Overview of Cystic Fibrosis, Genotyping Methods and NSQAP CF DNA PT

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APHL Molecular Training Workshop
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Molecular Test Helps Identify Babies with Cystic Fibrosis (CF)

- Inherited recessive disorder caused by mutations in the \textit{CFTR} gene
  - Thick, sticky mucus causes blockages in lungs and pancreas

- Primary screening test measures immunoreactive trypsinogen (IRT), a non-specific marker for pancreatic damage

- Second tier test identifies \textit{CFTR} mutations to increase specificity

- NBS Result: Decreased false positives
States performing a second-tier molecular assay (second-tier only)
Treatments for Cystic Fibrosis

- **Improve Protein Function**
  - FDA review of Kalydeco/Lumacaftor combination for people who have 2 copies of F508del

- **Airway Clearance**
  - Manual or mechanical techniques
  - Inhaled mucolytics or hypertonic saline

- **Antibiotics**
  - Oral, intravenous, or inhaled

- **Nutrition**
  - Pancreatic enzymes
  - Monitoring calories

Public Health Benefits of CF Newborn Screening

Percent of New Diagnoses Detected by Newborn Screening, 1990-2013

Median Predicted Survival Age, 1986–2013  In 5-Year Increments

*Cystic Fibrosis Foundation Patient Registry 2013 Annual Data Report, Bethesda, Maryland
© 2014 Cystic Fibrosis Foundation
Cystic Fibrosis Newborn Screening High False Positive Rate

- **Primary test for CF** – Immunoreactive trypsinogen (IRT)
  - Marker for pancreatic damage – not specific for CF
  - ↑ sensitivity = lower IRT cutoff
  - ↑ specificity = add 2nd tier mutation analysis

- **Current false positive rate** is as high as 94% depending on cutoffs and mutation panel – most of this is from carriers

- **Estimated incidence of CF by ethnic background**
  - White Americans ~1:3,000 (carrier rate = 1:28)
  - Hispanic Americans ~1:6,000 (carrier rate = 1:39)
  - African Americans ~1:10,000 (carrier rate = 1:51)

- **To ↓ false positives**, some programs have redefined a screen positive
CF NEWBORN SCREENING ALGORITHMS

IRT/IRT
IRT/DNA
IRT/IRT/DNA
IRT/DNA/EXTENDED GENOMIC ANALYSIS(EGA)

Screen positive: ↑ IRT and at least 1 CFTR mutation
Screen positive: ↑ IRT and at least 2 CFTR mutations
Algorithm 1: IRT/IRT

↑ IRT = collection of 2\textsuperscript{nd} sample for IRT testing

**Advantages**
- Carrier status is not determined
- Does not require genetic counseling
- Biochemical test easily incorporated into NBS laboratory

**Limitations**
- Best suited to second specimen states
- Complicating variables
  - IRT level variation (increasing age, sick and low birth weight, race/ethnicity)
  - Issues with assay kits have been documented
- Difficulty setting cut-off limits due to IRT variation
Algorithm 2: IRT/DNA

\[ \text{IRT} = \text{CFTR mutation detection from same sample} \]

**Advantages**
- Second specimen is not required
- Less time to final result (about 1 week)
- Improved detection sensitivity by \( \downarrow \) IRT cutoff
- Mutation detection \( \uparrow \) specificity

**Limitations**
- Increased cost for testing and genetic counseling
- Still high false positive rate due to CFTR carriers
- Mutation panel may not reflect population
Algorithm 3: IRT/IRT/DNA

↑ IRT = collection of 2\textsuperscript{nd} sample for IRT testing
↑ IRT = CFTR mutation detection from 2\textsuperscript{nd} sample

**Advantages**
- Improved detection sensitivity by ↓ IRT cut-offs
- Second IRT measurement ↓ number of samples for mutation detection = fewer carriers detected and cost savings

**Limitations**
- Best suited to second specimen states
- Delayed diagnosis due to wait time to collect 2\textsuperscript{nd} specimen
- Need for genetic counseling
- Mutation panel may not reflect population
Algorithm 4: IRT/DNA/EGA

IRT = CFTR mutation detection from same sample
1 mutation samples = gene sequencing
**Only 2 mutation samples are screen positive**

Advantages
- Carrier infants not sent for follow-up = ↓false positives
- Comprehensive picture of mutations in the population

Limitations
- More CRMS cases identified
- Need for genetic counseling
- Higher cost for two molecular tests
- Delayed diagnosis due to wait time for sequencing
CFTR Gene Structure
CFTR Gene Structure

http://image.tutorvista.com/content/feed/u509/CFTR%20GENE.JPG
CFTR Gene Structure

[Diagram showing CFTR gene structure with various segments labeled and mRNA length marked]

http://image.tutorvista.com/content/feed/u509/CFTR%20GENE.JPG
CFTR Gene Structure

[Diagram showing the structure of the CFTR gene, including transcription, splicing, mRNA, translation, and the roles of chloride ions, glycosylation, and the interaction with ATP and ADP.]
HGVS vs. Legacy Nomenclature

- Human Genome Variation Society guidelines facilitate uniform and standard nomenclature of DNA and protein sequence variants

- HGVS nomenclature recommends
  - Sequence variations should be described at the DNA level
  - DNA name: “g” for genomic or “c” for cDNA followed by nucleotide number(s) affected by the change
  - Protein name: “p” followed by the affected amino acid, the aa number and the substitution

- Legacy nomenclature
  - DNA names used for intron mutations, deletions, and insertions
  - Protein names used for both substitution and nonsense mutations
# CFTR HGVS Nomenclature

## Exon Changes

In the table below, the legacy exon numbers are compared to the new exon numbers according to the HGVS nomenclature. Each entry includes the xTAG CF Kit Name/Legacy Name, HGVS DNA Name, HGVS Protein Name, Legacy Exon or Intron, and Exon or Intron.

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<tr>
<th>xTAG CF Kit Name/Legacy Name</th>
<th>HGVS DNA Name</th>
<th>HGVS Protein Name</th>
<th>Legacy Exon or Intron</th>
<th>Exon or Intron</th>
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### CFTR HGVS Nomenclature

**Insertions**

**Ex:**

AGGTACCTG ATCGCTGAA

AGGTACCTGGATCGCTGAA
**CFTR HGPS Nomenclature**

### Deletions

**Ex:**

- AGGTACCTCTTGCTGAA
- AGGTACCT
- GCTGAA

### Table

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### Substitutions

Ex:  
AGGTACCTGATCGCTGAA  
AGGTACCTAATCGCTGAA
Assays Used in US to Detect Mutation(s) in CFTR

- **Single mutation detection (F508del)**
  - Gel based assays to discriminate size differences
  - Fluorescent detection Taqman assay

- **Multiplex mutation detection**
  - xTAG CF Assay – Luminex Corporation
    - xTAG 39
    - xTAG 60
  - InPlex CF Assay – Hologic (Invader technology)
    - InPlex – 32 mutations
    - InPlex – 40 mutations
  - DNA sequencing – Sanger or Next Generation
    - Unlimited within amplicons
Hologic InPlex Recall

**Issues**
- Reaction mixes in chamber 1-3 or 25-27 of InPlex cards leaked. No leaking between lanes was detected.
- Leaking was dominantly seen in Lanes 7 & 8 of the InPlex cards
- Impact should be limited to false positives

**Result**
- Current lots were recalled and the test has been permanently discontinued
- NBS labs using Hologic have to switch technologies quickly
Assays Used in US to Detect Mutation(s) in CFTR

- **Single mutation detection (F508del)**
  - Gel based assays to discriminate size differences
  - Fluorescent detection Taqman assay

- **Multiplex mutation detection**
  - xTAG CF Assay – Luminex Corporation
    - xTAG 39
    - xTAG 60
  - InPlex CF Assay – Hologic (Invader technology)
    - InPlex – 23 mutations
    - InPlex – 40 mutations
  - DNA sequencing – Sanger or Next Generation
    - Unlimited within amplicons
xTAG Cystic Fibrosis Assay Technology
Luminex Corp

- Multiplex PCR Rxn
- Amplicon Treatment
- Allele-Specific Primer Extension
- Bead Hybridization
- Reporter Addition
- Data Acquisition

https://www.luminexcorp.com/Products/Assays/ClinicalDiagnostics/xTAGCysticFibrosis/
When a dideoxy DNA base is incorporated, the DNA synthesis stops.
CFTR Next Generation DNA Sequencing

Target DNA with indices and sequence adaptors

Amplification of target regions on flow cell

DNA sequencing – after the addition of each fluorescent nucleotide, an image is taken to read the sequence

F508del heterozygote
Case Study of a Newborn with Elevated IRT

- DBS was detected with elevated IRT above the 4% Cutoff → Reflex to 2nd Tier Mutation Testing

- Initial Assay: Luminex xTAG 39
  - Two probes representing mutations Y1092X C>G and Y1092X C>A failed both the initial and repeat run
  - Repeat specimen was requested with the same results

- Secondary Assay: Inplex CF - 40
  - No mutations detected – both Y1092X probes gave a normal result
Case Study of Newborn with Elevated IRT

- DBS was detected with elevated IRT above the 5% Cutoff → Reflex to 2nd Tier Mutation Testing
- Assay: Luminex xTAG 39
  - Two probes representing mutations Y1092X C>G and Y1092X C>A failed both the initial and repeat run
  - Repeat specimen was requested with the same results
- Sample sent for DNA sequencing of Exon 20
  - Baby was “homozygous” for Y1092H T>C

![Graph showing genetic sequencing results](image-url)
The Good, The Bad and The Ugly...

Y1092H's proximity to Y1092X resulted in a failure of the Luminex Y1092X probes to hybridize
Is this Case Study Done??

- Was the baby **homozygous** or **hemizygous** for Y1092H T>C?
  - hemizygous is when there is only 1 member of a chromosome segment rather than the usual 2

- Could there be a large deletion of Exon 20???

- How could this be determined???
  - **Approach 1:** Sequence Exon 20 in both parents to see if they both have Y1092H T>C
  - **Approach 2:** Perform a molecular deletion assay such as MRC Holland’s MLPA which can detect 1 versus 2 copies of Exon 20
Case Study Take Home Messages

- Assay failures can offer important information
- No assay can catch everything
- Assays used in newborn screening labs do not detect most large deletions
- Know your state’s policies
  - What is your program responsible for and what is diagnostics responsible for in your state?
  - How do you communicate your findings in the most meaningful way to diagnostic partners?
NSQAP’s CF DNA Proficiency Program

- **Proficiency testing**
  - 5 blind coded specimens sent 4 times per year

- **Mutation panels available for method validation or verification**
  - CDC CF DNA repository contains 73 unique CFTR mutations (including ACMG 23) from 198 donors

- **Troubleshooting for molecular assays and DNA extraction**
Recent Modifications to CF DNA PT Program

- **2013**
  - Evaluations based on genotype and clinical assessment
  - Each allele counts as 5% and the clinical assessment counts for 10% of the score
  - Note: Sample was not evaluated if it contained a mutation not part of the program’s panel

- **2016**
  - Laboratories are evaluated for all 5 specimens based on their specific mutation panel
  - Note: Clinical assessments may differ from lab to lab based on the program’s mutation panel and/or screening algorithm
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</table>

\(^1\) Varies by the sequencing technology used and/or whether filters are applied to mask certain results

\(^2\) The 2183AA>G mutation is used for the interpretation of the 2184delA mutation and is not reported

\(^3\) Note that the InPlex-40+4 contains two non CF causing variants, I148T (c.443T>C) and D1270N (c.3808G>A) are not counted in these numbers
Cystic Fibrosis Key Points

- **CF** is caused by mutations in the **CFTR** gene (chromosome 7)
- Early detection of CF is associated with an increased lifespan
- 4 NBS algorithms used to detect CF
  - IRT/IRT (no molecular component)
  - IRT/DNA: elevated IRT $\rightarrow$ **CFTR** mutation(s)
  - IRT/IRT/DNA: elevated IRT $\rightarrow$ **CFTR** mutation(s)
  - IRT/DNA/EGA (elevated IRT $\rightarrow$ **CFTR** mutations $\rightarrow$ gene sequencing (only algorithm that defines screen positive with 2 **CFTR** mutations)
- **CF** newborn screening has a high false positive rate
Cystic Fibrosis Key Points cont.

- HGVS nomenclature describes the nature of the mutation (as opposed to legacy nomenclature)
  - Eg. F508del (legacy) vs. c.1521_1523delCTT (HGVS)

- Most NBS programs define a screen positive as ↑IRT and at least 1 CFTR mutation

- NSQAP’s CF DNA PT program is graded based on clinical assessment and correct genotype
Thank you!

Newborn Screening

Saving Lives.

Promoting Healthier Babies.

Protecting our Future.

For more information please contact Centers for Disease Control and Prevention

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