Newborn Screening for Cystic Fibrosis by Use of a Multiplex Immunoassay

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BACKGROUND: Since its beginnings, newborn screening for cystic fibrosis (CF) using an assay for immunoreactive trypsinogen (IRT) has been plagued by a high rate of false-positive results (screen positive, diagnosis negative), despite attempts to reduce this rate by use of altered cutoffs and second-tier DNA testing. IRT exists as 2 isoforms: IRT1 and IRT2, with IRT2 being more closely aligned with pancreatic disease, including CF. Assay standardization between programs is a continuing problem because the IRT assays currently in use variously recognize either 1 or both isoforms. Here we report the development of a multiplexed assay for both forms of IRT simultaneously.

METHODS: Using 2 different Luminex bead sets, we developed assays for each IRT isoform separately and then combined them. Using the sum of IRT1 and IRT2 values (IRT1 + IRT2), we compared the results with a CF kit currently in use.

RESULTS: In a sample set consisting of 16 cases confirmed positive for CF, we established a cutoff at >97 μg/L total IRT. Seven of 8 carriers with 1 CF mutation screen-positive by the standard method were also screen-positive by IRT1 + IRT2. Of 32 cases screen-positive by standard IRT, 11 were screen-negative by IRT1 + IRT2. None of these 11 cases had CF mutations identified by the screening program.

CONCLUSIONS: These data indicate that the multiplex method with specificity for 2 isoforms of IRT has performance comparable to that of a standard IRT method and the advantage of improved standardization by detection of the 2 isoforms.

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Newborn screening for cystic fibrosis (CF) has evolved following the report in 1979 by Crossly et al. (1) that blood immunoreactive trypsinogen (IRT) concentrations are increased in newborn infants with CF. There are several molecular forms of IRT; the 2 major forms secreted by exocrine cells of the pancreas are trypsinogen 1 (cationic trypsinogen or IRT1) and trypsinogen 2 (anionic trypsinogen or IRT2) (2, 3). Normally, the IRT1 form is present in higher concentrations; however, in pathologic conditions such as pancreatitis, the IRT2 form becomes predominant (4). Today 46 states provide newborn screening (NBS) for CF, all using IRT for the initial screen. In New York State in 2008, the most recent year with complete data, 1585 infants were screen-positive and 53 were confirmed to have CF, a ratio of 30:1 screen-positive to confirmed CF. In an effort to minimize the number of false-positive results, second-tier testing after an initial positive screen has been applied using several different protocols, such as retesting IRT positives on newly collected specimens or following up positive IRT tests with DNA analysis of the same first specimen (5).

Several investigators have developed immunoassays for IRT, and current commercial assays use both monoclonal and polyclonal antibodies for IRT (6–8). The heterogeneous nature of IRT and differing specificity of antibodies to the various components have raised concerns regarding the standardization and external QC of the assay. As noted by Li et al. (9), the lack of a universally acceptable IRT standard has made the comparison of absolute IRT values among commercial immunoassays difficult. As reported by Lafont et al. (10), trypsin exists in many forms in serum but is not recognized equally among immunoassays, thus contributing to the discordant results when comparing assays.

In the present study, we report development of a suspension array multiplexed immunoassay for the 2 specific isoforms of trypsinogen, IRT1 and IRT2. The specificity of the assay for the 2 isoforms allows development of external QC for the heterogeneous forms of IRT and allows for analysis of the IRT1-to-IRT2 ratio.
as a potential added parameter before referral for mutation analysis.

**Materials and Methods**

**ANTIBODY REAGENTS**

We coupled antitrypsin isoform–specific monoclonal antibodies to Luminex xMAP microspheres according to the instructions provided by Luminex (http://Luminexcorp.custhelp.com) with 100 μg IRT1 capture monoclonal antibody (HYB 021-08-02; Affinity Bioreagents) coupled to 5 × 10^6 Luminex carboxy microspheres, region 177 (L-100-C177-04). Similarly, 100 μg IRT2 capture monoclonal antibody (8607; Medix Biochemica) was coupled to 5 × 10^6 Luminex carboxy microspheres, region 183 (L-100-C183-04).

We biotinylated polyclonal detector antibody (K50900; Biodesign International) using the Fluoresporter biotin–XX labeling kit (Invitrogen) according to the manufacturer’s instructions. We used a sheep polyclonal antitrypsin with the biotin label from the manufacturer (BAF3586; R&D Systems). The 2 antibodies were combined to make the detector mix, with K50900 at a concentration of 5.0 μg/mL and BAF3586 at 125 μg/mL.

We determined the performance of the antibodies by titer to evaluate affinity and sensitivity and by cross-reaction tests to evaluate specificity. The concentration of each antibody was titrated to achieve optimal performance.

**ASSAY CALIBRATORS**

We used IRT1 calibrators from the standard method kit (MP Biomedical). These calibrators were prepared with IRT1 only (personal communication, MP Biomedical). IRT2 calibrators were prepared from recombinant IRT2 (R&D Systems). We treated serum with antitrypsin isoform–specific antibodies, we used antitrypsin isoform–specific specificity was tested for IRT1 and IRT2; as capture antibodies, we used antitrypsin isoform–specific monoclonal antibodies; in the multiplex assay, we de

**ASSAY PROCEDURE**

We prepared assay buffer (pH 7.4) containing PBS, 0.055% Tween 20, 0.05% sodium azide, and 0.2% gelatin. We added 1 mg/L aprotinin (Sigma Chemical Co.) to the assay buffer to prepare the spot elution buffer. The dried blood spots (a single 3-mm punch per well) were eluted overnight at room temperature in 100 μL elution buffer with gentle shaking. For the assay, we combined 75 μL sample eluate with 25 μL trypsin 1 and trypsin 2 bead mix to obtain 2000 microspheres per well for each of the analytes. The capture incubation was for 3 h at 37 °C with gentle shaking. We washed microspheres 3 times in 100 μL assay buffer then added 100 μL antitrypsin detector antibody mix to each well. The detector antibodies were incubated for 1 h at 37 °C with gentle shaking, and the microspheres were again washed 3 times with 100 μL assay buffer. For detection, 100 μL streptavidin phycoerythrin (Invitrogen, S-866) was added at 4 μg/mL and incubated for 30 min at 37 °C. We aspirated the assay plate and resuspended the microspheres in 100 μL Luminex sheath fluid for analysis.

Analysis and data collection were performed in multiplex acquisition mode on the Luminex 100 instrument. We used Luminex software LX100 IS 2.3 to calculate the results, expressed in median fluorescence intensity (MFI) of 100 microspheres of each set, and the software LiquiChip Analyser 1.0 (Qiagen) to analyze the data.

**SAMPLES**

All newborn specimens assayed were provided by the New York State Department of Health Newborn Screening Laboratory under Institutional Review Board (IRB) protocol number 07-016. In compliance with the New York State Department of Health IRB, no identifying information was transferred with the specimens—only the CF screening results (IRT and DNA status) were maintained. The IRT screening results were determined with the Blood Spot Trypsin MW ELISA (07596307; MP Biomedicals). In the NBS IRT assay, and in our assay, all samples were measured singly.

**Results**

We prepared calibrators with and without aprotinin and determined that calibrators without aprotinin had <60% recovery compared with calibrators with aprotinin. Also, we noted that the calibrators and buffer of the standard method (MP Biologicals kit) contain aprotinin.

**DEVELOPMENT AND OPTIMIZATION OF ASSAY**

Each immunoassay was developed separately and optimized for affinity, sensitivity, and specificity. The assays were then combined into a multiplex format. The specificity was tested for IRT1 and IRT2; as capture antibodies, we used antitrypsin isoform–specific monoclonal antibodies; in the multiplex assay, we detected no cross-reactivity between the antibodies to IRT1 and IRT2, as determined by calibration curves. Although the lower limit of quantification is not important in these assays (the program screens for greatly increased IRT), antibody concentrations were optimized to obtain sensitivity at 13 μg/L for IRT1 and 8
μg/L for IRT2. Using the mean of 8 independent measurements for each concentration of calibrators, we examined the assays’ precision profiles; the CVs were approximately 7% for the lower concentrations of IRT2 and 12% for the lower concentrations of IRT1. Standard curves were linear up to the highest standard concentration used in the IRTS assay (250 μg/L). We calculated intra- and interassay variations from controls with 3 levels of IRT concentrations (75, 195, 284 μg/L for IRT1; 31, 62, 125 μg/L for IRT2) and performed intra assay evaluations over a 3-week period using 14 plates, with the interassay variation calculated from each plate. At all concentrations, the interassay CV ranged from 6% to 14% for IRT1 and 9% to 10% for IRT2. The intraassay CVs for both ITR1 and IRT2 were 0% to 18% (data not shown).

CORRELATION STUDY
We compared samples analyzed in the IRT1-IRT2 assay with the values obtained from the standard method. The selection criteria for the NBS samples analyzed in the correlation study were specified for low to high IRT values: 3-mm punches from 168 blood spots with values determined by the NBS laboratory divided into 5 groups as shown in Table 1. Summed assay values (IRT1 + IRT2) compared with the standard method (IRTS) and had a correlation coefficient of 0.75 (Fig. 1). IRT1 + IRT2 [mean 63.8 (SD 62.6) μg/L] was lower than mean IRTS [92.9 (69.0) μg/L]. This is not surprising owing to the different formats of the assays and different antibodies (10). By the IRTS assay, 133 samples were screen-negative. Of the 35 samples screen-positive by the IRTS, 11 samples were screen-negative by the IRT1 + IRT2 method. However, each of these screen-negative cases by IRT1 + IRT2 had been confirmed to have no CF mutations by the screening program in its second-tier mutation analysis and was reported as negative for CF. Having no link to the original specimens, it was impossible to verify these findings.

Also, we tested the IRT2 spiked DBS calibrators in the standard method and found that none of the calibrators (ranging from 0–1000 μg/L IRT2) had IRTS measurements above background. Therefore, we conclude that this IRTS does not detect IRT2. This is of some concern, given that IRT2 has been reported to be increased in CF patients (1, 4).

POPULATION STUDY
We analyzed 597 population study samples on specimens from 4 consecutive days of the screening program; the distribution is shown in Fig. 2. Two cases in this population were screen-positive by the IRT1 + IRT2 criteria; of these, 1 case fell within the top 5%
of the IRTS method and had 1 CF mutation detected; the second case was screen-negative by the IRTS.

**SCREEN-POSITIVE SAMPLE EVALUATION**

To compare the IRT1/IRT2 assay with screen-positive results from the IRTS, we evaluated 164 samples that were determined to be IRTS screen-positive by the New York NBS laboratory, consisting of a total of 19 confirmed positive cases with 2 CF mutations, 8 confirmed positive cases with 1 CF mutation, and 137 cases confirmed as non-cystic fibrosis, with no CF mutations detected. The screen-positive cutoff established by the New York NBS laboratory for the standard method is a concentration ≥170 µg/L. The screen-positive sample evaluation shown in Table 2 indicated that a total trypsin (sum of IRT1 and IRT2) cutoff of >97 µg/L would be necessary to achieve 100% sensitivity for the confirmed disease population.

Table 3 shows the analysis of 8 single-mutation CF carrier samples that were screen-positive as established by the ≥170 µg/L cutoff for the IRTS assay. Seven of these carrier samples would also have been screen-positive by use of the IRT1 + IRT2 with cutoff of >97 µg/L.

### Table 2. Screen-positive sample evaluation.

<table>
<thead>
<tr>
<th>Two mutations, confirmed disease</th>
<th>IRT1, µg/L</th>
<th>IRT2, µg/L</th>
<th>IRT1 + IRT2, µg/L</th>
<th>IRT1:IRT2</th>
<th>IRT5, µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>F508del/G508del +G&gt;A</td>
<td>121</td>
<td>129</td>
<td>250</td>
<td>0.938</td>
<td>248</td>
</tr>
<tr>
<td>F508del/F508del</td>
<td>63.6</td>
<td>93.2</td>
<td>156.8</td>
<td>0.682</td>
<td>183.5</td>
</tr>
<tr>
<td>F508del/F508del</td>
<td>104</td>
<td>269</td>
<td>373</td>
<td>0.387</td>
<td>248</td>
</tr>
<tr>
<td>F508del/F508del</td>
<td>96.6</td>
<td>103</td>
<td>199.6</td>
<td>0.938</td>
<td>248</td>
</tr>
<tr>
<td>F508del/W1282X</td>
<td>104</td>
<td>81</td>
<td>185</td>
<td>0.701</td>
<td>226.5</td>
</tr>
<tr>
<td>F508del/N1303K</td>
<td>96.6</td>
<td>93.2</td>
<td>189.8</td>
<td>0.718</td>
<td>226.5</td>
</tr>
<tr>
<td>F508del/Undetected</td>
<td>104</td>
<td>129</td>
<td>233</td>
<td>0.682</td>
<td>183.5</td>
</tr>
<tr>
<td>F508del/R117H, 7T, 9T, var</td>
<td>67.6</td>
<td>33.6</td>
<td>101.2</td>
<td>2.012</td>
<td>194.3</td>
</tr>
<tr>
<td>F508del/F508del</td>
<td>131</td>
<td>129</td>
<td>260</td>
<td>1.016</td>
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</tr>
<tr>
<td>F508del/N1303K</td>
<td>131</td>
<td>129</td>
<td>260</td>
<td>1.016</td>
<td>248</td>
</tr>
<tr>
<td>F508del/Undetected</td>
<td>154</td>
<td>354</td>
<td>508</td>
<td>0.435</td>
<td>248</td>
</tr>
<tr>
<td>F508del/R117H, 7T, 9T, var</td>
<td>67.6</td>
<td>33.6</td>
<td>101.2</td>
<td>2.012</td>
<td>194.3</td>
</tr>
<tr>
<td>F508del/F508del</td>
<td>131</td>
<td>129</td>
<td>260</td>
<td>1.016</td>
<td>248</td>
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<tr>
<td>F508del/N1303K</td>
<td>154</td>
<td>354</td>
<td>508</td>
<td>0.435</td>
<td>248</td>
</tr>
<tr>
<td>F508del/Undetected</td>
<td>154</td>
<td>354</td>
<td>508</td>
<td>0.435</td>
<td>248</td>
</tr>
</tbody>
</table>

* Cutoff >97 µg/L.

### Table 3. Carriers (1 CF mutation) screen-positive by IRTS.

<table>
<thead>
<tr>
<th>One mutation</th>
<th>IRT1, µg/L</th>
<th>IRT2, µg/L</th>
<th>IRT1 + IRT2, µg/L</th>
<th>IRT1:IRT2</th>
<th>IRT5, µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>R553X</td>
<td>45.4</td>
<td>70.7</td>
<td>116.1</td>
<td>0.642</td>
<td>197.9</td>
</tr>
<tr>
<td>F508del</td>
<td>159</td>
<td>289</td>
<td>448</td>
<td>0.550</td>
<td>226.5</td>
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<tr>
<td>D1152H</td>
<td>223</td>
<td>187</td>
<td>410</td>
<td>1.193</td>
<td>226.5</td>
</tr>
<tr>
<td>3120 + 1G&gt;A</td>
<td>254</td>
<td>790</td>
<td>1044</td>
<td>0.322</td>
<td>226.5</td>
</tr>
<tr>
<td>A455E</td>
<td>59</td>
<td>130</td>
<td>189</td>
<td>0.454</td>
<td>186.3</td>
</tr>
<tr>
<td>F508del</td>
<td>87.4</td>
<td>111</td>
<td>198.4</td>
<td>0.787</td>
<td>213.7</td>
</tr>
<tr>
<td>711 + 1G&gt;T</td>
<td>44.3</td>
<td>51</td>
<td>95.3</td>
<td>0.869</td>
<td>174.7</td>
</tr>
<tr>
<td>F508del</td>
<td>57.5</td>
<td>70.1</td>
<td>127.6</td>
<td>0.820</td>
<td>226.5</td>
</tr>
</tbody>
</table>

* Cutoff >97 µg/L.
Of the 137 cases that were screen-positive by the IRTS assay but had no CF mutations detected, 26 would have been screen-negative using the IRT1 + IRT2 cutoff of >97 μg/L, a reduction of 19% in the false-positive rate in this selected study population.

Analysis of 3 cases of confirmed disease with an IRTS value below the 170 μg/L cutoff is shown in Table 4. Two of the 3 cases would have been screen-positive using the IRT1 + IRT2 assay criteria, with values >97 μg/L.

Discussion

The false-positive rate in newborn screening for CF has remained persistently high, despite numerous attempts to lower it (5). To reduce this, 1 unexplored approach, separate analysis of the 2 isoforms of trypsin, was examined in these studies. The goal in this study was the development of a multiplexed assay for CF using the 2 major trypsinogen isoforms that would meet screening standards for clinical accuracy when compared with current commercial NBS IRT assays.

The correlation study showed substantially equivalent performance of the assays in segregation of a screen-positive population. Importantly, of the 11 discrepant cases that were screen-positive in the IRTS assay but had no mutations detected by the screening program, all were screen-negative in the IRT1 + IRT2 assay, suggesting a greater specificity for the multiplex assay. (The current protocol of New York State NBS program considers samples with IRT values ≥170 μg/L as screen-positive regardless of mutation analysis results.) Using the IRT1 + IRT2 cutoff of >97 μg/L, we achieved a reduction of 19% in the false-positive rate in this selected study population.

Analysis of a screen-positive population with confirmed disease indicated that a cutoff of ≥97 μg/L in the IRT1 + IRT2 assay would be needed to achieve 100% sensitivity for these samples. Although this cutoff is substantially lower than that developed for the IRTS method of 170 μg/L, it is nearer to the cutoff of 112 μg/L reported for a monoclonal antibody–based method for total IRT (8). Li et al. (9) reported recoveries in their IRT spiked preparations between 45% and 60% as measured by 2 commercial immunoassays. It is likely that more specific immunoreactivity is observed when measuring the 2 isoforms individually in a multiplex assay as reported here.

Cystic fibrosis carriers have been shown to have higher IRT values than the normal population (11, 12). In a screening program in which the goal is detect disease and not carrier status, discrimination of disease state from carrier state could be of great help. In these studies, use of the sum of IRT1 + IRT2 was unable to discriminate the carrier population, with 7 of 8 carriers who were screen-positive by the IRTS assay noted also to be screen-positive by the IRT1-IRT2 criteria. Moreover, the ratio of IRT1 to IRT2 was unable to distinguish carriers from unaffected, as proposed by Itkonen et al. (4).

In 3 individuals identified with confirmed disease who had an IRTS below the cutoff (per NBS protocol), 2 were screen-positive by our assay criteria (Table 3). More studies are needed to determine whether these results indicate that the IRT1 + IRT2 assay has greater sensitivity.

This study demonstrates that the IRT1 + IRT2 multiplexed assay for CF has substantial equivalence in detecting screen-positive specimens compared with the standard IRT method. Thus, this multiplexed assay for the 2 IRT isoforms has equal sensitivity in detecting CF and could offer improved specificity over the standard single-analyte methods used in newborn screening. The specificity of the antibodies for the 2 isoforms might also provide advantages in the standardization and preparation of external QC materials (9).

Perhaps most importantly, the multiplex format allows additional biomarkers, e.g., pancreatitis-associated protein (13), to be added in the future to improve the specificity of this assay. Building on our previous work with multiplex assays (14–16), the assay described here brings us a step closer to even more comprehensive multiplex assays for newborn screening; the combination of this CF assay with immunoassays for congenital hypothyroidism and congenital adrenal hyperplasia into a single multiplex assay would...
bring benefits, serving as a substitute for the 3 current immunoassays used by NBS, thereby saving time in the screening laboratory, specimen usage, and perhaps cost.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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Consultant or Advisory Role: None declared.
Stock Ownership: None declared.

References