these mutations are presented in the same sample.

In addition to the mutations listed in Tables 4 and 5, 8 additional mutations in our CF DNA DBS repository that cannot be detected by any of the IVD genotyping assay were observed. These mutations can only be detected by Sanger and some of the next-generation sequencing methods. They include the following mutations: 124del123bp (c.-9_14del123), 185+4A>T (c.53+4A>T), F311del (c.933_935delCTT), 1288insTA (c.1153_1154dupTA), 2105-2117del13insAGAAA (c.1973_1985del13insAGAAA), L967S (c.2900C>G), M1101R (c.3302T>G), and S1235R (c.3705T>G). There was 100% concordance between all methods where the mutation was run, however, more complex mutations such as the 2105-2117del13insAGAAA (c.1973_1985del13insAGAAA) had to be visually inspected and manually called for both the AmpliSeq and Accel-Amplicon assays, and the 124del123bp (c.-9_14del123) was analyzed with no primer trimming for Accel-Amplicon (Note: only the normal sequence of this mutation was sequenced using the AmpliSeq assay in this study).

A summary of DNA quantity inputs, single-run capacity and assay time requirements, data analysis software, and mutation reports for each method is presented in Table 3. DNA quantity inputs are not reported for the xTAG CF and InPlex CF kits because DNA is not typically quantified prior to use in newborn screening laboratories, rather each run uses 5 μL of a 50-μL Generation DNA extraction from a 3.2-mm DBS punch. Library prep time, which is only reported for next-generation sequencing assays, includes PCR setup and run time, whereas PCR setup and run time is only reported for genotyping assays. Analysis time for Sanger sequencing and next-generation sequencing is not included in this table because it may vary depending on the software and pipeline utilized. The OpenArray mentioned with TaqMan Genotyping was not used in this study, however, it is included in the table as it is an available option. All methods except Sanger sequencing were executed using DNA extracted from a 3.2-mm punch taken from a DBS using the Generation DNA extraction method.

**Discussion**

All US states and many international laboratories screen their newborn population for CF with the majority using second-tier CFTR mutation detection assay as part of their screening algorithm. Although these programs have been very effective, CF newborn screening has a low-positive predictive value with >90% false-positives. The reason for the low-positive predictive value is that most programs use a panel of only 23 to 40 CF-causing mutations, and a screen-positive sample only has to contain 1 CFTR mutation. As CF is a recessive disease, carriers of a single CFTR mutation are initially flagged as false-positives. In order to increase the positive predictive value of the CF screen to reduce the burden on the CF care centers, some newborn screening programs are exploring more comprehensive mutation detection assays. The goal of a screen positive being defined as babies with elevated IRT and 2 CF-causing mutations. A 2-mutation detection strategy could increase the false-negative rate if the panel of CFTR mutations was not sufficiently large enough to address the spectrum of mutations across diverse ethnic populations. Using variants identified by CFTR2 project, a study by Baker et al found that a panel of 162 mutations was not sufficiently comprehensive to capture all babies identified by the current algorithm in Wisconsin. Expanding the CFTR2 panel of mutations to 276 mutations and variants, 2 babies with known mutations in the Wisconsin study would still have been reported as false negatives. With an increasingly diverse ethnic population occurring in the most US state populations, it is expected that a predefined expanded panel of mutations approach will likely be insufficient to define a screen positive as 2 CFTR mutations, resulting in the continued high false-positive rates.

To address the limitations of genotyping panels, gene sequencing methods enable every base within the CFTR gene to be screened. Currently, there are 2 distinct sequencing methodologies used. The older, more established method uses Sanger sequencing and is currently being used for newborn screening in the US state of California. Next-generation sequencing is the second and more recent technology that enables more comprehensive and higher throughput screening of CF samples.
Table 3. Comparison of Method Input DNA Required, Mutations Detected, Number of Reactions, Run Time, and Run Capacity.¹

| Method | Variants Detected | No. Reactions | DNA Volume Used in Study² | Average DNA Qty Used Per Reaction (Min-Max)³ | Library Prep Time | PCR Setup and Run Time | Post-PCR Processing/Run Time | Instrument Run Time | Total Run Time | Single-Run Capacity⁴ | System Dedicated Software | Mutation Report |
|--------|-------------------|---------------|---------------------------|---------------------------------------------|------------------|------------------------|--------------------------|-------------------|---------------|------------------|------------------------|----------------|
| Sanger | Unlimited in regions amplified | 42 PCR rxns/86 cycle sequencing | 42 µL (1 µL/PCR rxn) | 5-10 ng | NA | 3 hours | 4 hours | 14.5 hours | 21.5 hours | Nine 384-well plates (+2,96-well plates with varying run conditions) | Y Seqscape | Does not distinguish variants from defined mutations—requires expert interpretation |
| Ion AmpliSeq CFTR Community Panel | Unlimited in regions amplified | 2 Pools | 12 µL (6 µL/pool) | 18.2 ng (0.76-8.98 ng/µL) | 4.6 hours | NA | 6-7 hours | 2-7 hours (dependent on chip size) | 12-20 hours | Eight 314 chip (500×) Forty-nine 316 chip (500×) Ninety-six 318 chip (500×) | Y Coverage Analysis Variant Caller | CFTR2 defined mutations and variants listed in Hotspot file are annotated (Legacy and HGVS)—novel mutations and variants require expert interpretation |
| Swift Biosciences Accel-Amplicon CFTR Panel | Unlimited in regions amplified | 1 Pool | 5-10 µL | 19.3 ng (0.55-5.21 ng/µL) | 2.5 hours | NA | 1.5 hours | 28 hours | 32 hours | Forty-eight Nano flow cell (400×) Forty-eight Micro flow cell (1600×) | Y² Free ware: Cutadapt, BWA-MEM, FreeBayes, GATK | Does not distinguish variants from defined mutations—requires expert interpretation |
| MiSeqDx Cystic Fibrosis 139-Variant Assay | 139 | 1 Pool | 5 µL | 8.95 ng (0.55-3.88 ng/µL) | 5 hours | NA | 3 hours | 28 hours | 36 hours | Forty-eight flow cells | Y MiSeq Reporter | Defined mutations identified |
| Hologic InPlex CF Molecular Test 40:1-4 | 42-2 variants | 1 rxn | 5 µL | Not quantified | NA | 2.5 hours | 1 hour | 5 minutes | 3.5 hours | 8 Cards | Y Call Reporting Software | Defined mutations identified |
| Lumine xTAG CF39v2 kit | 39-4 variants | 1 rxn | 5 µL | Not quantified | NA | 2 hours | 3.5 hours | 1 hour | 6.5 hours | Ninety-six 96-well plates Forty-eight 96-well plates | Y TDAS CFTR | Defined mutations identified |
| Lumine xTAG CF60v2 kit | 60-4 variants | 1 PCR rxns/2 ASPE rxns | 5 µL | Not quantified | NA | 2 hours | 4 hours | 1 hour | 7 hours | Forty-eight 96-well plates | Y TDAS CFTR | Defined mutations identified |
| Thermo Fisher Scientific TaqMan SNP Genotyping | 44 | 45 rxns | 45 µL² (1 µL/mutation) | 1.59 ng (0.62-4.82 ng/µL) | NA | 15 minutes - 96-well plate 30 minutes - 384-well plate 30 minutes - OpenArray plate | NA | ~2 hours - 96-well plate ~2 hours - 384-well plate ~4 hours - OpenArray plate | Two 96-well plates Eight 384-well plate Forty-eight OpenArray plates (64 assay format) | Y Geno typer | Defined mutations identified—some mutations require interpretation using multiple probes (intron 9.5/7/9T and 1507del and F508del region) |

Abbreviations: ASPE, allele specific primer extension; CF, cystic fibrosis; CFTR, CF transmembrane regulator; DBS, dried blood specimen; HGVS, human genome variation society; NA, not available; NBS, newborn screening; PCR, polymerase chain reaction; rxn, reaction; SNP, single-nucleotide polymorphism.

¹The run times in this table are based on our laboratory’s experience, however it does not include analysis of the data. The Bioinformatics are described in the materials and methods.

²Typically DNA is not quantitated prior to use in NBS labs, so some of the quantities are estimated based on average known concentration of DNA extracted from adult DBS.

³The single-run capacity indicates the number of samples that can be loaded onto an instrument at a time.

⁴The Accel-Amplicon CFTR panel is still in development, the company plans on offering a Bioinformatic pipeline (T. Harkins, personal communication, March 29, 2016).

⁵OpenArray was not performed in this study. The manufacturer recommended input is 2.5 µL of DNA extracted from DBS.
Although these approaches solve the issue of being able to identify uncommon mutations particularly in minority populations,\(^ {34}\) it creates a new issue, which is the identification of babies who do not have CF but rather CRMS.\(^ {35}\) The Cystic Fibrosis Foundation describes CRMS as infants with hypertrypsinogenemia on newborn screening who have sweat chloride values <60 mmol/L and up to 2 CFTR mutations, at least 1 of which is not clearly categorized as CF causing.

This study demonstrates that NSQAP's CF DNA DBS repository is appropriate for use with CF screening assays as they are performed today both in the United States and internationally. These repository samples would also support next-generation sequencing assays for CFTR if programs choose to modify their screening algorithms to require 2 CFTR mutations. The input DNA is a critical component to all molecular tests and the DNA extraction methods reported from NSQAP participants range from a very crude methanol boil preparation to a more purified extraction involving column purification. The majority of US programs use a commercially available simple purification that involves wash steps followed by a boil step (Table 1). In addition, the CF DNA DBS repository samples used in this study have a lower DNA yield than newborn DBS because they were made from adult blood that has a lower average white blood cell count than newborns (7.4\(^ {10}\) 6 per mL of blood vs 1.9\(^ {10}\) 7 per mL of blood, respectively).\(^ {37}\)

### Table 4. ACMG Recommended Mutations found in the CF DNA DBS Repository Samples as Characterized by Next-Generation Sequencing and Mutation Analysis.

| Mutation (Legacy Name) | Mutation (HGVS) | AmpliSeq CFTR Community Panel | Sanger | Accel-Amplicon CFTR Panel | CF 139- Variant Assay | InPlex CF 40+4 | xTAG CF39v2 kit | xTAG CF60v2 kit | TaqMan SNP Genotyping |
|------------------------|-----------------|-------------------------------|--------|--------------------------|----------------------|---------------|----------------|----------------|----------------------|
| F508del                | c.1521_1523delCTT | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| I507del                | c.1519_1521delATC | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| G542X                  | c.1624G>T        | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| G8SE                   | c.254G>A         | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| R117H                  | c.350G>A         | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| 621+1G>T               | c.489+1G>T       | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| 711+1G>T               | c.579+1G>T       | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| R334W                  | c.1000C>T        | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| R347P                  | c.1040G>C        | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| A455E                  | c.1364C>A        | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| 1717-1G>A              | c.1585+1G>A      | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| R560T                  | c.1679G>C        | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| R553X                  | c.1657C>T        | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| G551D                  | c.1652G>A        | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| 1898+1G>A              | c.1766+1G>A      | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| 2184delIA              | c.2052delIA      | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| 2789+5G>A              | c.2657+5G>A      | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| 3120+1G>A              | c.2988+1G>A      | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| R1162X                 | c.3484C>T        | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| 3459delC               | c.3528delC       | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| 3849+10kbC>T           | c.3717+12191C>T  | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| W1282X                 | c.3846G>A        | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| N1303K                 | c.3909C>G        | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| F508C                  | c.1523T>G        | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| T5                     | c.1210-12[5]     | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| T7                     | c.1210-12[7]     | +                             | +      | +                        | +                    | +             | +              | +              | +                    |
| T9                     | c.1210-12[9]     | +                             | +      | +                        | +                    | +             | +              | +              | +                    |

Abbreviations: ACMG, American College of Medical Genetics; CF, cystic fibrosis; CFTR, CF transmembrane regulator; CR, conditionally reported with an R117H present; ND, not distinguishable; nr, not reported but used for correct mutation interpretation; SNP, single-nucleotide polymorphism; +, mutations detected; ++, not assayed in this study but detectable by method.

\(^{a}\)Boldface entries indicates ACMG mutations.
this study. The next-generation sequencing approaches used in this study were selected to test the utility and robustness of crude low-concentration DNA with different next-generation sequencing platforms and library preparation assays. Although the US Food and Drug Administration (FDA)-approved CF 139-Variant Assay uses next-generation sequencing technology, it reports a genotype for a defined panel of mutations and variants. The mutation panel is not diverse enough to define a positive newborn screen positive as having 2 CFTR mutations; Illumina does offer an FDA-approved CFTR gene sequencing assay that was not tested in this study. Given the similar technology and workflow, it is anticipated that this method would also work well with DNA extracted from DBS. The CFTR gene next-generation sequencing assays tested in this study were the AmpliSeq CFTR Community Panel on the Ion Torrent PGM and the Accel-Amplicon CFTR Panel on the MiSeq. The Accel-Amplicon CFTR Panel can also be made for use with the Ion Torrent PGM.

### Table 5. Mutations Excluding ACMG Recommended Mutations Found in the CF DNA DBS Repository Samples as Characterized by Next-Generation Sequencing and Mutation Analysis.

| Mutation (Legacy Name) | Mutation (HGVS) | Sanger | AmpliSeq CFTR Community Panel | Accel-Amplicon CFTR Panel | CF 139-Variant Assay | InPlex CF 40þ4 xTAG CF39v2 kit | xTAG CF60v2 kit | TaqMan SNP Genotyping |
|------------------------|-----------------|--------|-------------------------------|---------------------------|----------------------|-----------------------------|----------------|----------------------|
| 394delTT               | c.262_263delTT   | +      | +                             |                           | +                    | +                          | +             | +                   |
| A559T                  | c.1675G>A        | +      | +                             |                           | NA                   | NA                         | +             | AV                  |
| S549N                  | c.1646G>A        | +      | +                             |                           | NA                   | NA                         | +             | +                   |
| 2181AA>G               | c.205_2052delAinsG | +    | +                             |                           | +                    | +                          | +             | +                   |
| 2307insA               | c.2175_2176insA  | +      | +                             |                           | +                    | +                          | +             | +                   |
| Y1092X                 | c.3276C>A or c.3276C>G | +    | +                             |                           | +                    | +                          | +             | +                   |
| 387delA                | c.3744delA       | +      | +                             |                           | +                    | +                          | +             | +                   |
| 390insT                | c.3773dupT       | +      | +                             |                           | +                    | +                          | +             | +                   |
| CFTR del2,3            | 5940–273+10250del21kb |     |                               |                           |                      |                            |               |         |
| E60X                   | c.178G>T         | +      | +                             |                           | +                    | NA                         | +             | +                   |
| R75X                   | c.223C>T         | +      | +                             |                           | NA                   | NA                         | +             | AV                  |
| 406-1G>A               | c.274-1G>A       | +      | +                             |                           | +                    | +                          | +             | +                   |
| L206W                  | c.617T>G         | +      | +                             |                           | +                    | +                          | +             | +                   |
| 935delA                | c.803delA        | +      | +                             |                           | NA                   | NA                         | +             | +                   |
| Q493X                  | c.1477C>T        | +      | +                             |                           | +                    | +                          | +             | 1                   |
| Q890X                  | c.2666C>T        | +      | +                             |                           | +                    | +                          | +             | +                   |
| 1677delTA              | c.1545A_1546delTA | +    | +                             |                           | +                    | +                          | +             | +                   |
| 2055del9A              | c.1932_1931del9insA | +    | +                             |                           | NA                   | NA                         | +             | +                   |
| R1158X                 | c.3472C>T        | +      | +                             |                           | +                    | +                          | +             | +                   |
| R1066C                 | c.3196C>T        | +      | +                             |                           | +                    | +                          | +             | +                   |
| W1089X                 | c.3266G>A        | +      | +                             |                           | +                    | +                          | +             | +                   |
| D1152H                 | c.3454G>C        | +      | +                             |                           | NA                   | NA                         | +             | +                   |
| 3791delC               | c.3659delC       | +      | +                             |                           | +                    | +                          | +             | +                   |
| D1270N                 | c.3808G>A        | +      | +                             |                           | NA                   | +                          | +             | +                   |
| Q39X                   | c.1115C>T        | +      | +                             |                           | +                    | +                          | +             | +                   |
| 663delT                | c.531delT        | +      | +                             |                           | +                    | +                          | +             | +                   |
| P205S                  | c.613C>T         | +      | +                             |                           | +                    | +                          | +             | +                   |
| 1154 insTC             | c.1022_1023insTC | +    | +                             |                           | +                    | +                          | +             | +                   |
| 1248_1G>A              | c.1116_1G>A      | +      | +                             |                           | +                    | +                          | +             | +                   |
| L467P                  | c.1400T>C        | +      | +                             |                           | +                    | +                          | +             | +                   |
| S492F                  | c.1475C>T        | +      | +                             |                           | +                    | +                          | +             | +                   |
| I812-1G>A              | c.1680-1G>A      | +      | +                             |                           | +                    | +                          | +             | +                   |
| 2184insA               | c.2052dupA       | +      | +                             |                           | +                    | +                          | +             | +                   |
| 3121-1G>A              | c.2989-1G>A      | +      | +                             |                           | +                    | +                          | +             | +                   |
| 3272-26A>G             | c.3140-26A>G     | +      | +                             |                           | +                    | +                          | +             | +                   |
| R1066H                 | c.3197G>A        | +      | +                             |                           | +                    | +                          | +             | +                   |
| W1204X                 | c.3611G>A or c.3612G>A | +    | +                             |                           | +                    | +                          | +             | +                   |
| G1244E                 | c.3731G>A        | +      | +                             |                           | +                    | +                          | +             | +                   |

Abbreviations: AV, assay available but not evaluated in this study; CF, cystic fibrosis; CFTR, CF transmembrane regulator; NA, not available in assay; SNP, single-nucleotide polymorphism; +, mutations detected; +*, specimen containing mutation not assayed but normal region sequenced in this study.
The general trend of increasing population diversity, new technology introductions allowing for expanded mutation screening or gene sequencing, and the varying sizes of newborn screening programs are just 3 of many factors contributing to the complexities of newborn screening for CF. In addition, the current newborn screening algorithms have a high false-positive rate, prompting some programs to consider whether the definition of a CF screen positive should be redefined. The NSQAP offers quality assurance tools and services to newborn screening laboratories as they explore transitioning from one technology to another to meet their changing needs. The CF DNA DBS repository provides laboratories with representative samples with rare CFTR mutations for robust testing and evaluation purposes. The study presented here is a comprehensive characterization of these DBS samples and highlights their utility for a diverse range of methods being used in CF newborn screening today as well as next-generation sequencing assays that can be used in the future.

Acknowledgments
The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention. Use of trade names and commercial sources is for identification only and does not constitute endorsement by the US Department of Health and Human Services or US Centers for Disease Control and Prevention. Special thanks to manufacturer collaborators Clarence Lee, PhD and Warren Tom from Life Technologies (now Thermo Fisher Scientific); Tim Harkins, PhD and Julie Laliberte, PhD, from Swift Biosciences; and Junko Stevens, PhD, Jordan Lang, and Lauren Tracey from Thermo Fisher Scientific. We also thank our partners who have helped collect patient samples for the CF DNA DBS repository which includes Dr Phillip M. Farrell from the University of Wisconsin School of Medicine and Public Health, Dr Peter Mogayzel from the CF Center at Johns Hopkins Medical Institutions, Dr Michael Konstan from Rainbow Babies and Children’s Hospital and Case Western Reserve University School of Medicine, Martin Kharrazi, PhD from the California Department of Public Health, and Charlene Sacramento from Sequoia Foundation. In addition, we thank the patients and families who generously donated blood. Without them, this program would not be possible.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Stephanie Foster was funded by the Research Participation Program at the Centers for Disease Control and Prevention (CDC), National Center for Environmental Health’s Division of Laboratory Sciences, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and CDC.

References
1. Grosse SD, Boyle CA, Botkin JR, et al. Newborn screening for cystic fibrosis: evaluation of benefits and risks and recommendations for state newborn screening programs. MMWR Recomm Rep. 2004;53(RR-13):1-36.
2. Dijk FN, Fitzgerald DA. The impact of newborn screening and earlier intervention on the clinical course of cystic fibrosis. Pediatr Respir Rev. 2012;13(4):220-225.
3. Crossley JR, Elliott RB, Smith PA. Dried-blood-spot screening for cystic fibrosis in the newborn. Lancet. 1979;1(8114):472-474.
4. Hammond KB, Abman SH, Sokol RJ, Accurso FJ. Efficacy of statewide neonatal screening for cystic fibrosis by assay of trypsinogen concentrations. N Engl J Med. 1991;325(11):769-774.
5. Riordan JR, Rommens JM, Kerem B, et al. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science. 1989;245(4922):1066-1073.
6. Welsh MJ, Ramsey BW, Accurso FJ, Cutting GR. Cystic Fibrosis. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The Metabolic and Molecular Bases of Inherited Disease. Vol 3. 8th ed. New York, NY: McGraw-Hill; 2001:5121-5188.
7. Comeau AM, Parad RB, Dorkin HL, et al. Population-based newborn screening for genetic disorders when multiple mutation DNA testing is incorporated: a cystic fibrosis newborn screening model demonstrating increased sensitivity but more carrier detections. Pediatrics. 2004;113(6):1573-1581.
8. Gregg RG, Wilford BS, Farrell PM, Laksova A, Hasseder M, Mischler EH. Application of DNA analysis in a population-screening program for neonatal diagnosis of cystic fibrosis (CF): comparison of screening protocols. Am J Hum Genet. 1993;52(3):616-626.
9. Ranieri E, Lewis BD, Gerace RL, et al. Neonatal screening for cystic fibrosis using immunoreactive trypsinogen and direct gene analysis: four years’ experience. BMJ. 1994;308(6942):1469-1472.
10. Grody WW, Cutting GR, Klinger KW, Richards CS, Watson MS, Desnick RJ. Laboratory standards and guidelines for population-based cystic fibrosis carrier screening. Genet Med. 2001;3(2):149-154.
11. Baker MW, Grosse M, Hoffman G, Rock M, Levy H, Farrell PM. Optimal DNA tier for the IRT/DNA algorithm determined by CFTR mutation results over 14 years of newborn screening. J Cyst Fibros. 2011;10(4):278-281.
12. Kay DM, Maloney B, Hamel R, et al. Screening for cystic fibrosis in New York State: considerations for algorithm improvements. Eur J Pediatr. 2016;175(2):181-193.
13. Kharrazi M, Yang J, Bishop T, et al. Newborn screening for cystic fibrosis in California. Pediatrics. 2015;136(6):1062-1072.
14. Tluczek A, Koscik RL, Farrell PM, Rock MJ. Psychosocial risk associated with newborn screening for cystic fibrosis: parents’ experience while awaiting the sweat-test appointment. Pediatrics. 2005;115(6):1692-1702.
15. La Pean A, Farrell MH, Eskra KL, Farrell PM. Effects of immediate telephone follow-up with providers on sweat chloride test timing after cystic fibrosis newborn screening identifies a single mutation. J Pediatr. 2013;162(3):522-529.
16. Castellani C, Picci L, Scarpa M, et al. Cystic fibrosis carriers have higher neonatal immunoreactive trypsinogen values than non-carriers. Am J Med Genet A. 2005;135(2):142-144.
17. Wells J, Rosenberg M, Hoffman G, Anstead M, Farrell PM. A decision-tree approach to cost comparison of newborn screening strategies for cystic fibrosis. *Pediatrics*. 2012;129(2):e339-e347.

18. Baker MW, Atkins AE, Cordovado SK, Hendrix M, Earley MC, Farrell PM. Improving newborn screening for cystic fibrosis using next-generation sequencing technology: a technical feasibility study. *Genet Med.* 2016;18(3):231-238.

19. Pratt VM, Caggana M, Bridges C, et al. Development of genomic reference materials for cystic fibrosis genetic testing. *J Mol Diagn.* 2009;11(3):186-193.

20. Tsui L. Cystic Fibrosis Mutation Database. 2010; Web site. http://genet.sickkids.on.ca/. Updated April 25, 2011. Accessed January 26, 2016.

21. Li L, Zhou Y, Bell CJ, Earley MC, Hannon WH, Mei JV. Development and characterization of dried blood spot materials for the measurement of immunoreactive trypsinogen. *J Med Screen.* 2006;13(2):79-84.

22. Earley MC, Laxova A, Farrell PM, et al. Implementation of the first worldwide quality assurance program for cystic fibrosis multiple mutation detection in population-based screening. *Clin Chim Acta.* 2011;412(15-16):1376-1381.

23. Newborn Screening Quality Assurance Program (NSQAP) Cystic Fibrosis Mutation Detection. Web site. https://www.cdc.gov/labstandards/pdf/nsqap/nsqap_CysticFibrosisMDFeb2016.pdf. Published February 1, 2016.

24. Robinson JT, Thorvaldsdottir H, Winckler W, et al. Integrative genomics viewer. *Nat Biotechnol.* 2011;29(1):24-26.

25. Thorvaldsdottir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief Bioinform.* 2013;14(2):178-192.

26. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. journal.* 2011;17(1):10-12.

27. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics.* 2009;25(14):1754-1760.

28. Garrison E, Marth G. Haplotype-based variant detection from short-read sequencing[published online July 24, 2012]. *Genomics.* 2012.

29. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* 2010;20(9):1297-1303.

30. Cordovado SK, Hendrix M, Greene CN, et al. CFTR mutation analysis and haplotype associations in CF patients. *Mol Genet Metab.* 2012;105(2):249-254.

31. (CLSI) CaLSI. *Newborn Screening for Cystic Fibrosis: Approved Guideline.* Wayne, PA: Clinical and Laboratory Standards Institute; 2011.

32. Sosnay PR, Siklosi KR, Van Goor F, et al. Defining the disease liability of variants in the cystic fibrosis transmembrane conductance regulator gene. *Nat Genet.* 2013;45(10):1160-1167.

33. Clinical and Functional Translation of CFTR (CFTR2). Web site. http://www.cftr2.org/. Accessed February 9, 2016.

34. Kammesheidt A, Kharrazi M, Graham S, et al. Comprehensive genetic analysis of the cystic fibrosis transmembrane conductance regulator from dried blood specimens—implications for newborn screening. *Genet Med.* 2006;8(9):557-562.

35. Prach L, Koepeke R, Kharrazi M, et al. Novel CFTR variants identified during the first 3 years of cystic fibrosis newborn screening in California. *J Mol Diagn.* 2013;15(5):710-722.

36. Cystic Fibrosis Foundation practice guidelines for the management of infants with cystic fibrosis transmembrane conductance regulator-related metabolic syndrome during the first two years of life and beyond. *J Pediatr.* 2009;155(6 suppl): S106-S116.

37. Engorn B, Flerlage J, eds. *The Harriet Lane Handbook: A Manual For Pediatric House Officers.* 20th ed. Philadelphia: Elsevier Saunders; 2015.

38. Hughes EE, Stevens CF, Saavedra-Matiz CA, et al. Clinical sensitivity of cystic fibrosis mutation panels in a diverse population. *Hum Mutat.* 2016;37(2):201-208.

39. Loukas YL, Thodi G, Molou E, Georgiou V, Dotsikas Y, Schulpis KH. Clinical diagnostic Next-Generation sequencing: the case of CFTR carrier screening. *Scand J Clin Lab Invest.* 2015;75(5):374-381.

40. Letterova MI, Shen P, Odegard JI, et al. Next-generation molecular testing of newborn dried blood spots for cystic fibrosis. *J Mol Diagn.* 2016;18(2):267-282.