Implementing Next Generation Sequencing as a Third-Tier Newborn Screen for Cystic Fibrosis in New York State
Disclosures

Illumina provided reagents for 2014 validation study and a loaned instrument.
Cystic Fibrosis (CF) NBS in New York State

IRT–DNA algorithm

IRT immunoassay
MP Biomedicals, Perkin Elmer

Mutation panel
Abbott, Hologic, Luminex

2MUT 65.2%* (100%**)
1MUT 1.7%* (4.5%**)
VHIRT 0.6%* (1.1%**)

Referral

2013–2016
850 infants referred annually
23–36* confirmed CF (51–59**)

PPV
Overall 3.7%* (7.2%**)
2MUT 65.2%* (100%**)
1MUT 1.7%* (4.5%**)
VHIRT 0.6%* (1.1%**)

*CF
**CF, CRMS, Possible CF, 2MUT/sweat negative
Only infants with a final diagnosis assigned are included in PPV.
The problem

Current algorithm: low PPV (high FPR)
- impact on families
- healthcare cost

Mutation panels not comprehensive

Diverse NYS CFTR mutation spectrum
- 439 infants with CF
  - 160 different variants
  - 95% rare/private
- need to refer 1MUT (29%), VHIRT (6%)

Sequence 1MUT, VHIRTs and refer only 2MUT
- Sanger labor intensive, expensive
- NGS???
A possible solution?

Illumina MiSeqDx Cystic Fibrosis Clinical Sequencing Assay (CSA)

- FDA-cleared IVD
- amplicon-based
- next generation sequencing assay
- 27 CFTR exons, intron/exon boundaries, 2 deep intronic
- point mutations, small ins/del, 2 large del, intron 8 polyTG/T

Goals
1. Validate DBS.
2. Assess clinical validity.
3. Assess logistics, potential for implementation.
Technical validation, N=266 DBS

- DNA extracted from 1 x 3-mm DBS
- Genotyped using CSA, validated using independent methods
- Sanger sequenced >10 samples (to assess false negative rate)

- 94.7% (252/266) samples w/ 100% call rate on first pass
- Median read depth = 9,550X (5–121,666X)
- Excellent (not perfect) concordance
  - 1 recurrent false positive/position fail (p.Q1035K)
  - 1 false negative (c.1679+1.6kbA>G)
  - 1 recurrent reporting issue (c.1973_1985del13ins5)
  - 1 recurrent mutation causing sample fail (homozygous c.1817_1900del84)

<table>
<thead>
<tr>
<th>DNA</th>
<th>CSA Specs</th>
<th>DBS DNA tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extr Method</td>
<td>Any validated</td>
<td>Saavedra-Matiz, 2013, Clin Chem</td>
</tr>
<tr>
<td>Conc. (A260/A280)</td>
<td>50 ng/µl</td>
<td>~1 – 6 ng/µl</td>
</tr>
<tr>
<td>Amount</td>
<td>250 ng</td>
<td>~5 – 30 ng</td>
</tr>
</tbody>
</table>
Clinical validation

Illumina 139 Variant Assay
- Panel sensitivity = 86.0%
- Refer 2MUT only → 75.4% CF

Illumina Clinical Sequencing Assay (CSA)
- Panel sensitivity = 96.8%
- Refer 2MUT only → 94.1% CF

Comprehensive Genotyping*
- Sensitivity = 99.2%
- Refer 2MUT only → 98.6% CF

*complete Sanger, del/dup exons 1-27, gap PCRs

Cost-benefit analysis of adding supplemental assays
94.1% – 98.6%

Diverse NYS CFTR mutation spectrum
- 160 variants among 439 CF patients

All before taking FN due to low IRT into account
# New York State IRT-DNA-SEQ algorithm

Panel: CSA–Supplemental (CSA-S)

<table>
<thead>
<tr>
<th>Variant(s)</th>
<th># alleles</th>
<th>Method(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outside coding region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1679+1643G&gt;T 9-bp downstream of</td>
<td>3</td>
<td>bioinformatics</td>
</tr>
<tr>
<td>c.1679+1634A&gt;G (1811+1.6KbA&gt;G)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Validated for del/ins ≤3 bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1817_1900del84 (1949del84)</td>
<td>3</td>
<td>gap-PCR / qPCR</td>
</tr>
<tr>
<td>Large del/dup</td>
<td></td>
<td></td>
</tr>
<tr>
<td>exon 2 del/dup</td>
<td>5</td>
<td>qPCR</td>
</tr>
<tr>
<td>exon 17b del/dup</td>
<td>3</td>
<td>qPCR</td>
</tr>
<tr>
<td>Positions missed (see FDA recall)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.3615delC (3747delC)</td>
<td>1</td>
<td>bioinformatics</td>
</tr>
</tbody>
</table>

**CSA sensitivity = 96.8%**
Refer 2MUT only → 94.1% CF

+15 CF alleles

**CSA-S sensitivity = 98.5%**
Refer 2MUT only → 97.5% CF

# alleles detected using supplemental panel among 439 CF patients
Before taking FN due to low IRT into account
Strategy and significance

Fall 2017: implementation of IRT-DNA-SEQ

Third-tier genotyping labs at Wadsworth
- CSA: Applied Genomics Technologies Core
- Supplemental: Newborn screening lab

Molecular dx at screening
↓ false positive referrals (900 → 100)
↓ healthcare costs
impact on families

Molecular dx at screening
↓ false positive referrals (900 → 100)
↓ healthcare costs
impact on families

- Low throughput (6/run)
- Long run time (~2.5 days)
- $$$
Prospective blind pilot study

- Specimens tested, assessed and referred using current IRT-DNA CF NBS algorithm
- 1MUT and VHIRT blinded and tested using CSA-S
- Assess
  - impact on referral rate
  - infrastructure – handling, batching, testing, reporting
  - effect on turnaround time (TAT)
Prospective blind pilot study

1/23/17 – 4/03/17

IRT-DNA

<table>
<thead>
<tr>
<th>Tier 1 IRT</th>
<th>Tier 2 Luminex-39</th>
</tr>
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<tbody>
<tr>
<td>45,388</td>
<td>2,560</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
</tr>
<tr>
<td>1</td>
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<table>
<thead>
<tr>
<th>VHIRT</th>
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<tbody>
<tr>
<td>42</td>
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<table>
<thead>
<tr>
<th>Screen Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,420</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total Referrals</th>
</tr>
</thead>
<tbody>
<tr>
<td>140</td>
</tr>
</tbody>
</table>

IRT-DNA-SEQ

<table>
<thead>
<tr>
<th>Tier 1 IRT</th>
<th>Tier 2 Luminex-39</th>
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<tbody>
<tr>
<td>45,388</td>
<td>2,560</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Tier 3 Illumina CSA-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>140</td>
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<table>
<thead>
<tr>
<th>Variants</th>
</tr>
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<tbody>
<tr>
<td>2</td>
</tr>
<tr>
<td>1 Variant (Carrier)</td>
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<table>
<thead>
<tr>
<th>Screen Negative</th>
</tr>
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<tbody>
<tr>
<td>87</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Total Referrals</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
</tr>
</tbody>
</table>

81.4% reduction in referrals

114 unnecessary referrals
Variant interpretation and reporting

Variant classification
- ACMG guidelines
- Databases used: CFTR2, SickKids, EmVClass, ClinVar, PubMed, Google/Scholar, ExAC/gnoMAD

Modifications to mailers
- Screening sensitivity
- Genetic counseling, recurrence risk, family planning

<table>
<thead>
<tr>
<th>Variant Types</th>
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<tbody>
<tr>
<td>CF-causing</td>
</tr>
<tr>
<td>Pathogenic</td>
</tr>
<tr>
<td>Likely pathogenic</td>
</tr>
<tr>
<td>Varying clinical consequence</td>
</tr>
<tr>
<td>Unknown significance</td>
</tr>
<tr>
<td>Variant of uncertain significance (VOUS)</td>
</tr>
<tr>
<td>Non CF-causing</td>
</tr>
<tr>
<td>(available upon request)</td>
</tr>
<tr>
<td>Benign</td>
</tr>
<tr>
<td>Likely benign</td>
</tr>
<tr>
<td>Polymorphism</td>
</tr>
<tr>
<td>(not reported)</td>
</tr>
</tbody>
</table>
Prospective blind pilot study

140 infants: 26 referrals, 87 carriers, 29 screen neg
59 different CFTR variants

15 CF-causing (on NYS panel)
  6 CF-causing
  1 Likely pathogenic (splicing, nonsense, del/dup/ins)

7 Varying clinical consequences
3 Unknown significance
12 VOUS (missense, synonymous, non-coding)

5 Not CF-causing
5 Benign/likely benign
5 Common polys (not reported)

22 VOUS

| p.R74W*     | p.I37L |
| p.D1270N*   | p.I807T |
| p.V201M*    | p.R851Q |
| p.F640=*    | p.R31L |
| p.R117H;7T  | p.S912L |
| 5T;13TG     | p.Y1014C |
| 5T;12TG     | p.N1148K |
| 5T;11TG     | p.P1332= |
| p.R1070Q    | p.M1354T |
| p.R170H     | p.C1355F |
| p.L467F     | p.D1445N |

*typically in cis
Considerations – Turnaround time (TAT)

Blind Pilot Study
- 3 (2 – 3) runs/week
- 5 (2 – 6) samples/run

Batching schema (6 samples per run)
- 3 instruments
- 6 samples/run + pos + neg
- day, # specimens, # held

Turnaround time IRT-DNA-SEQ
- IRT days 1-2
- DNA days 2-3
- DNA confirmation days 3-4
- CSA-S days 3-6
- CSA-S confirmation days 6-7
- Report days 6-7

Confounders: batching, fails, confirmations, day, holidays
Considerations - Troubleshooting

Company can’t provide guidance for off-label use of products (FDA)

CSA protocol modifications
- Increase DNA input volume from 5 µl to 15 µl
- Scrutinize quality metrics if cluster density <300
- Quantitate library preps using Qubit
- Triple amount pooled library (9 µl → 27 µl) sequenced if library yields <1.0 ng/µl
- Eliminate normalization step?
What’s next?

Go-live mid-Fall 2017

Custom Illumina TruSeq Custom Amplicon panel
- MiSeqDx (RUO/Dx mode), MiSeq (RUO)
- CFTR coding, targeted non-coding, del/dup
- Higher throughput (96 vs 6 per run)
- Virtual 2\textsuperscript{nd} tier (read panel CF-causing variants) $\rightarrow$ unmask 3\textsuperscript{rd} tier if 1MUT or VHIRT
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**NYS Newborn Screening Program**

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- Michael Palumbo, BA

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- Matt Shudt, BS

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