Development of Internal Controls for the Luminex Instrument as Part of a Multiplex Seven-Analyte Viral Respiratory Antibody Profile

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The ability of the Luminex system to simultaneously quantitate multiple analytes from a single sample source has proven to be a feasible and cost-effective technology for assay development. In previous studies, my colleagues and I introduced two multiplex profiles consisting of 20 individual assays into the clinical laboratory. With the Luminex instrument’s ability to classify up to 100 distinct microspheres, however, we have only begun to realize the enormous potential of this technology. By utilizing additional microspheres, it is now possible to add true internal controls to each individual sample. During the development of a seven-analyte serologic viral respiratory antibody profile, internal controls for detecting sample addition and interfering rheumatoid factor (RF) were investigated. To determine if the correct sample was added, distinct microspheres were developed for measuring the presence of sufficient quantities of immunoglobulin G (IgG) or IgM in the diluted patient sample. In a multiplex assay of 82 samples, the IgM verification control correctly identified 23 out of 23 samples with low levels (<20 mg/dl) of this antibody isotype. An internal control microsphere for RF detected 30 out of 30 samples with significant levels (>10 IU/ml) of IgM RF. Additionally, RF-positive samples causing false-positive adenovirus and influenza A virus IgM results were correctly identified. By exploiting the Luminex instrument’s multiplexing capabilities, I have developed true internal controls to ensure correct sample addition and identify interfering RF as part of a respiratory viral serologic profile that includes influenza A and B viruses, adenovirus, parainfluenza viruses 1, 2, and 3, and respiratory syncytial virus. Since these controls are not assay specific, they can be incorporated into any serologic multiplex assay.

The Luminex (Austin, Tex.) Multi-Analyte Profiling (Lab-MAP) technology is based on microscopic polystyrene particles called microspheres that are internally labeled with two different fluorophores. When excited by a 635-nm laser, the fluorophores emit light at different wavelengths, 658 and 712 nm. By varying the 658-nm/712-nm emission ratios, an array of up to 100 different fluorescent profiles has been created. Using precision fluidics, digital signal processors, and advanced optics, the unique Luminex 100 analyzer classifies each microsphere according to its predefined fluorescent emission ratio. Thus, multiple microspheres coupled to different analytes can be combined in a single sample. A third fluorophore coupled to a reporter molecule allows for quantitation of the interaction that has occurred on the microsphere surface.

The Luminex 100 system has been shown to be a feasible and cost-effective technology for assay development. Our institute has validated two multiplex assays for use in the clinical laboratory, one that includes a profile of six cytokines and one that includes a profile of pneumococcal antibodies of 14 different serotypes (J. W. Pickering, T. B. Martins, R. W. Greer, M. C. Schroder, M. E. Astill, C. M. Litwin, and H. R. Hill, submitted for publication). Other published applications of the current Luminex format include analysis of single-nucleotide polymorphisms (5, 8) and mutation screening (1). With the Luminex instrument’s ability to classify up to 100 distinct microspheres, we now have the ability to add true internal controls to determine correct sample and reagent addition, identify interfering substances such as heterophile antibodies (7) and rheumatoid factors (RFs), and monitor instrument parameter performance. During the development of a seven-analyte serologic viral respiratory profile, internal controls were investigated to determine if the correct sample was added and if interfering RF was present in the sample. When reporting negative results, a concern among technicians in a clinical laboratory is whether the patient sample was actually added to the reaction mixture. The patient sample may be left out of the reaction mixture due to human or automated instrument pipetting errors, sample clots, or other factors. These sampling errors generally go undetected in standard laboratory assays. Because of the multiplexing ability of the Luminex, true internal controls for the validation of sample addition can now be added to each individual well or reaction. To accomplish this, a goat anti-human immunoglobulin M (IgM) or anti-human IgG antibody is coupled to a specific microsphere that can be added to IgM- or IgG-specific serologic assay panels. This coupled microsphere then binds IgM or IgG isotypes present in the patient’s serum. If the patient sample is present, it will be detected by the anti-human IgM or IgG reporter conjugate, generating a semi-qualitative result.

A second control was developed to detect significant levels of interfering IgM RFs. RFs represent one of the most serious problems in IgM testing (3). RFs are autoimmune antibodies, usually of the IgM class, which recognize human IgG. In anti-
body testing, specific IgG present in the serum binds to anti-GEN, presenting a site for the anti-IgG IgM RF to bind. The IgM is then recognized by the labeled anti-IgM conjugate, giving rise to a false-positive result (Fig. 1). Traditional enzyme immunoassay (EIA) methods for IgM antibody testing typically employ an absorbent in the sample diluent consisting of a goat anti-human IgG antibody to minimize potential RF IgM interference. In our assay, an RF control was developed by coupling human IgG to a specific Luminex microsphere. If RFs are present in the patient sample, they will bind to the human IgG. The reporter anti-human IgM antibody added next to detect specific respiratory viral IgM antibodies will also detect the potentially interfering IgM RFs.

**MATERIALS AND METHODS**

**Coupling of microspheres.** A Luminex multiplex panel of nine analytes, including seven viral antigens and two internal controls, was developed. Partially purified antigens for adenovirus, respiratory syncytial virus, influenza A virus (Flu A), influenza B virus, and parainfluenza viruses 1, 2, and 3 were purchased from Chemicon International (Temecula, Calif.) and BioWhittaker (Walkersville, Md.) and covalently coupled to carboxylated Luminex microspheres. For the sample addition control, 1 μg of goat anti-human IgG (Sigma Chemical, St. Louis, Mo.) per ml or 5 μg of goat anti-human IgM (Sigma Chemical) per ml was coupled to specific microspheres. The interfering RF control was developed by coupling 50 μg of human IgG (Sigma Chemical) per ml to another microsphere. Coupling of the antigens or antibodies to the carboxylated microspheres was accomplished using a two-step carboximide reaction (4). Carboxylated microspheres were activated for 20 min at 6.25 × 10⁶/ml in phosphate-buffered saline (PBS; pH 6.1) with 5 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide–HCl and N-hydroxysuccinimide (Pierce, Rockford, Ill.) per ml. Activated microspheres were then washed by centrifugation with PBS, pH 7.2, and incubated with the previously described antigens and antibodies for 1 h at room temperature on a rocker. The coupled microspheres were then washed twice with PBS (pH 7.2)–0.05% Tween 20 and re suspended in 1 ml of PBS (pH 7.2)–0.1% bovine serum albumin–0.05% sodium azide. The microspheres were then incubated for 30 min on a rocker to allow blocking of the unreacted sites and stored at 4°C in the PBS-bovine serum albumin-sodium azide mixture.

**Luminex-multiplexed IgG and IgM serology assays.** The nine different analyte-coupled microspheres were mixed together at a concentration of 1.0 × 10⁷ copies of each microsphere/ml. Fifty microliters of the microsphere mixture was added to 100 μl of diluted serum (1:100 in PBS-Tween 20) for a final concentration of 5,000 copies of each individual microsphere (45,000 total) per reaction. Serum samples and microspheres were incubated for 20 min at room temperature using a 96-well microtiter plate on a shaker. This was followed by the addition of 50 μl of R-phycocerythrin-conjugated anti-human IgG or IgM (Jackson ImmunoResearch, West Grove, Pa.) to each well of the microtiter plate. Following a second 20-min incubation on the shaker, the microtiter plate was placed in a Luminex 100 instrument with an XY platform (automated microtiter plate handler), on which the microspheres were counted and analyzed. The amount of antibody bound to the microspheres was determined with anti-human IgG or IgM conjugated to phycoerythrin. When the microspheres were excited at a wavelength of 532 nm, phycoerythrin emitted light at a wavelength of 575 nm. The mean fluorescence intensity (MFI) at 575 nm is directly proportional to the amount of antibody bound to the microspheres. Since the analyte specificity and position of each bead classification in the array are known, a single fluorescent reporter molecule can be used to measure antibody levels for all nine microspheres.

For the serum addition validation control, a calibrator was established to identify patient samples with less than 20 mg of IgM antibody per dl. The normal range for total IgM for individuals 10 years and older is 60 to 253 mg/dl. A semiquantitative result was then established using the calibrator, in which a value of 20 mg of IgM units (MU) equals 20 mg of IgM antibody per dl. The calibration curve was diluted normal human serum with bovine serum albumin until a value of 20 mg/dl was obtained by my standard nephelometric quantitative method. For the RF interference control, a calibrator was created by pooling an RF IgM-positive serum sample with RF-negative bank serum samples until a value of 10 IU/ml was obtained based on a commercial EIA method (Zeus Scientific, Raritan N.J.). By dividing the Luminex MFI value of the unknown sample by the calibrator, an index value (IV) was established to identify any samples containing >10 IU of RF IgM (IV, 1.0 or greater) per ml as having potentially interfering concentrations of RF.

A calibrator for the serologic viral respiratory immunogold assays was developed by pooling slightly positive serum samples. The reactivities of the calibrator and serum samples used in this study were determined with commercially available IgM-specific viral respiratory EIA kits obtained from Immuno-Biological Laboratories (Stuttgart, Germany) and by in-house complement fixation assays. The MFI value obtained by the Luminex technology for the patient sample was divided by the MFI value of the calibrator to calculate an IV. An IV of 1.0 or greater indicated that significant concentrations of IgM antibody to the specific viral antigen were detected, and the result was considered positive. An IV of less than 1.0 was considered negative.

**RESULTS**

For the serum addition validation control, the established calibrator was used to identify patient samples with less than 20 mg of IgM antibody per dl. Eighty-two patient samples were tested on the Luminex as part of a nine-analyte multiplex IgM serological assay, which included the seven viral respiratory antigens and two internal controls. Fifty-nine of the 82 patient samples were reported as valid or acceptable for the presence of at least 20 mg of IgM antibody per dl, indicating that the correct sample (serum) was submitted and that the sample was diluted correctly. Twenty-three of the 82 samples were incorrectly pipetted, receiving only 1/10 of the required sample.

![FIG. 1. False-positive reaction caused by RF interference.](http://cvi.asm.org)
The data shown in Table 2 were taken from the same multiplex assay as described above but show the results with the Flu A antigen-coupled microsphere. Similar to the previous results, samples 6 and 21 were IgM negative for Flu A when they were run under normal conditions (IVs, 0.4 and 0.3, re-

TABLE 1. Results from a multiplex IgM assay including seven viral respiratory antigens and two internal controls showing initial and absorbed results for adenovirus, an RF interference control, and a serum addition control

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial multiplex run</th>
<th>Absorbed with anti-IgG diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenovirus IV&lt;sup&gt;a&lt;/sup&gt;</td>
<td>RF control IV&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 (adenovirus IgG&lt;sup&gt;+&lt;/sup&gt;, IgM&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>0.4 (−)</td>
<td>0.5 (−)</td>
</tr>
<tr>
<td>6 (adenovirus IgG&lt;sup&gt;−&lt;/sup&gt;, IgM&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>0.8 (−)</td>
<td>0.1 (−)</td>
</tr>
<tr>
<td>2 (spiked with RF)</td>
<td>3.0 (+)</td>
<td>4.8 (+)</td>
</tr>
<tr>
<td>6 (spiked with RF)</td>
<td>2.3 (+)</td>
<td>4.8 (+)</td>
</tr>
</tbody>
</table>

<sup>a</sup> IVs of 1.0 were considered positive (see symbols in parentheses).
<sup>b</sup> An IV of 1.0 = 10 IU of IgM per ml.
<sup>c</sup> 20 MU = 20 mg of IgM antibody per dl.
body detection for IgG serologic assays was also developed and performed similarly.

Results with a semiquantitative RF control showed good correlation with the results of an EIA ($r^2 = 0.88$), and in a run of samples from 52 patients, the Luminex assay correctly identified 32 of 32 samples (100%) as having elevated or significant levels (>10 IU/ml) of IgM RF. The effectiveness of the assay with the RF control was further shown by spiking viral respiratory serum samples with RF. By testing samples in a standard serum diluent and a diluent containing anti-human IgG as an absorbent, it was possible to distinguish true IgM-positive samples from false-positive samples caused by interfering RF. Testing patient samples directly for the presence of RF has several advantages over the traditional serologic method of treating all samples with an anti-human IgG absorbent. Commercial IgG absorbents are expensive, adding to the cost $1.75 to $2.50 per patient sample. The cost of adding an RF control on the Luminex is less than $0.25 per sample. Since only a small percentage of the population possesses IgM RFs, treating every sample with an IgG absorbent adds significantly to the cost of an assay. The IgG absorbents have also been shown to remove as much as 20% of the IgM isotypes from serum samples (2). This could potentially cause false-negative results on low-level IgM-positive samples. Additionally, the Luminex internal RF control provides additional information to the clinician by reporting a semiquantitative result for the presence of IgM RFs rather than trying to indirectly block RF interference with an IgG absorbent.

Even though these internal controls were developed in conjunction with the viral respiratory profile, they are not assay specific. They could, therefore, be incorporated in any serologic multiplex assay to ensure that sufficient serum has been added to the reaction mixture or to determine the presence and possible interference of RF in IgM antibody assays.

The multiplexing ability of the Luminex instrument is proving to be a powerful platform for the development of multiple-analyte profiles which require fewer reagents, a smaller volume of patient sample, and lower costs than those of traditional diagnostic methodologies. By employing additional microspheres as internal controls, quality control parameters can now be included for each individual patient or reaction. Internal controls could be developed to ensure that correct concentrations of each individual reagent (sample, conjugate, substrate) have been added and that patient samples are free from known interfering substances such as RFs, heterophile anti-
bodies, bilirubin, hemolysis, and other substances. Internal controls could also be utilized to increase precision and accuracy by monitoring instrument fluctuations, allowing intra- and interassay normalization.

REFERENCES

terence in the solid-phase radioimmunoassay of rubella-specific IgM antibod-

$N$-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling
ington, D.C.