# Benefits of multiplex testing for the differential diagnosis of COVID-19, Flu and RSV in a busy flu season

This year's winter season is anticipated to be a difficult one as experts warn of a "twindemic" with surges of both influenza and COVID-19 expected. The drop in seasonal temperature combined with people staying indoors traditionally creates a sharp increase in transmission of winter respiratory viruses such as flu and RSV. However, last year due to social distancing and mask wearing policies, the flu season experienced a reprieve with historically low rates of influenza recorded. In contrast, this year as communities re-open and many of those policies are relaxed, experts predict large increases in transmission for COVID-19 and flu simultaneously. For a medical system that has already been under tremendous strain for the past 18 months, this news is concerning. Using tools such multiplexing assays for flu, COVID and RSV to diagnose and treat patients faster and more effectively becomes a necessary next step.

Multiplex testing and syndromic panels save significant time and resources by testing for multiple diseases using a single sample. Flu, COVID-19, RSV and other respiratory diseases cause similar symptoms and differential diagnosis is not possible without a laboratory test. Combination testing provides physicians the unique opportunity to quickly and accurately diagnose a patient and provide the right treatment. This is important for respiratory infections such as COVID-19, RSV and flu, which require different treatments and put different patient groups, such as babies or the elderly, at risk for serious complications if mistreated or left untreated.

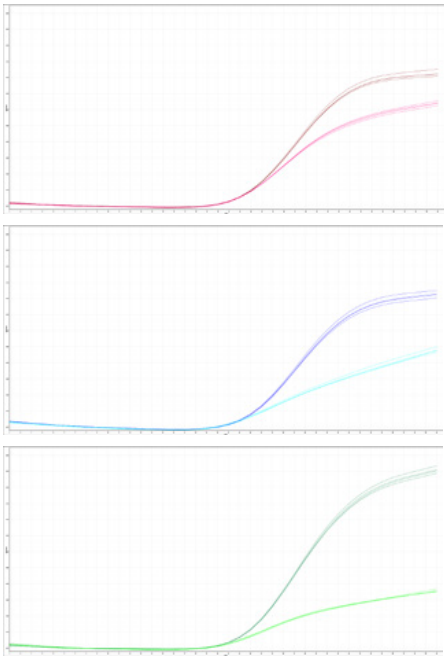
Several assay techniques are used for multiplex testing including immunoassay-based rapid antigen detection and nucleic acid amplification using molecular methods. Throughout the COVID-19 pandemic, molecular testing has remained predominant test used for diagnosis due to its high sensitivity and specificity. However, molecular tests require a laboratory to perform, involve complex logistics for sample transportation and are vulnerable to bottlenecks in machine capacities and reagent supplies, all impacting the turn-around-times which can be up to several days. In contrast, rapid antigen assays are highly scalable, can be used at the point of care (such as a school or airport) and can provide a result in less than 10-15 minutes. Yet their sensitivity is lower (>80% as compared to RT-PCR) (CDC 2021) or more, they are prone to cross-reactivity, and they have a higher incidence of false positive and false negative results compared to molecular tests. Depending on the available resources, accuracy required and level of urgency, one testing method may prove to be more useful than another for a given situation.

## Multiplex Molecular Testing

Molecular multiplex assays have the ability to significantly lower the cost of testing multiple targets at one time. Compared to singleplex reactions, multiplex requires less reagents, less time, and less labor to achieve the same result. However, the performance of a molecular assay depends on its ability to accurately detect low levels of a pathogen, and in multiplex analysis, this challenge grows substantially. The overall variability in the levels of the targets can result in preferential amplification of one target over another, as well as PCR drift—stochastic variation caused by low template concentration. Variability in the physicochemical characteristics of the amplified sequences, its length, GC content, flanking regions and secondary structures also may add to the imbalance of the reaction and impact on Ct values (Jong, 2013).

To overcome these challenges, it is important to use a master mix optimized for multiplexing to ensure that Ct value remains the same and the assay retains its high sensitivity for each of its targets. Meridian's Fast 1-Step RT-qPCR Mix (Catalog MDX032) is designed for multiplex RT-qPCR diagnostic tests and for high-throughput, automated platforms. The amount of polymerase, dNTP, and Mg2+ has been optimized and the mix's buffering capacity (to stop the pH changing) has been enhanced to provide optimal performance.

Fig. 1 Multiplex vs Singleplex 1-Step RT-qPCR assays using Meridian's Fast 1-Step RT-qPCR Mix (MDX032)



A 100-fold dilution of human RNA was used with four probes, using a TaqMan prime/probe set and Fast 1-Step RT-qPCR Mix (MDX032) (singleplex dark red, quadruplex light red), qScript 1-step RT-qPCR mix (singleplex dark blue, quadruplex light blue) and QuantiFast Probe RT-qPCR mix (singleplex dark green, quadruplex light green). The conditions were 48°C 10 min followed by 95°C for 5 min and 35 cycles of 95°C 10s, 60°C 10s, 72°C 5s. The results show Fast 1-Step RT-qPCR Mix is highly efficient in multiplexing, reactions containing single and 4 probes giving the same Ct, unlike qScript and QuantiFast.

Another major concern for multiplexing assays, especially ones on high-complexity platforms that involve the manipulation of amplified PCR products, is the potential for false-negative results from PCR inhibition or false-positive results from PCR amplicon carryover. Most assay protocols require DNA or RNA extraction prior to testing in order to remove PCR inhibitors found in clinical samples such as nasopharyngeal or sputum specimens. However, these purification methods are problematic, can cause sample loss, and are not completely effective at removing all inhibitors. In addition, during the height of the first wave of the COVID-19 pandemic, the huge demand for molecular testing created an RNA extraction reagent shortage. Faced with the challenge of overcoming this supply shortage, assay manufacturers looked for alternative methods that rely on direct detection, entirely avoiding the need to purify the DNA or RNA.

To address these issues, Meridian focused on creating inhibitor-tolerant molecular master mixes that allow clinical crude specimens to be run directly on a PCR machine, without performing purification or extraction first. Meridian's first direct detection mix, Inhibitor-Tolerant RT-qPCR Mix (Catalog MDX016) was designed for qualitative multiplex assays using crude lysates or inhibitor-rich samples from different sources. The mixes can be used for direct amplification to substantially reduce steps in the assay workflow and improve assay turn-around times.

## POCT Molecular Assays & Multiplexing

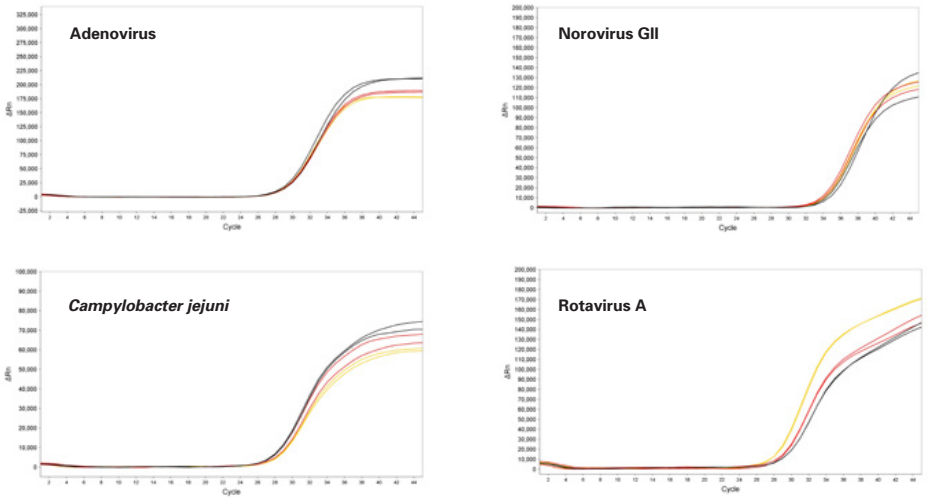
Molecular testing methods require skilled technicians, sophisticated lab equipment, and cold shipment and storage to maintain the shelf-life of the product. Although these challenges are manageable in a centralized large laboratory, there are not suitable in the field where point of care (POCT) assays are needed.

Meridian has developed two reagent POCT solutions for molecular assays. The first is a group of lyophilization compatible mixes (Lyo-Ready™) that are glycerol-free and fully optimized with a specialized blend of lyo-excipients and which only require the addition of primers and probes to complete the assay. The formulation can be used as a liquid mix for an instant multiplex RT-qPCR diagnostic test or lyophilized into cakes or beads to create an ambient-temperature stable assay. The Lyo-Ready™ 1-Step RT-qPCR Mix (Catalog MDX024) and Lyo-Ready™ 1-Step RT-qPCR Virus Mix (Catalog MDX062) are widely used formulations in regulated assays around the world. The virus mix (Cat #MDX062) is specifically optimized for amplification of RNA or DNA viruses with a high secondary structure (reverse transcriptase remains active at 55-60°C).

The second POCT solution is Meridian's new Air-Dryable™ Direct RNA/DNA product line which combines the benefit of inhibitor-tolerance with the ability to air-dry the liquid mix to create an ambient-temperature stable assay. Unlike lyophilization, air-drying is relatively quick, inexpensive, and can be performed in-house with an oven.

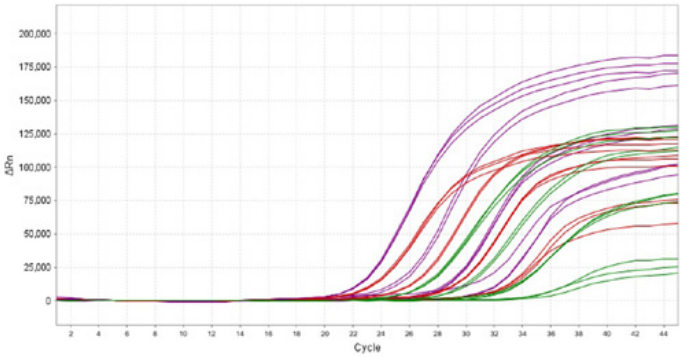
Novel sample-specific solutions of Air-Dryable™ mixes have been designed to

Fig. 2 Efficient multiplex reactions in the presence of inhibitors (20% stool) using Inhibitor-Tolerant RT-qPCR Mix (MDX016)




Amplification profiles of 4 pathogens (RNA: Norovirus and Rotavirus, DNA: Adenovirus, *C. jejuni*) in a multiplex reaction containing 5% (yellow), 10% (red) and 20% (black) stool extract. The results demonstrate the multiplexing capability of Inhibitor-Tolerant RT-qPCR Mix (MDX016) in the presence of inhibitors found in stool (up to 20% final volume).

Fig. 3 Multiplex Analysis of DNA and RNA Viral Targets Using Lyo-Ready™ 1-Step RT-qPCR Virus Mix



Respiratory Syncytial Virus (RSV) (green), Rotavirus A (purple) and Dengue Type 2 Virus (DENV-2) (red) were amplified in a single multiplexed RT-qPCR assay using inactivated crude viral lysates and lyophilized Lyo-Ready™ 1-Step RT-qPCR Virus Mix (MDX062). The result illustrates that Lyo-Ready™ 1-Step RT-qPCR Virus Mix can be used in a multiplex RT-qPCR assay to detect several low-copy number RNA targets simultaneously from a single sample.

overcome the inhibitors present in saliva/sputum, blood, urine and stool. The new Air-Dryable™ Direct RNA/DNA qPCR Saliva mix (Catalog MDX131) is a unique alternative for multiplexing flu/COVID/RSV assays and is optimized for the sensitive detection of RNA targets from crudely processed saliva or sputum samples and only requires the addition of primers and probes to complete the assay. The mix can be used in a liquid format or oven-dried to create a highly sensitive, ambient-temperature stable, multiplex assay.

Air-Dryable™ Direct RNA/DNA qPCR SALIVA (Catalog MDX131) can tolerate complex inhibitors in crude saliva such as sputum, mucin, UTM swabs. Syndromic respiratory panels have adopted this master mix to detect COVID-19, Flu, RSV, Mycoplasma pne, etc), because of its sensitivity to less than 10 copies and its compatibility with liquid or dry assay formats.

## Immunoassays

Although molecular tests are considered the gold standard for viral respiratory detection, they have limitations in accessibility, scalability, and point-of-care applications. In contrast, lateral flow immunoassays are perfectly suited for field-based testing. Rapid antigen assays are highly scalable, can be stored at room temperate, require minimal training, and can provide a result in less than 10-15 minutes.

However, immunoassays have challenges with multiplex testing due to the overall variability in the levels of targets present in the patient sample. Compared to singleplex immunoassays, multiplex versions must have a wider dynamic range in order to detect targets that are present at radically different concentrations. Although multiplex ELISA assays are reported to maintain linearity better than singleplex ELISA assays (over three or even five orders of magnitude), a key component to an assay's performance in the selection of antibodies. The antibodies selected for a multiplex assay must be highly sensitive in order to detect both low and high abundance targets, and they must be very specific so that the antibodies do not cross-react with each other or with other proteins in the assay mixture or patient sample.

## Immunoassay Testing for COVID-19, Flu A/B and RSV

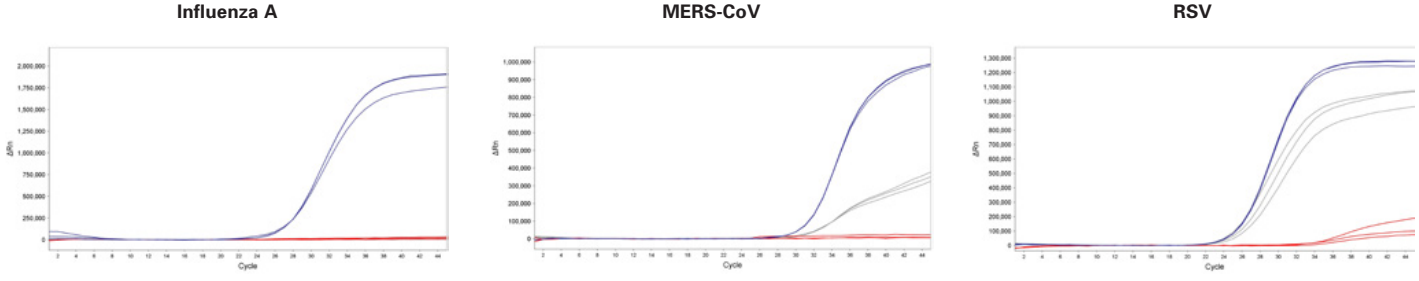
For COVID-19, the nucleocapsid (N protein) is the main protein targeted for rapid antigen testing as it is also the most abundant protein present on the surface of the SARS-CoV-2 virus and can easily be detected at low viral loads. Meridian's highly sensitive antibody pair to SARS-CoV-2 Nucleocapsid Protein (Catalog 9548 and 9547) is proven to detect all of the WHO Variants of Concern (Alpha B.1.1.7, Beta B.1.351, Gamma P.1/P.2 and Delta B.1.617.2) and is used in FDA and CE-marked commercial rapid antigen tests around the world. The antibodies detect a conserved region N protein and they do not exhibit any cross-reactivity to influenza A/B, RSV or seasonal coronavirus strains.

For influenza, immunoassay testing typically detects the main circulating seasonal strains of both influenza type A and B. Influenza viruses are RNA viruses that are prone to antigenic drift and reassortment, which enables news strains of the virus to emerge each year. Rapid assays for influenza typically detect the nucleoprotein (NP) which is one of the more conserved proteins in the influenza virus and subsequently less likely to undergo mutations that lead to antigenic drift (which in turn can cause the functional components of an assay to not recognize a current influenza strain). Meridian offers several high performing antibody pairs for both influenza A and B that are ideal for rapid diagnostic testing and that do not cross-react with RSV or SARS-CoV-2 antigens.

RSV is recognized as one of the most common causes of childhood illness and can lead to serious illnesses such as bronchiolitis and pneumonia in infants and older adults. In fact, almost two out of every one hundred children younger than six months of age with an RSV infection are at risk for hospitalizaton (CDC, 2021). Structurally, RSV virus consists of three main proteins and the fusion protein (which is responsible for fusion to the host membrane) is the current leading target for diagnostic assays and the majority of vaccines and immunotherapies under development. This is due to the protein's unique aspects in that it is only one of only two antigens that induce an RSV-neutralizing antibody response, it has a high degree of sequence conservation among RSV strains (>90%) (Meng, 2014) and it is highly immunogenic. Meridian offers several antibody pairs targeting the fusion protein which do not cross-react with SARS-CoV-2 or influenza and are for rapid antigen testing solutions.

While immunoassay multiplexing offers numerous efficiency advantages, it also introduces several technical challenges that make assay design more complicated. One of the biggest challenges is controlling for interference between the various antibodies and proteins in the assay. Sample dilution can help with limiting interference caused

Fig. 4 Respiratory Multiplex Testing Using Air-Dryable™ Direct RNA/DNA qPCR Saliva (MDX131)




Three respiratory pathogens, Influenza A, Middle East Respiratory syndrome coronavirus (MERS-CoV) and Respiratory Syncytial Virus (RSV) were amplified in a triplex qPCR assay in the presence of 35% Universal Transport Media (UTM) with 50% artificial sputum swab. The results illustrate that a higher performance was achieved with Air-Dryable™ Direct RNA/DNA qPCR Saliva (blue) compared to an inhibitor-tolerant RT-qPCR mix (TaqPath™ 1-step Multiplex Mix) from supplier T (red) and supplier Q (grey) (Ultra-Plex™ 1-Step Tough Mix).

by proteins and other substances present in complex sample types. However, in order to remove potentially interfering particles including endogenous antibodies such as heterophilic antibodies (HA) (e.g. HAMA) and rheumatoid factor (RF), it is important to incorporate immunoassay blockers into the assay design. Double mouse monoclonal assays such as those for rapid respiratory antigen tests, are specifically prone to HAMA and RF interference and require a specialized blocker to ensure the assay's accuracy.

Meridian manufactures in multikilogram scales of passive blockers such as Mouse IgG and animal serums as well a proprietary active blocker, TRU Block™, which contains specific binders directed against all types of heterophilic interference including HAMA and RF. Once bound to the interfering antibodies, TRU Block™ prevents further binding of HA to other assay components through steric hindrance. Active blockers can typically be used in lower concentrations than passive blocking reagents, which minimizes the reduction in assay signal commonly associated with passive blockers.

Overall, immunoassay multiplexing calls for carefully chosen reagents that allow the antibodies to work together to produce an accurate, meaningful result. Interfering factors must be minimized to prevent a false positive or a false negative error.

## Conclusion

Multiplex assays that combine the detection of several targets at once have several advantages including their high-throughput potential, ability to provide more results per sample, and lower reagent consumption (i.e., miniaturization). All of these benefits translate to a lower price-per-data point compared to traditional singleplex assays.

With the reopening of most countries and relaxing of social distancing and mask wearing strategies, the circulation of influenza, COVID-19 and RSV will rebound and likely exceed normal levels for some time. Given many respiratory disease symptoms overlap, it is not possible to reliably differentiate an infection with one of these viruses on clinical grounds alone. Multiplex testing addresses the higher demand for screening between infections due to SARS-CoV-2, influenza A/B and RSV during what is expected to be higher than normal flu season. Molecular multiplex testing offers high sensitivity and specificity, detecting infected individuals early in the course of the disease and enabling adequate time for the appropriate treatment. However, molecular tests require sophisticated equipment, experienced technicians and generally have a turn-around time of 1-5 days. In contrast, immunoassay rapid antigen assays are easy to use and can provide a result in less than 10-15 minutes, but they are considered to have a lower performance and are less sensitive. Each testing type, molecular or immunoassay, has advantages and disadvantages and they can be used independently from one another or in a testing algorithm that leverages the advantages of both. By employing multiplexing assays for the flu, COVID and RSV, patients will be able to be treated faster and more effectively, regardless of the testing type employed.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