

Clinical Sensitivity of Cystic Fibrosis Mutation Panels in a Diverse Population

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ABSTRACT: Infants are screened for cystic fibrosis (CF) in New York State (NYS) using an IRT-DNA algorithm. The purpose of this study was to validate and assess clinical validity of the US FDA-cleared Illumina MiSeqDx CF 139-Variant Assay (139-VA) in the diverse NYS CF population. The study included 439 infants with CF identified via newborn screening (NBS) from 2002 to 2012. All had been screened using the Abbott Molecular CF Genotyping Assay or the Hologic InPlex CF Molecular Test. All with CF and zero or one mutation were tested using the 139-VA. DNA extracted from dried blood spots was reliably and accurately genotyped using the 139-VA. Sixty-three additional mutations were identified. Clinical sensitivity of three panels ranged from 76.2% (23 mutations recommended for screening by ACMG/ACOG) to 79.7% (current NYS 39-mutation InPlex panel), up to 86.0% for the 139-VA. For all, sensitivity was highest in Whites and lowest in the Black population. Although the sample size was small, there was a nearly 20% increase in sensitivity for the Black CF population using the 139-VA (68.2%) over the ACMG/ACOG and InPlex panels (both 50.0%). Overall, the 139-VA is more sensitive than other commercially available panels, and could be considered for NBS, clinical, or research laboratories conducting CF screening.

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KEY WORDS: cystic fibrosis; CFTR; newborn screening; mutation panel; clinical sensitivity; next-generation sequencing; NGS

Introduction

Cystic fibrosis (CF; MIM #219700) is a chronic, multisystem disease affecting epithelia of the respiratory tract, exocrine pancreas, intestine, hepatobiliary system, exocrine sweat glands, and male genital tract. CF results from mutations in the CF transmembrane conductance regulator gene, *CFTR* (MIM #602421), which affects chloride transport and flow of water into and out of cells [Riordan et al., 1989; Rommens et al., 1989]. Thick, sticky mucus accumulates and clogs organs, leading to, among other chronic problems, persistent respiratory infections and pancreatic obstruction, hindering pancreatic enzymes from breaking down food and absorbing nutrients. CF is one of the most common autosomal-recessive genetic diseases in North America, affecting an estimated 30,000 children and adults in the US, with an overall incidence of one in every few thousand births.

CF has been screened by the New York State (NYS) newborn screening (NBS) program since October 2002. Since 2009, all state NBS programs in the US include CF in their panels. Early diagnosis allows careful monitoring and early preventative care that is primarily aimed at preventing infection, ensuring proper nutrition, and improving quality of life [Farrell et al., 2001]. The current NYS algorithm for CF NBS includes a first-tier screen for serum immunoreactive trypsinogen (IRT) in newborn dried blood spot (DBS) specimens, followed by molecular analysis using a 39-mutation panel in infants with elevated IRT (top 5%). NBS programs in states using IRT-DNA algorithms determine the number and type of mutations included on the state panel, which can be dependent on state population demographics, commercial availability, and cost. Some programs screen only for p.F508del (delF508), the most

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common *CFTR* mutation worldwide; others target the 23 mutations currently recommended for screening by the American College of Medical Genetics and Genomics (ACMG) [Watson et al., 2004] and the American Congress of Obstetricians and Gynecologists (ACOG) [ACOG, 2011]; many include up to 40 *CFTR* mutations; and at least one, California, includes complete *CFTR* sequencing in infants with high IRT and only one panel mutation [Prach et al., 2013; Grosse et al., 2004]. More than 2,000 *CFTR* variants have been documented in the “SickKids” Cystic Fibrosis Mutation Database (now referred to as CFTR1) (<http://www.genet.sickkids.on.ca>). Rare *CFTR* mutations are known to exist in the diverse NYS CF population [Kay et al., 2015]. In the absence of complete *CFTR* analysis, which is labor intensive and expensive, all possible relevant mutations cannot be tested. To maximize screening sensitivity, the NYS NBS program refers all infants with at least one panel mutation or an extremely elevated IRT (VHIRT; since 2010, highest 0.1%) in the absence of mutations to CF Specialty Care Centers (SCC) for confirmatory diagnostic testing. Because IRT specificity for CF is low, many healthy carriers of single *CFTR* mutations and healthy infants with VHIRT are referred each year [Kay et al., 2015].

In November 2013, Illumina received 510K clearance from the US Food and Drug Administration (FDA) for its MiSeqDx next-generation sequencing (NGS) system, which includes a panel targeting 139 *CFTR* variants. The MiSeqDx Cystic Fibrosis 139-Variant Assay (henceforth 139-VA) is the most comprehensive panel of validated *CFTR* variants commercially available, and includes the 23 mutations currently recommended by the ACMG/ACOG [Watson et al., 2004], and all variants classified as CF causing in the CFTR2 database (<http://www.cftr2.org>) as of August 2013.

The goal of this study was to assess the 139-VA, with respect to feasibility and clinical validity. We also sought to determine whether the 139-VA could be integrated into the NYS NBS algorithm for CF, either replacing the existing 39-mutation panel, or as a third-tier in which infants with high IRT and less than two mutations on the InPlex panel could be reflex tested using the 139-VA.

Materials and Methods

Procedures for CF screening in NYS using an IRT-DNA algorithm have been described [Kay et al., 2015]. Briefly, the ImmuChem Trypsin-MW enzyme-linked immunosorbent assay testing kit (MP Biomedicals, Santa Ana, California) was used to identify infants with elevated IRT (top 5%). From 2002 to 2007, the Abbott/Celera Molecular Cystic Fibrosis Genotyping Assay (henceforth Abbott panel) was used to screen specimens with the top 5% IRT for 31 mutations. In May 2007, the 39-mutation Hologic InPlex CF Molecular Test (henceforth, InPlex panel) replaced the Abbott panel. The p.F508C and p.D1270N variants included on the InPlex panel were blinded because they are not pathogenic mutations, and c.3067_3072delATAGTG/p.I1023_V1024del (3199del6) was reflex tested in p.I148T carriers. Infants with at least one panel mutation or with VHIRT (top 0.2% or top 0.1%) in the absence of mutations were referred to a NYS CF SCC for confirmatory diagnostic testing.

Subjects

This retrospective study included infants with CF who were born and screened in NYS between October 2002 and December 2012. Infants with *CFTR*-related metabolic syndrome (CRMS), a *CFTR*-related disorder, possible CF or who screened negative but were later found to have CF (false negatives) were excluded from this study. To ensure the most accurate and up-to-date diagnoses were used,

the NBS CF dataset was compared with NYS infants included in the national CF patient registry maintained by the Cystic Fibrosis Foundation (CFF) [MacKenzie et al., 2014]. The two datasets were matched using date of birth, gender, final diagnosis, sweat chloride test results, date of sweat test, specialty care center, and *CFTR* mutations. This study was approved by the NYS Department of Health Institutional Review Board (IRB).

Demographic and clinical data were obtained from NBS records and the CFF patient registry. Data were available from SCC chart reviews for a small proportion of cases. Race/ethnicity data from the CFF was used for all who could be matched to registry records; otherwise (i.e., for the unverified cases), race/ethnicity was obtained from the NBS Guthrie card, which can be subject to error [Kay et al., 2015]. The availability of sweat chloride data was typically limited to a single test result reported to the NBS program or to the CFF patient registry.

Physicians are mandated to report diagnoses for all screen positive infants to the program, but, as we have described previously [Kay et al., 2015], NBS is typically limited to short-term follow-up, in which the primary goal is to receive and document a diagnosis, thereby “closing the case.” Unless informed anecdotally, there is no protocol for active longer-term follow-up, to correct errors in collecting or recording data, to track diagnoses for infants lost to follow-up, or to obtain information on diagnosis changes for infants with previous inconclusive results. Therefore, in this study, data were analyzed using two diagnostic classification groups. Comparison of NBS to CFF records allowed a second measure of confidence in the diagnosis. The first group, henceforth referred to as “confirmed CF” cases included 392 infants with CF diagnoses reported to and documented in NBS records, and also documented in the CFF’s patient registry. This group included infants for whom there was additional documentation supporting a diagnosis of CF. The second group, henceforth referred to as “all CF,” included all 392 confirmed CF cases in the first group, plus 47 additional cases with “unverified CF.” The unverified CF case group comprised all diagnosed cases in the NBS dataset that could not be matched with certainty to the CFF registry database. Using the more stringent inclusion criteria ensured a CF diagnosis and therefore two mutations were expected in every case. The less stringent criteria allowed us to assess the mutation spectrum in the complete NYS dataset, which includes the less certain diagnoses, but also those who did not consent to be included in the registry. To avoid biasing data, once a subject was categorized as confirmed or unverified, this designation did not change, and the unverified case group therefore included some infants with two CF-causing mutations that later could have been considered verified diagnoses. Data were analyzed with and without the unverified cases. Mutation frequencies in the CFTR2 dataset, comprising more than 35,000 individuals with CF, were obtained from supplementary data files published by Sosnay et al. (2013).

In all, 164 infants with CF (140 confirmed and 24 unverified) were genotyped using the 139-VA (24 had zero, 136 had one, and four had two NYS panel mutations). These 164 infants included all 160 who were born in NYS between 2002 and 2012, screened positive, and were found to have CF, but did not carry two *CFTR* mutations on the NYS panel. Specimens from an additional 97 infants born between 2002 and 2012 who screened negative for CF plus 57 specimens used for control purposes were also genotyped using the 139-VA. The 57 control specimens included a subset of infants with CRMS, possible CF, and infants who had false negative CF screens due to low IRT. Data from 163/164 specimens (one failed genotyping) from infants with CF and 45/57 control specimens (those that had been genotyped using the NYS panel and the 139-VA) were used for cross-platform validation studies. For comparison, and to allow us

to assess prevalence in the full NYS CF population, 275 infants with two NYS panel mutations (252 confirmed and 23 unverified CF) were included in the sensitivity and race/ethnicity-specific analyses, but were not actually genotyped using the 139-VA.

Genotyping

Archived DBS were retrieved for all subjects, deidentified, and relabeled with a unique ID number. DNA was extracted from one 3-mm DBS punch using a laboratory-developed method routinely used by the NYS program [Saavedra-Matiz et al., 2013]. Genotyping was carried out by the Applied Genomics Technology Core (AGTC) at the Wadsworth Center. For each sample, 5 μ l DNA was genotyped using MiSeqDx Cystic Fibrosis 139-Variant Assay kits on the Illumina MiSeqDx system using manufacturer specifications outlined in the MiSeqDx Cystic Fibrosis 139-Variant Assay Reference Guide (Part #15038349 Rev. A). Use of nonpurified and nonquantitated DNA extracted from DBS was considered an off-label use of the system. DNA concentration was not estimated. Each run contained 48 samples, including a no template control and at least one positive control DBS of known genotype. Data were analyzed using Illumina's MiSeq Reporter v.2.2.3.1. Read depth was obtained from amplicon coverage files.

Mutation Validation

Variants detected using the 139-VA were Sanger sequenced for validation. Mutations also present on the NYS panel were not sequenced because they had already been genotyped by the NBS program using a clinically validated screening assay. PCR primers used to amplify the 27 *CFTR* exons, exon/intron boundaries, one deep intronic mutation, and promoter region are included as online Supporting Information (Supp. Table S1). For all reactions, the PCR protocol started with a 5-min denaturation at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at varying primer annealing temperatures (Supp. Table S1), 30 sec at 72°C, and a final extension for 5 min at 72°C. Reactions consisted of LightCycler DNA Master HybProbe mix (Roche Applied Science, Indianapolis, IN), MgCl₂ (Supp. Table S1), 0.066 μ M TaqStart Antibody (Clontech Laboratories, Mountain View, CA) when indicated, 0.2 μ M forward primer, 0.2 μ M reverse primer, 4 μ l extracted DNA (diluted 1:4 in water), and PCR-grade water to a final volume of 25 μ l. PCR reactions were purified by adding 2 μ l of ExoSAP-IT enzyme (USB Affymetrix, Santa Clara, CA) to 5 μ l PCR product. Samples were incubated at 37°C for 15 min, then 80°C for 15 min. Cycle sequencing was performed using 2 μ l of the ExoSAP-IT-purified PCR product, 0.5 μ l BigDye Terminator Ready Reaction Mix v.3.1 (Applied Biosystems, Foster City, CA), sequencing buffer, 0.18 μ M M13 forward or M13 reverse primer, and deionized water for a final volume of 20 μ l. Cycling parameters included denaturation at 95°C for 5 min, followed by 25 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. Samples were sequenced in both forward and reverse directions. Centri-Sep gel filtration plates (Princeton Separations, Adelphia, NJ) were used to remove unincorporated dNTPs and primers. Samples were sequenced on a 3130xl/Genetic Analyzer (Applied Biosystems) and sequences were analyzed using SeqScape v.2.6 and FinchTV using NCBI reference sequences NC_000007.13 and NM_000492.3. Variants are reported using current HGVS nomenclature. Nucleotide numbering is based on cDNA sequence and reported using the A of the ATG translation initiation codon as +1. If different from HGVS nomenclature, legacy nomenclature is also provided in parentheses.

Intron 8 poly T tract length was validated by comparison to NYS panel data. Intron 8 poly TG tract length was not validated because it is not tested in our laboratory or other screening laboratories in general, since it is not typically considered a mutation on its own. The CFTRdele2,3 mutation was validated using a previously described PCR-based assay (Supp. Table S1) [Dörk et al., 2000].

Results

Validation Study

In order to assess input DNA requirements, specimens from 14 CF cases were each genotyped on the 139-VA in triplicate using a total volume of 5 μ l extracted DNA (two replicates; $N = 28$ total tests) or 2.5 μ l DNA diluted 1:1 in water (one replicate; $N = 14$ tests), regardless of and without prior quantitation of the input DNA. The DNA extraction method used typically yields 100–600 ng DNA at a concentration of 1–6 ng/ μ l [Saavedra-Matiz et al., 2013]; therefore, we estimate 2.5–30 ng DNA was used for each assay. Despite using approximately 10-fold less DNA than recommended by the manufacturer (250 ng), this initial run was successful. Four DNA controls (25 or 50 ng high-quality commercial genomic DNA, each run in duplicate) and two water blanks passed with call rates (proportion of successfully genotyped variants on the panel) of 100% and 0%, respectively. Of samples with 5 μ l input DNA, 27/28 passed with 100% call rates and 1/28 failed (call rate = 97.78%; threshold for pass $\geq 99.0\%$). The heterozygous mutation known to be present in this sample was called correctly, and its corresponding duplicate passed with a call rate of 100%. Among samples run with 2.5 μ l input DNA, 9/14 passed with 100% call rates, 1/14 passed with a 99.26% call rate, and 4/14 failed.

For the validation study, we specifically included samples already known from prior testing to carry mutations on the 139-VA. All 12 mutations on both the InPlex panel and the 139-VA that were expected in these specimens were correctly identified. We identified five additional mutations not on the InPlex panel, and each was verified by Sanger sequencing with 100% concordance. There were no false positive or false negative calls. Results were concordant across all replicates. Based on the results of the validation study, 5 μ l DNA was used for all subsequent runs.

Technical Evaluation

Excluding the validation study, seven plates containing DNA extracted from 318 DBS were genotyped using the Illumina 139-VA. One plate initially failed due to contamination in the no template control. The contamination was attributed to plate seals popping in the thermocycler during oligonucleotide hybridization, and the plate passed upon rerun. Results were obtained for 317/318 specimens (99.7%); one specimen that failed genotyping due to low call rates twice (call rates <1%, 65.2%) was attributed to an issue with poor DNA quality/low DNA extraction yield; this sample was not run a third time. Among the 317 passing specimens, complete genotyping results (100% call rates) were obtained for 99.1% of specimens (314/317) on the first attempt. One locus each failed in two different specimens (99.3% call rates) and a third specimen failed completely (0% call rate). Complete genotypes were obtained for all three specimens upon rerun. The median read depth for the 317 specimens (best run for each infant that passed) across 82 amplicons was 2,017 (range 4–41,509), and the mean depth \pm standard deviation was 2,433 \pm 1,970. Among nearly 26,000 amplicons

Table 1. Characteristics of Infants with CF

	Confirmed CF	Unverified CF
Total infants	392	47
Gender		
Male	49.5% (194)	44.7% (21)
Female	50.5% (198)	55.3% (26)
Race/ethnicity ^a		
White	76.3% (299)	72.3% (34)
Black	5.1% (20)	4.3% (2)
Hispanic	15.3% (60)	23.4% (11)
Asian	1.0% (4)	0% (0)
Other/unknown	2.3% (9)	0% (0)

^aRace/ethnicity data from CFF records used when available; otherwise, race/ethnicity was obtained from the newborn screening Guthrie card.

(317 individuals x 82 *CFTR* amplicons each), only 51 in total (0.2%) had coverage <100X, and five (0.02%) had coverage <20X.

Among the 317 individuals genotyped, 56 different mutations representing a total of 244 mutant alleles and two polyTG/T calls were reported by MiSeq Reporter software; however, no mutations were detected for 390 alleles. Twenty-four different mutations, corresponding to 176 mutant alleles detected, were present on the NBS panel in use at the time of screening, and all results were concordant between platforms. Thirty-one different point mutations, corresponding to 65 mutant alleles that were not on the NYS panel at the time of screening, were validated by Sanger sequencing with 100% concordance. The 21-kb *CFTR*dele2,3 mutation was detected in three individuals, and all were validated using a PCR-based assay. Twenty-seven specimens were completely Sanger sequenced for all 27 *CFTR* exons and tested for the *CFTR*dele2,3 deletion. All mutations found by Sanger sequencing were concordant with calls made by the 139-VA, and there were no apparent false positive or false negative calls made by the 139-VA.

Clinical Evaluation

Genotypes from the NYS panel were available for all 439 infants with CF identified via NBS (Table 1). Excluding the sample that failed, 163 of these 439 infants were genotyped using the 139-VA (Table 2). The 275 infants with two known NYS panel mutations that were not genotyped using the 139-VA and the one sample that failed genotyping are included in analyses of the NYS CF mutation spectrum.

Table 2. Number Likely Pathogenic Mutations Detected by NYS Mutation Panels Compared with the Illumina 139-VA

Referral type	NYS referral algorithm ^a		Illumina 139-variant assay ^b	
	Confirmed CF	Unverified CF	Confirmed CF	Unverified CF
Two mutations	256	23	300 (53 + 247)	31 (8 + 23)
One mutation	114	22	79 (73 + 6 ^c)	14 (14 + 0)
VHIRT, 0 mutations	22	2	13 (13 + 0)	2 (2 + 0)
Overall	392	47	392 (139 + 253 ^c)	47 (24 + 23)

^aBased on the algorithm and mutation panel in use at the time of referral (2002–2007, 31 mutation Abbott Molecular Cystic Fibrosis Genotyping Assay and 2007–2012, 39 mutation Hologic InPlex CF Molecular Test; see *Materials and Methods* for details). Variant of uncertain clinical significance, p.R117H, in the absence of 5T ($N = 7$ infants), was considered a mutation.

^bMost infants with CF of known etiology (i.e., two mutations on the NYS NBS mutation panel) were not retested using the 139-VA. The number of infants (tested + not tested) using the 139-VA is indicated in parentheses.

^cThe one sample that failed genotyping using the 139-VA is included in the group not tested using the 139-VA.

Table 2 shows the number of mutations on the NYS panel and on the 139-VA, in NYS infants with CF. Among the infants classified as confirmed CF, 256 were referred for two NYS panel mutations; 251/256 had two mutations on the 139-VA and five had one; these five infants carried p.D1152H as their second mutation. p.D1152H is not on the 139-VA, and was also not on the Abbott panel used when these infants were screened, but is on the current NYS InPlex panel. Of 114 infants referred for one NYS panel mutation, the second CF mutation was identified in 46, but 68 still had only one mutation on the 139-VA. The second mutation was p.E60* (E60X) in 2/46 infants, which was not on the Abbott panel in use at the time of screening, but is on the current InPlex panel. Three infants referred for one mutation included in the group of 68 with a second mutation not detected on the 139-VA are known to carry p.D1152H as their second mutation. Finally, of the 22 infants with no NYS panel mutations referred for VHIRT, three specimens had two, six had one, and 13 still had no mutations on the 139-VA. Overall, 300 NYS infants with confirmed CF had two 139-VA mutations, 79 had one, and 13 had none. Among the infants with unverified CF, 31 had two 139-VA mutations, 14 had one, and two had none. Samples from 97 screen negative infants were also run on the 139-VA, and one was found to be a heterozygous carrier of p.F508del (delF508). No other mutations were identified in screen negative infants.

Clinical Sensitivity of Mutation Panels in NYS

Table 3 shows the clinical sensitivity of the current 39-mutation InPlex panel used in NYS compared with the Illumina 139-VA. Most (79.7%) of the mutations in CF patients, henceforth CF alleles, are included on the InPlex panel (631/784 confirmed plus 69/94 unverified CF alleles). An additional 63 confirmed and seven unverified CF alleles not on the InPlex panel were detected by the 139-VA, but eight p.D1152H alleles were not, resulting in a net increase of 55 CF alleles overall. Clinical sensitivity of mutation panels among the major race/ethnic groups in NYS CF cases is shown in Table 4.

Common CF Mutations in NYS

There are 138 distinct CF-causing mutations on either the 139-VA or the current NYS panel (including p.R117H and p.I148T with reflex testing for c.3067_3072delATAGTG/p.I1023.V1024del [3199del6]; Supp. Table S2). Supp. Table S3 lists all mutations detected in at least one NYS CF patient in this study. p.F508del (delF508) was the most common (56.4% of all NYS CF alleles). Approximately 80% of mutations segregating in the NYS CF population are included on the InPlex panel currently in use. Six mutations, each of which is on the ACMG-recommended panel, were found at an allele frequency between 1% and 5%. Thirty-six additional mutations were found on at least two alleles each (minor allele frequency [MAF] = 0.2%–0.9%), and 19 mutations were each detected once (MAF = 0.1%). Based on all mutations tested in this study, the second, third, and fourth most common mutations in our CF population, respectively, were p.W1282* (W1282X; 22 alleles), p.G542* (G542X; 19 alleles), and p.R117H (19 alleles; regardless of intron 8 polyTG/T tract length) in the White; c.2988+1G>A (3120+1G→A; four alleles), p.A559T (three alleles) and p.R1066H (two alleles) in the Black; and c.2988+1G>A (3120+1G→A; 11 alleles), p.G542* (G542X; 4 alleles), p.L206W (three alleles), and p.I507del (delI507; three alleles) in the Hispanic population. Other than p.F508del (delF508), the only mutations detected in the Asian population were p.W1282* (W1282X) and p.S492F (one allele each), and five individual mutations were each only detected once in the other/unknown population group.

Table 3. Clinical Sensitivity^a of Mutation Panels in NYS Infants with CF Identified via NBS and in Published Datasets

	p.F508del (delF508)	ACMG-23	InPlex-39 ^b	Illumina 139-VA
Confirmed NYS CF alleles	57.4% (53.9%–60.9%) 450/784	76.8% (73.8%–79.7%) 602/784	80.5% (77.7%–83.3%) 631/784	86.6% (84.2%–89.0%) 679/784
All NYS CF alleles	56.4% (53.1%–59.7%) 495/878	76.2% (73.4%–79.0%) 669/878	79.7% (77.1%–82.4%) 700/878	86.0% (83.7%–88.3%) 755/878
CFTR2 CF alleles ^c	70.3% (69.9%–70.6%) 49,740/70,777	87.8% (87.6%–88.0%) 62,138/70,777	90.4% (90.2%–90.6%) 63,997/70,777	95.0% (94.8%–95.1%) 67,230/70,777
Pan-ethnic US CF alleles ^d	66.3%	83.9%	Unknown	Unknown

^aClinical sensitivity measured as the proportion of pathogenic *CFTR* alleles detected by the panel. For each group, the sensitivity is shown on the top line, 95% confidence interval for the estimate in parentheses on the middle line, and the allele counts (number mutations/total number alleles) on the bottom line. Totals differ between Table 2 (considers the number of mutations reported at the time of referral) and Table 3 (considers mutations included on the panel, resulting in three additional p.E60* [E60X] and three additional p.D1152H alleles not included on the Abbott panel in use at the time of screening, overall).

^bExcludes non-CF-causing variants p.D1270N, p.F508C, and p.I148T (in the absence of c.3067_3072delATAGTG/p.I1023_V1024del [3199del6]).

^cEstimates derived from published CFTR2 data [Sosnay et al., 2013]. Does not include p.I148T alleles ($N = 99$; because it is unknown that proportion, if any, also harbor c.3067_3072delATAGTG/p.I1023_V1024del [3199del6]), p.D1270N, p.F508C, or c.3717+4A>G (3849+4A>G; present on the InPlex panel but not in CFTR2). All CFTR2 p.R117H alleles are included (intron 8 polyTG/T tract status was unknown).

^dEstimates from data in published tables [Palomaki et al., 2004; Watson et al., 2004].

Table 4. Clinical Sensitivity^a of Mutation Panels in NYS Infants with CF Identified via NBS, Stratified by Race/Ethnicity

	p.F508del (delF508)	ACMG-23	InPlex-39	Illumina 139-VA
Overall	56.4% (53.1%–59.7%) 495/878	76.2% (73.4%–79.0%) 669/878	79.7% (77.1%–82.4%) 700/878	86.0% (83.7%–88.3%) 755/878
White	61.9% (58.2%–65.6%) 412/666	81.8% (78.9%–84.8%) 545/666	85.7% (83.1%–88.4%) 571/666	91.0% (88.8%–93.2%) 606/666
Black	34.1% (20.1%–48.1%) 15/44	50.0% (35.2%–64.8%) 22/44	50.0% (35.2%–64.8%) 22/44	68.2% (54.4%–81.9%) 30/44
Hispanic	40.1% (32.1%–48.2%) 57/142	59.9% (51.8%–67.9%) 85/142	63.4% (55.5%–71.3%) 90/142	71.1% (63.7%–78.6%) 101/142
Asian	50.0% (15.4%–84.6%) 4/8	62.5% (29.0%–96.0%) 5/8	62.5% (29.0%–96.0%) 5/8	75.0% (45.0%–100%) 6/8
Other/unknown	38.9% (16.4%–61.4%) 7/18	66.7% (44.9%–88.4%) 12/18	66.7% (44.9%–88.4%) 12/18	66.7% (44.9%–88.4%) 12/18

^aClinical sensitivity measured as the proportion of pathogenic *CFTR* alleles detected. For each group, the sensitivity is shown on the top line, 95% confidence interval for the estimate in parentheses on the middle line, and the allele counts (number mutations/total number alleles) on the bottom line.

Results for the race/ethnicity-specific clinical sensitivity analysis were similar when including only infants with confirmed CF (data not shown).

Discussion

The population for this study was drawn from >2.5 million infants screened for CF by the NYS NBS program, from the onset of screening in October 2002 through 2012. Four-hundred thirty-nine infants with CF, including 392 confirmed cases and 47 with unverified CF, representing all major race/ethnic groups in NYS were included, allowing comprehensive assessment of the sensitivity of mutation panels in the diverse NYS CF population.

We have shown that genotyping using DNA extracted from DBS on Illumina's MiSeqDx platform using the 139-VA is robust and reliable, even using approximately 10-fold less DNA than recommended. Furthermore, the samples genotyped in this study were derived from archived blood spots dating back as far as 2002, and although the DNA extraction method used is routinely used by our program for a range of applications [Saavedra-Matiz et al., 2013],

the DNA is not purified, so the 139-VA chemistry is clearly robust and at least somewhat tolerant of impurities. There was perfect concordance between mutations detected on the 139-VA, the Abbott or Hologic InPlex assay in use at the time of screening, and Sanger sequencing. The Illumina protocol is straightforward, albeit with far longer run times compared with some existing methodologies such as the InPlex assay. Based on our experience, the total turnaround time (from DNA in-hand to genotype availability) for the 139-VA is 39.5 hr with sample processing time of 9.5 hr, including hands-on sample processing time of 2.5 hr, plus instrument run time of 30 hr (32 hr including post-run wash cycles), whereas the InPlex assay turnaround time is approximately 4.25 hr, with a sample processing time of 3.5 hr, including hands-on sample processing time of 45 min, plus instrument run time of 45 min.

The Wisconsin NBS program evaluated an early release (investigator use only; IUO) version of the Illumina 139-VA [Baker et al., 2015]. The current commercially available, FDA-cleared Illumina 139-VA used in our study includes 134 CF-causing variants (<http://www.cftr2.org>), one variable consequence mutation (p.R117H) (<http://www.cftr2.org>), plus conditional reporting of intron 8 polyTG/T tract length in the presence of p.R117H, and conditional reporting of p.I506V, p.I507V, and p.F508C when p.F508del (delF508) is homozygous. The IUO version of the panel included 162 variants, encompassing the 134 CF-causing variants listed above, p.R117H, with full, rather than conditional reporting of polyTG/T status, plus an additional nine mutations with varying consequences, seven variants that were still under evaluation by CFTR2 (one, p.I1234V, is now reported as CF-causing in CFTR2), and nine non CF-causing variants (Supp. Table S2) [Baker et al., 2015].

The IUO assay was validated using DNA extracted from 67 DBS from the Wisconsin NBS program and the Centers for Disease Control and Prevention (CDC), using two methods (commercial extraction protocol and a laboratory-developed method). The samples were known to carry 48 different mutations included on the IUO assay, and all were correctly identified. Excluding intron 8 polyTG/T tract status, in the current study, we validated a total of 56 different mutations, also finding 100% concordance, increasing the number of CF-causing mutations validated using DNA extracted from DBS (albeit using a different extraction method) on the 139-VA from 44/134 to 68/134.

We have also assessed the clinical validity of the panel, demonstrating >6% increased sensitivity compared with the InPlex panel currently used by NYS and some other NBS programs, and 9.8% increased sensitivity compared with the 23-mutation ACMG panel, also used by some NBS programs. However, the increased sensitivity must be interpreted in the context of the CF referral algorithm. In NYS, infants with two InPlex mutations, one InPlex mutation, or ultrahigh IRT (VHIRT) in the absence of InPlex mutations are referred for follow-up sweat testing. From 2010 to 2013, the positive predictive value (PPV) for CF screening in NYS overall was 4.3% (average of 923 infants referred annually), ranging from 75.5% in infants with two mutations (31 referrals/year), 1.9% with one mutation (657 referrals/year), to 0.7% in infants with VHIRT (234 referrals/year) [Kay et al., 2015]. An ideal mutation panel would allow modification of the NYS algorithm to reduce the number of unnecessary referrals (false positive screens) by eliminating the need for referral of infants with one mutation and/or VHIRT. The ultimate goal is to refer only babies at highest risk to have CF, sparing families the cost of diagnostic work-up and stress and anxiety associated with waiting for a diagnosis for a potentially life-limiting disorder [Moran et al., 2007; Tluczek et al., 2011]. Most of the ~60 additional mutations identified in this study, which included all NYS CF cases identified via screening, were detected in infants who were already known to carry one InPlex mutation (i.e., the 139-VA detected their second mutation). Considering confirmed plus unverified CF cases, over the 9.5-year period of this retrospective study, including false negative IRT screens reported to the program, clinical sensitivity for CF screening overall was 96.9%. If the 135 mutations included on the 139-VA had been used since the onset of CF screening instead of the 31-mutation Abbott or 39-mutation InPlex panels, and if only infants with two mutations were referred (eliminating the one mutation and VHIRT referral categories), sensitivity would decrease to 73.1%, because 108 additional infants with CF would have been considered screen negative. Even if infants with at least one mutation on the 139-VA panel were referred (eliminating only the VHIRT referral category), sensitivity would decrease to 93.6%, with 15 additional false negative screens. The reduction in sensitivity was similar considering confirmed CF cases only. Therefore, although the 139-VA does, in fact, increase sensitivity of screening, the program could not tolerate an algorithm modification because of the unacceptable increase in the number of infants who would be missed. Complete sequencing in infants with high IRT and zero to one *CFTR* mutation as a third-tier screen could allow for an algorithm modification, if the cost savings associated with eliminating false positive screens compensates for the cost of sequencing, which is labor intensive and expensive.

Screening for 23 recommended ACMG mutations is reported to account for 83.9% of CF alleles in a pan-ethnic US population [Palomaki et al., 2004; Watson et al., 2004]. However, the CF mutation spectrum is race/ethnicity and geographically dependent, and ACMG panel sensitivity estimates ranged from 48.9% in Asian-Americans, to 94.0% in the US Ashkenazi Jewish population. States like New York, California, and Massachusetts with large cities and immigrant populations may include more genetic diversity than can be captured by any panel with a limited number of mutations. The InPlex panel was thought to increase sensitivity in non-White populations, including mutations not on the ACMG-23 panel reported to be common in other race/ethnic groups, such as c.3744delA (3876delA) in US Hispanics [Watson et al., 2004]. Our data show a modest 3.5% increase in sensitivity of the InPlex-39 mutation panel (79.7%) compared with the ACMG-23 panel (76.2%). Additional non-ACMG-23 CF-causing mutations present on the InPlex panel were only detected in the White (26 alleles; nine different mutations)

and Hispanic (five alleles; three different mutations) CF populations. Sensitivity in the Black and Asian groups was unchanged.

Though the PPV is low (<1%), use of a “failsafe” VHIRT referral category in NYS permits early identification of CF in infants with two rare mutations, especially in the Hispanic population [Kay et al., 2015]. However, Black infants are enriched among infants without CF (false positive screens) referred for VHIRT. If a more comprehensive mutation panel allowed elimination of at least the VHIRT referral category, some of these race/ethnic disparities could be reduced or possibly eliminated. Although sensitivity of the 139-VA was still highest in the White population (91.0%) and lowest in the other/unknown group (66.7%), the largest gain in sensitivity over the current NYS panel was for the Black population (18.2% increase), followed by Asian (12.5% increase), Hispanic (7.7% increase), and White populations (5.3% increase), whereas no additional mutations were detected in the other/unknown group (0% increase). The expected clinical sensitivity of the 139-VA was 95.0%, based on data from approximately 35,000 CF patients in the CFTR2 dataset [Sosnay et al., 2013]. Ninety-five percent of patients with ancestry data in the CFTR2 dataset were listed as Caucasian, compared with 76% of all NYS CF patients who were listed as White. Therefore, increased race/ethnic diversity in NYS likely contributes to decreased 139-VA sensitivity in the NYS CF population, which was 86.0% overall and 91.0% in Whites. Use of the 139-VA also allows identification of five mutations that are currently approved for treatment in patients age two and older using Ivacaftor (Kalydeco™) that are not included on the ACMG panel (p.S549N, p.S549R), or both ACMG and InPlex panels (p.G178R, p.G1244E, p.S1251N).

We have identified mutations recurrent in the NYS CF population that are not included on the ACMG panel. The most common of those that are not on the current NYS InPlex panel, p.L206W, is a milder CF mutation, most often reported to be associated with pancreatic sufficient disease (<http://www.cftr2.org>), but also associated with CBAVD [Clain et al., 2005]. In NYS, eight infants were compound heterozygous for p.L206W and another mutation; six with p.F508del (delF508), one with p.R553* (R553X) and one unknown. Six were in the confirmed CF group, and two were unverified (one is now known to have been reclassified as CRMS). Three were Hispanic and five were White. Median sweat chloride in these eight infants was 60 (range 39–64). At 0.8%–0.9%, the frequency of this mutation is higher in the NYS CF population than in the CFTR2 dataset (0.2%) [Sosnay et al., 2013], but may be primarily associated with nonclassic CF. Other recurrent mutations not on the NYS and/or ACMG panel included c.1327_1330dupGATA (1461ins4; detected in two White and one Black CF patient), p.E60* (E60X; one Hispanic and four White), p.A559T (three Black), p.S549N (two Hispanic and one White), c.3744delA (3876delA; two Hispanic and one White), and p.R1066H (one Hispanic and two Black infants); and c.948delT (1078delT), c.1022_1023insTC (1154insTC), c.2490+1G>A (2622+1G>A), c.2052dupA (2184insA), p.R347H, p.R1158* (R1158X), c.3140-26A>G (3272-26A>G), c.54-5940_273+10250del (CFTRdele2,3), only detected in White infants. All other non-NYS panel mutations detected using the 139-VA were found on only one or two alleles.

p.D1152H, also thought to be associated with milder CF [Burgel et al., 2010; Terlizzi et al., 2015], was included in the IUO version of the 139-VA, but, based on its designation as a mutation of varying clinical significance in CFTR2, was not included in the final version of the panel. We identified eight infants with p.D1152H among the 439 with CF; all were compound heterozygous with p.F508del (delF508), classified as confirmed CF, and White. Sweat test results were available for six, and the median sweat chloride was 34 (range 19–57); therefore, it is likely that these infants were diagnosed as CF

based on the presence of two panel mutations, as opposed to having diagnostic sweat test results [Farrell et al., 2008]. Eighteen additional NYS infants identified via NBS are known to be compound heterozygous for p.D1152H and another mutation, and one is homozygous for p.D1152H. These infants were not included in this study because they were not diagnosed as CF. Fourteen compound heterozygotes (with p.F508del [delF508], p.W1282* [W1282X], or c.3717+12191C>T [3849+10kbC→T]) and the infant homozygous for p.D1152H were classified as CRMS, possible CF or a *CFTR*-related disorder, and the median sweat chloride in these infants was 22 (range 12–42). The remaining five infants (compound heterozygous with p.F508del [delF508], p.W1282* [W1282X], or p.R117H) were classified as “No CF” (sweat chloride for four infants with data ranged from 20 to 35). Our data support the variable phenotype associated with p.D1152H, though the current clinical phenotype for these infants is unknown.

Strengths of this study include the large population-based ascertainment of CF cases, representing all infants identified by NBS in the racially/ethnically diverse NYS population over a 9.5-year period. The large population provided us the opportunity to assess and compare the sensitivity of four approaches to molecular CF screening—p.F508del (delF508) by itself, 23 mutations recommended for screening by the ACMG, 39 mutations on the InPlex panel used by other US NBS programs using IRT-DNA algorithms, and the newest, most comprehensive targeted panel available, the Illumina 139-VA. We were also able to assess the mutation spectrum in the major NYS population groups, permitting identification of several recurrent mutations that may be useful to other NBS programs with similar population demographics.

Limitations of this study include problems that most, if not all large NBS programs without complete centralized medical records encounter, such as lack of detailed clinical phenotype information, incomplete data, an inability/difficulty in reliably tracking infants/diagnoses through time, and potential inaccuracy of race/ethnicity in both the NBS dataset [Kay et al., 2015] and the CFF patient registry. Confidence in diagnoses was strengthened by matching infants with CF in the NBS database to NYS infants in the CFF patient registry, and data were analyzed including and excluding the cases that could not be verified by comparison to registry records.

Most NBS programs screen for CF using an IRT-DNA algorithm, including a mutation panel consisting of one to 40 mutations. To the best of our knowledge, only California routinely screens more than 40 mutations [Prach et al., 2013], although the Wisconsin NBS program is now pilot testing an expanded mutation panel [Wisconsin, 2015]. There are other larger mutation panels available for clinical *CFTR* testing by commercial laboratories, but such panels are not routinely used in the NBS setting. Use of the 139-VA should be feasible in NBS laboratories with existing molecular capabilities, but the increased sensitivity of the assay must be assessed in the context of a longer turnaround time and additional cost per sample (list price for Illumina assay/reagents is approximately 1.4 times the cost of the InPlex assay/reagents). The NYS program also has the advantage of being at the Wadsworth Center, which has a molecular core facility (Applied Genomics Technologies Core; AGTC) with existing infrastructure and expertise with various NGS platforms and technologies and other ongoing public health projects using NGS.

Overall, the 139-VA identified a net of 55 more mutations than the InPlex panel, but the PPV for CF screening in NYS would not change if the InPlex assay was replaced with the 139-VA, because all babies with one mutation or VHIRT/no mutations would still need to be referred, to ensure overall CF NBS sensitivity >95%. The

139-VA is more sensitive than other commercially available panels, and could be considered for NBS, clinical, or research laboratories conducting CF screening.

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