CASE STUDY OF MOLECULAR ASSAY VALIDATION: 2ND TIER QUALITATIVE SCREENING

March 11, 2015

Rachel Lee, PhD

TEXAS Department of State Health Services

Which Mutations?

- Targeted Mutation Panel or Gene Sequencing?
- Purpose of 2nd tier test
- Mutation Detection Rate
- False Negatives
- False Positives and Carriers
- Population

Texas CF Mutation Data

	No. of Alleles	% Total Alleles
Total for Texas (1426 pts)	2852	100%
No Mutation for 1 and 2	446	15.6%
1,2 or Both Not Identified	274	9.6%
Identified Alleles	2132	74.8%

Information provided by Dr. John Saito and Dr. Donna Beth Willey-Courand

TX CF Mutation Data (Cont) 2132 Identified Alleles

Mutations	No. of alleles	Mutations	No. of alleles	Mutations	No. of alleles
ΔF508	1664	R347P	1	2307insA	2
ΔI507	12	711+1G-T	0	3876delA	5
G542X	64	1898+1G-A	7	2183AA-G	5
G551D	59	2184delA	4	1677delTA	2
W1282X	19	1078DelT*	1	D1152H	2
N1303K	35	3849+10kbC-T	12	G330X	1
R553X	20	2789+5G-A	8	L206W	3
621+1G-T	23	3659delC	11	R1158X	2
R117H	25	I148T*	3	Q493X	11
1717-1G-A	15	3120+1G-A	10	3905insT	6
A455E	3	delF311	1	V520F	2
R560T	5	R1066C	2	1717-1G-T	1
R1162X	11	S549N	6	S549R	1
G85E	5	W1089X	5	Y1092X	1
R334W	3	1812-1G>A	1	3120G-A	2
				Other mutations	51

* Deleted from the ACMG recommended panel (Watson et al 2004 Genetics in Medicine 6(5) 387-391)

TEXAS Department of State Health Services

ACMG-23 Panel

- Recommended by ACMG for routine diagnostic and carrier testing
- Mutation Detection Rate
  - Non-Hispanic Caucasian 88.3%
  - African American 69%
  - Hispanic American 57%

Moskowitz et al, 2008, Gene Review,
http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=cf

Expected Proportion of Abnormal Alleles Detected

Mutation Detection Rate	Proportion of CF patients for which a given number of abnormal alleles is detected		
	2 Abnormal Alleles	1 Abnormal Allele	0 Abnormal Allele
98%	96%	4%	0%
95%	90%	10%	0%
90%	81%	18%	1%
85%	72%	26%	2%
80%	64%	32%	4%
75%	56%	38%	6%
70%	49%	42%	9%
60%	36%	48%	16%
50%	25%	50%	25%
40%	16%	48%	36%
30%	9%	42%	49%

Moskowitz et al, 2008, Genetics in Medicine 10(12): 851-868

Comparison of Mutation Panels

	Texas Data	ACMG Panel (23)	CA Panel (38)	Selected Commercial CF DNA Testing Kits				Custom Panel (45)
				Lumine x xTAG (39+4)	Hologic Extended Panel (40+2)	Elucigene CF-US (44+1)	Asuragen Expand (47)	
Total # of Identified Alleles	2132	2016	1974	≥2045	≥2055	≥2038	≥2041	2081
% of Identified Alleles	100%	94.56%	92.59%	≥95.92%	≥96.39%	≥95.59%	≥95.73%	97.6%
% of Total Alleles	75%	70.69%	69.21%	≥71.7%	≥72.05%	≥71.45%	≥71.56%	72.97%

TEXAS Department of State Health Services

Which Method?

- Cost
- Existing methods
- Existing equipment
- Expertise
- TAT
- Capacity/Throughput/Automation
- LIMS interface
- Multiplexing or tier approach
- Algorithm (e.g. IRT/IRT/DNA or IRT/DNA)

Cystic Fibrosis NBS in TX

- Implemented statewide December 1, 2009
- IRT/IRT/DNA methodology
  - 1st screen elevated IRT/2nd screen elevated IRT/DNA
  - IRT fixed cutoff:
    - 60 ng/mL in blood for infants <21 days at the time of specimen collection
    - 46.5 ng/mL in blood for infants 21 days or older at the time of specimen collection
  - CFTR mutation panel – Hologic (40+2)
    - 1 or 2 mutations identified – Abnormal CF screen
    - 0 mutation identified - Normal

‘Failsafe’ Protocols in TX

- Ultra-high IRT levels (>150 ng/mL blood) but 0 mutations
- If 1st screen is elevated & no or unacceptable second specimen received by 30 days of age, the first screen is reflexed to DNA
- 1st normal IRT or no 1st screen with 2nd screen elevated IRT is reflexed to DNA

Materials Used for Validation

- Collecting specimens needed for method development and validation
- Must have representatives for each mutation on panel or cannot report that mutation
- Received specimens from diagnosed cases from Wisconsin, Michigan, North Carolina, and Indiana
- Received QC materials from CDC
- Missing 4 mutations on panel (3849+4A>G, S549R A>C, Y122X, and Y1092X C>G)

Method Development / Optimization

- Instrument installation
- Testing process – workflow evaluation
  - Punch
  - Extraction protocol
  - Testing protocol
  - Data analysis / Result interpretation
- Staff training
- Troubleshooting
- LIMS interface, modification and validation
- PT program enrollment

Validation Plan–Hologic Inplex 40+4

- Accuracy
- Precision
- Sensitivity
- Specificity
- Reportable range
- Reference range
- Stability study
- Carryover study

Validation Runs

- A total of 33 specimens that encompassed a high percentage of the targeted genotypes were tested in the DNA Analysis laboratory using the CFTR InPlex assay.
- Day 1 - the specimens were extracted and tested in triplicate by one technician (tests #1, #2 and #3).
- Day 2 - the specimens were analyzed by another technician at two different times in one day (tests #4 and #5).
- Day 3 - the specimens were tested again by a third technician (test #6).

Accuracy

- Determined by comparing the CFTR InPlex results from each specimen with results from the reference laboratories. In order for specimen CFTR InPlex results to be acceptable, they must be in at least 90% agreement with the reference laboratories results.

Mutation	Calls per Mutation	Reference Laboratory Results		CFTR InPlex Calls			Agreement		
		Positive	Negative	Positive	Negative	Indeterminate	Positive	Negative	Overall
394delTT	192	3	189	3	189	0	100%	100%	100%
621+1G>T	192	12	180	12	180	0	100%	100%	100%

Precision

- Determined by assessing the day-to-day, run-to-run, within-run, and operator variation. In order for CFTR InPlex results to be acceptable, repeat testing of the specimens over time should give consistent results (>90% agreement) and they should not be time or technologist dependent.

Specimen #	Mutations and Polymorphisms Identified by Reference Laboratories	Agreements between calls for each mutation/polymorphism on the CFTR InPlex test		
		Within Run Agreement (42 calls per sample x 3 repeats)	Run-to-run (Within Day) Agreement (42 calls per sample x 2 runs)	Day-to-day and Between Operator Agreement (42 calls per sample x 3 days/operators)
NC-1	711+1G>T	100%	100%	100%
IN-6	1078delT	100%	100%	100%
CDC-165	2789+5G>A ΔF508 IVS8-7T/9T	100%	100%	100%
CDC-164	3905insT 1248+1G>A IVS8-7T/7T	100%	98.8%	99.2%

Analytical Sensitivity

- Genomic DNA extracts of 3 newborn screening specimens (one with high signal, one medium, and one low) were subjected to a series of dilutions (1:1, 1:2, 1:4,, 1:8, and 1:16) and analyzed by CFTR InPlex assay.

Dilution	**CFTR InPlex Calls**		
	High Signal (Specimen# 20092734097)	Medium Signal (Specimen# 20092974616)	Low Signal (Specimen# 20092905445)
1:1	Normal	3120+1G>A	Normal
1:2	Normal	3120+1G>A	Low Signal
1:4	Normal	3120+1G>A	Low Signal
1:8	Normal	Low Signal	Low Signal
1:16	Normal	Low Signal	Low Signal

Clinical Sensitivity

- Percent of specimens with the targeted condition whose test values are positive

$$[TP/ (TP+FN)] \times 100\%$$

Analytical Specificity

- Determined by evaluating the cross-over signals within the multiplex panel and the ability to discriminate similar and adjacent mutations, such as dF508 and dI507 alleles or G551D and R553X alleles.

Specimen #	Mutations and Polymorphisms Identified by Reference Laboratories	Mutation Name (Invader Results)	
CDC-16	1717-1G>A G551D	G551D (HET)	R553X (Normal)
CDC-8	1717-1G>A R553X	G551D (Normal)	R553X (HET)
CDC-163	3120+1G>A S549N IVS8-7T/7T	S549N (HET)	S549R T>G (Normal)
NC-4	S549R T>G	S549N (Normal)	S549R T>G (HET)
NC-3	R347H	R347H (HET)	R347P (Normal)
CDC-25	R347P R1066H IVS8-7T/7T	R347H (Normal)	R347P (HET)

Analytical Specificity (cont)

- Analytical specificity due to interfering substances was not tested because interfering substances will result in non-amplification of the patient's DNA. There would not be a reportable result, and the specimen would be considered unsatisfactory.
- The presence of such substances was not encountered during the method evaluation period.
- A variety of components in clinical specimens and DNA extraction solutions have been reported to interfere with the enzymatic reactions in amplification processes, including heme and its by-product, heparin, and sodium dodecyl sulfate.

Clinical Specificity

- Percent of specimens without the targeted condition whose test values are negative

$$[TN/ (TN+FP)] \times 100\%$$

Reportable Range and Reference Range

- Reportable Range – Since the CFTR InPlex assay is a qualitative test, the reportable result for each sample is “Normal”, “HET (heterozygous)”, “MUT (homozygous)” or “EQ (equivocal)”.
- Reference Range (normal value) - Since the CFTR InPlex assay is a qualitative test, the normal value of the tested specimens should be “Normal” or “0 Mutation Identified”.

Stability Study

- Determine if the current TX newborn screening specimen acceptance criterion of 13 days after Date of Collection is applicable and how long the mutations are stable at room temperature storage. Ten newborn specimens that were received within 1 or 2 days after Date of Collection were selected. Punches were made, extracted, and tested on the same day (Day 1), Day 5, Day 8, Day 12, Day 19, Day 26, Day 40, month 3, month 6, .month 9, and month 12.

Carryover Study

- Determine potential cross contamination caused by using the same puncher head to punch samples without cleaning between punches

Other Considerations

- Scale up (workflow, coordination with 1st tier)
- Result notes (interpretation, recommendation)
- Method limitation
- Write SOP
- Reporting / follow-up algorithm
- Inform and educate healthcare providers

Problems encountered

- Low signals
  - Poor extraction
  - Low genomic DNA
  - Homozygous mutation on the same codon
- Equivocal
- Het cannot be confirmed
- Instrument malfunction
- Missed cases
- Lack of control materials for all mutations

Take Home Messages….

- Each NBS program has different needs - define your goals
- Need help? Just ask
- Be familiar with CLIA and CAP requirements and CLSI guidelines on validation
- Document…..document…document