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Principles of the xTAG™ respiratory viral panel assay (RVP Assay)

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1. Background

Multiplexed nucleic assays enable the simultaneous detection of multiple analytes and minimize the cost per result while maintaining high analytical and clinical sensitivity and specificity. The Luminex bead microarray based Universal array platform delivers on these desirable characteristics for both genotyping and infectious disease based assays. The platform utilizes a validated array comprised of 100 spectrally distinct beads, each of which is coupled to a unique oligonucleotide primer sequence comprising the Luminex Universal array (Bortolin et al., 2004). The use of this array for clinical genotyping has been well documented (Bortolin et al., 2004; Strom et al., 2005; Zhu et al., 2007) and most recently its utility for infectious disease testing has been exemplified (Mahony et al., 2007).

Generally genotyping assays require the amplification and interrogation of all alleles present in the test. Infectious disease testing on the other hand, as is the case for the Luminex xTAG™ RVP assay, generally detects the presence of one or more target sequences when present in a clinical sample.

The xTAG™ RVP assay provides clinicians with new tools with greater sensitivity and specificity and improved positive and negative predictive values in detecting respiratory viruses compared to current infectious disease diagnostic laboratory standards which rely upon culture and fluorescence techniques. By providing broader target coverage in the same assay, multiplexing methods obviate the diagnostic doubt that occurs when a symptomatic patient tests negative by less comprehensive methods. The Luminex Molecular Diagnostics (LMD) Respiratory Viral Panel (xTAG™ RVP) is a multiplexed nucleic acid assay covering almost all viruses and some subtypes associated with respiratory or influenza-like-illness. It is based on LMD’s proprietary universal array platform and allows detection of 20 distinct respiratory viruses and subtypes for three of these viruses in a single reaction. The target analytes of the assay are shown in Table 1.

This is one of the first examples of how multiplexing technology can be applied to infectious disease diagnostics providing significantly improved clinical utility as well as potential savings in overall health costs. The etiology of respiratory infections has been attributed to a wide range of pathogens including viruses, bacteria and mycoplasmas (approximately 80% viral). Due to the similarity in presentation of respiratory illness, it is essentially impossible to identify a specific cause on the basis of symptoms alone. Since this information is essential for optimal patient treatment as well as resource management, e.g. identification of patients during outbreaks, multiplex tests such as xTAG™ RVP are likely to be increasingly adopted in the evolution of the diagnostic laboratory.

In addition to these benefits, xTAG™ RVP has utility in epidemiological studies (including analyses of archived specimen banks), public health and surveillance programs. The broad spectrum of viral species covered will enable investigators to rule-in or rule-out suspected respiratory viral types and subtypes that might pose severe public health problems. Moreover, the ability to distinguish the highly

| Virus        | Subtypes     | Results |
|--------------|--------------|---------|
| Influenza A  | H1, H3, H5   | 4       |
| Influenza B  | –            | 1       |
| RSV A        | –            | 1       |
| RSV B        | –            | 1       |
| Coronas      | SARS, NL63, 229E, OC43, HKU1 | 5       |
| Parainfluenzas | 1, 2, 3, 4  | 4       |
| Metapneumo   | –            | 1       |
| Entero/Rhino | –            | 1       |
| Adenovirus   | –            | 1       |
| **Total**    | **19**       |         |

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For Influenza A, 1 for each subtype.
pathogenic flu A H5 sub-type from other common H sub-types makes it particularly relevant to current concerns with respect to a possible avian flu pandemic.

2. Overview of the assay

The xTAG™ RVP assay can be divided into two main junctures: a coupled multiplex Reverse Transcription-Polymerase Chain Reaction (RT-PCR) to generate target-specific amplicons followed by a multiplex Target-Specific Primer Extension (TSPE). The target-specific primers are chimeric primers containing the target sequence juxtaposed to a Universal Tag sequence which allow for sorting on the Luminex®xMAP® instrument (Bortolin et al., 2004). A summary of this process is outlined in Fig. 1.

Viral nucleic acids are extracted from a nasopharyngeal sample and a multiplex RT-PCR reaction is carried out under optimized conditions in a single multiplex (16-plex) PCR resulting in amplicons for each of the viruses/subtypes present in the sample. The amplicons sizes range from 107 bp to 402 bp (Table 2). Subsequent to amplification, the amplicons are treated with Shrimp Alkaline Phosphatase (SAP) to inactivate any unincorporated nucleotides (particularly dCTP), and with Exonuclease I (EXO) to degrade any unutilized single stranded primers.

The treated amplicons are subjected to multiplex TSPE reaction to detect viral targets present in the sample. In this step, each virus target is specifically hybridized to Target-Specific Primer (TSP) possessing a unique DNA tag. A DNA polymerase extends perfectly formed complements and simultaneously incorporates biotin-dCTP into the extension product (Strom et al., 2005; Zhu et al., 2007). Following the TSPE reaction, the extension products are added directly to microwells containing an anti-Tag coupled bead array. The beads, each of which contains an anti-tag sequence unique to e specific viral target, are spectrally distinguishable from each other. Biotinylated extension products hybridizing onto the bead surface are detected with a fluorescent reporter molecule (typically streptavidin-phycocerythrin). Each tagged primer hybridizes only to its unique anti-tag complement (Bortolin et al., 2004); therefore, each bead detects a specific virus, through the bead/anti-tag/tagged primer association. The beads are sorted and analyzed by the Luminex® 100 IS or 200 IS instrument.

The Luminex® instrument contains two lasers: one identifies the fluorescent bead signature, and the other identifies the presence or absence of primer extension products through the phycocerythrin reporter (Figure 2). All viruses, internal control and reaction control are identified in a single multiplex reaction. The data generated by the Luminex® 100 IS and 200 IS systems are analyzed by the Tag-IT™ Data Analysis Software RVP-I (TDAS RVP-I) to

| Table 2 | Amplicon sizes |
|--------|---------------|
| Amplicon | Size |
| RSV | 238 |
| Corona | 211–220 |
| Influenza A | 245 |
| Influenza B | 243 |
| Parainfluenza 1 | 172 |
| Parainfluenza 2 | 174 |
| Parainfluenza 3 | 242 |
| Parainfluenza 4 | 245 |
| Metapneumovirus | 163 |
| Adenovirus | 107 |
| Enterovirus | 107 |
| H1 | 114 |
| H3 | 107 |
| Avian Flu-H5 | 172 |
| MS2 Phage | 122 |
| Lambda Phage | 152 |

Fig. 1. Summary of xTAG™ RVP Assay procedure.

Fig. 2. Bead classification and Extension product detection on the Luminex instrument.
provide a report summarizing which viruses are present in the sample, if any. Detailed reports with raw data are also available.

3. Detailed assay description

The xTAG™ RVP is a bead array-based system for detecting the presence/absence of viral DNA/RNA in clinical specimens. The oligonucleotide primer/probe components of the xTAG™ RVP have been designed to specifically target unique regions in the RNA/DNA of each virus listed in Table 1. Amplified products are then sorted and analyzed on the Luminex® xMAP instrument, which generates signals based on the acquisition of spectrally distinguishable data. The raw signals are median fluorescence intensities (MFI) which are acquired in a Luminex® Output.csv file that is subsequently analyzed by the software component of the xTAG™ RVP to establish the presence or absence of all viral types/subtypes for which a Luminex® microsphere control has been included within the reaction mixture. A portion of the extension products will not interact with the conjugate and hence not yield a fluorescent signal.

3.1. PCR primer mix and amplification primer design

The viral target specific amplification primers (16 pairs of primers) incorporated into the multiplex-PCR primer mix have been designed to amplify conserved regions of the viral types/subtypes. In addition to the viral targets listed in Table 2, amplification primer pairs for both MS2 phage (internal control) and lambda phage (PCR control) have been included within the reaction mixture. The MS2 phage, derived from a conserved region within the MS2 phage genome, is added to the sample and simultaneously extracted. MS2 was chosen as a suitable control because it most closely resembles viruses whose genomes are composed of RNA. The RNA based genome of MS2 serves as both a nucleic acid extraction control and an internal control for all steps of the assay, particularly the reverse transcription/PCR. Specimens should be handled in an appropriate manner to maintain integral-infectious virus prior to the addition of the MS2. The lambda phage DNA specifically acts as a PCR external control since it is not subjected to extraction and is not reverse transcription dependent as is the MS2. In addition to the aforementioned controls it is recommended that a negative control be run with every batch of samples as well as external positive controls representing viruses probed by the xTAG™ RVP be included in routine laboratory control procedures in accordance with local regulations.

The amplification products once generated are treated with Shrimp alkaline phosphatase (SAP) and Exonuclease 1 (Exo). This reaction is conveniently performed in the sample reaction tube so that neither transfer or product purification is necessary. The PCR amplification product is then subjected to a Target Specific Primer Extension (TSPE) reaction described below.

3.2. Target specific primer (TSP) mix

The TSP mix consists of 21 target specific primers, biotin-dCTP and dATP, dTTP and dGTP. Target dependent hybridization occurs only if an amplicon derived from the viral target is present in the original specimen. The TSP primers have been critically chosen to bind conserved regions within the viral targets. Subtype specific primers have been designed to differentiate between various viral subtypes, for example RSV-A vs RSV-B or between the multiple coronas present. When extension occurs, the strand will become biotinylated by virtue of the biotin-dCTP present in the reaction mixture. A portion of the extension products is then combined with the anti-tag-conjugated bead array to allow tag/anti-tag specific hybridization. The tag/anti-tag reaction is highly specific as they have been designed to be minimally cross-reacting among each other and to be maximally divergent from NCBI database sequences. Bead hybridized sequences representing extension products are detected using a streptavidin-phycoerythrin reporter molecule that is added to the reaction mix. Non-extended products will not interact with the conjugate and hence not yield a fluorescent signal.

3.3. Coupled bead mix

The 21-bead array represents a suspension containing a defined set of Luminex® microspheres. Each microsphere population in this set is spectrally distinguishable from all
other microsphere populations in the set when read on the Luminex® xMAP system. This feature is the basis on which median fluorescent intensity (MFI) signals recorded in the Luminex® Output.csv file are sorted. The intensity of each recorded signal (Note: one MFI signal is recorded for each bead population in the Bead Mix) is a function of the degree to which the streptavidin-phycocerythrin reporter molecule interacts with the bead within the population. This, in turn, is a function of the highly specific tag/anti/tag hybridization between the coupled beads and the TS primers which have incorporated biotinylated dNTPs (Figure 4).

### 3.4. xTAG™ RVP data analysis software

To ease data analysis, proprietary software designed and developed by Luminex Molecular Diagnostics Inc (LMD) is provided with the xTAG™ RVP assay. The software component of the system applies analysis algorithms to the MFI signals recorded in the Luminex® Output.csv file and reports a qualitative result for each viral type/subtype/ control discriminated by the assay.

The xTAG™ RVP has been designed to generate unique PCR products for each of the targets described above with the exception of targets falling within the Coronavirus, RSV, Enterovirus and Rhinovirus families. For the corona and RSV viruses, the targets are amplified with non-subtype specific PCR primers. Subtyping occurs during the TSPE step through the use of appropriately discriminating subtype-specific target specific primers. Enterovirus and Rhinovirus are two separate targets that will generate signals on the same Luminex® microsphere population (single amplicon called Entero/Rhino). The discrimination of Parainfluenza subtypes occurs at both the PCR and TSPE step.

The detection of Influenza A subtypes is achieved by amplifying conserved regions of the matrix gene common to all subtypes and target specific regions of the hemagglutinin gene (3 sets of PCR primers for the three listed subtypes, H1, H3, H5). Therefore flu targets, when present, will yield two signals for this viral type, one for flu A and a second for one of the present subtypes.

### 4. xTag™ RVP versus other test methods

In one study comparing xTAG™ RVP assay with DFA and cell culture, the RVP assay was far more sensitive (98% vs 69%) in a set of 294 NP specimens where 183 were considered positive (Mahony et al., 2007). The increased detection rate was attributable to increased sensitivity of the RVP test relative to DFA/cell culture and the fact that the assay can detect a broader range of viral targets.

Real time PCR is currently the most widely used nucleic acid test in the infectious disease laboratory. While this method can be very sensitive, less labor intensive and relatively fast, it suffers from one critical setback. The multiplexing capability of this technology is limited by the number of fluoros available (at most 6 dyes). This requires labs to run multiple assays on multiple instruments to obtain similar results to that obtained with RVP. In addition, data from the multiple runs have to be collated, compiled and analyzed in order to issue a clinical report.

In order to evaluate performance against real-time detection, xTAG™ RVP analysis was carried out on sample extracts at the same time real-time analysis was performed. By carrying out parallel analysis, we were able to control for possible variation in sample integrity as well as copy number. Table 3 shows sample data. As can be seen, xTag RVP is comparable to real-time PCR. For samples with Ct values of 35 or less, xTAG™ RVP gave strong signals as well as high signal to noise ratios (≥50:1). Between Ct values of 36 and 40, xTAG™ RVP was able to detect most targets. Beyond Ct values of 40, in a few cases xTAG™ RVP gave low but clear signals above background. However the clinical relevance of such samples is unknown.

### 5. Conclusions and Summary

The xTAG™ RVP assay is a valuable tool for diagnosis and management of respiratory viral illness. The multiplexed nature of this assay offers simultaneous identification of 19 distinct viruses, thereby assisting with patient treatment. In addition, xTAG™ RVP has clear utility in epidemiology and both public health and surveillance applications.
Conflict of interest statement
The authors are employees of Luminex Molecular Diagnostics.

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