Multiplexed detection and differentiation of SARS-CoV-2, influenza A, and influenza B

Workflow, instrument compatibility, and performance of the OPTI SARS-CoV-2/Influenza A/B RT-PCR Test

R. Chan[1], C. Carol[1], S. Tiwari[2], L. Gow[2], L. Plourde[2], M. Angelichio[2]
1. IDEXX Reference Laboratories, Inc., Markham, Ontario, Canada
2. IDEXX Laboratories, Inc., Westbrook, Maine, USA

Introduction

The OPTI* SARS-CoV-2/Influenza A/B RT-PCR Test is a multiplexed real-time fluorescent reverse transcription polymerase chain reaction (RT-PCR) test for the qualitative detection and differentiation of RNA from SARS-CoV-2, influenza A, and influenza B in nasopharyngeal specimens from patients.

The target regions include:

- SARS-CoV-2: two separate targets in the N gene (N1 and N4)
- Influenza A: matrix protein
- Influenza B: nuclear export protein
- Internal Sample Control: human RNase P

The test contains everything needed for testing 500 samples, including the target mix, master mix, a positive control for all three targets and the internal control, and PCR-grade water (negative control).

Figure 1. Test contents (500 reactions)

Test workflow

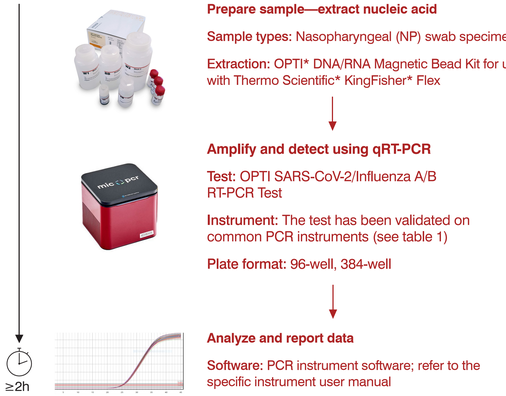

Figure 2. Overview of the OPTI SARS-CoV-2/Influenza A/B RT-PCR Test workflow, showing steps from sample preparation to data reporting. Results can be available in as little as 2 hours, including RNA extraction. **Note:** Time to results depends on the number of samples and the instrumentation available.

Instrument compatibility

Two different versions of the test allow for broad instrument compatibility. Table 1 indicates which test version is compatible with which validated qPCR cyclers. Both versions use the same SARS-CoV-2, influenza A, influenza B, and RNase P primers and probes. The only difference is the version 2 influenza B detection occurs in a different fluorescent channel and therefore requires a master mix that does not contain ROX.*

Table 1. qPCR instruments validated for use with the OPTI SARS-CoV-2/Influenza A/B RT-PCR Test (versions 1 and 2)

OPTI SARS-CoV-2/Influenza A/B RT-PCR Test Version 1, 99-57014	OPTI SARS-CoV-2/Influenza A/B RT-PCR Test Version 2, 99-57015
Applied Biosystems* 7500 Applied Biosystems* QuantStudio* 5	Roche LightCycler* 480 II Bio-Rad CFX96 Touch* Real-Time PCR Detection System Bio Molecular Systems Mic qPCR Cycler

Assay performance

The data shown below correspond to the first version of the OPTI SARS-CoV-2/Influenza A/B RT-PCR Test (product code 99-57014). Results for product code 99-57015 are available upon request.

Clinical evaluation

The performance of the OPTI SARS-CoV-2/Influenza A/B RT-PCR Test was evaluated using archived clinical nasopharyngeal (NP) swab samples in viral transport medium. Results for SARS-CoV-2 detection (n=164) were compared to results from a highly sensitive, FDA-authorized molecular assay. Results for influenza A (n=143) and B detection (n=143) were compared to results from a highly sensitive, FDA-cleared molecular assay. Table 2 summarizes the results.

Table 2. Clinical evaluation results (96-well format)—diagnostic sensitivity (PPA) and diagnostic specificity (NPA)

Target	PPA (95% CI)	NPA (95% CI)
SARS-CoV-2	100% (91.4%–100%)	100% (97.0%–100%)
Influenza A	100% (92.9%–100%)	100% (96.0%–100%)
Influenza B	100% (93.9%–100%)	99.4% (95.6%–100%)

PPA: positive percent agreement
NPA: negative percent agreement
CI: confidence interval

Analytical sensitivity and efficiency of PCR

Analytical sensitivity represents the smallest amount of analyte in a sample that can accurately be measured by an assay. The efficiency of PCR is defined as the number of folds of target molecules that are copied in one PCR cycle. Typically, desired amplification efficiencies range from 90% to 110%. Table 3 and table 4 show the analytical sensitivity and efficiency of PCR results, respectively.

Table 3. Analytical sensitivity per target

Target	Analytical sensitivity (RNA copies per reaction)
SARS-CoV-2 N1	10
SARS-CoV-2 N4	1
Influenza A	10
Influenza B	1
RNase P	10

Table 4. Efficiency of PCR

Target	Efficiency of PCR
SARS-CoV-2 N1	108%
SARS-CoV-2 N4	103%
Influenza A	99%
Influenza B	104%

Inclusivity (analytical reactivity)

Inclusivity of the OPTI SARS-CoV-2/Influenza A/B RT-PCR Test for all SARS-CoV-2 (N1, N4), influenza A, and influenza B sequences has been demonstrated through both *in silico* and wet analyses of the primer and probe sequences (table 5). A multisequence alignment (MSA) was generated from influenza A and influenza B sequences in the GISAID database submitted between December 23, 2019 and October 23, 2020 and compared for identity to the test primers and probes. A multisequence alignment (MSA) was generated for SARS-CoV-2 sequences in the GISAID CoV database sequences submitted between September 1, 2021 and December 20, 2021 and compared for identity to the test primers and probes. Only full-length, high-coverage sequences were used.

Table 5. *In silico* analysis of primers and probe alignment to published database sequences

Target	Primer/Probe	Alignment (perfect match)
SARS-CoV-2 (N1, N4) n = 115,332	Forward primer	N1: 99.7%; N4: 98.2%
	Probe	N1: 98.1%; N4: 99.5%
	Reverse primer	N1: 99.6%; N4: 98.1%
Influenza A n = 75,000	Forward primer	98.7%
	Probe	94.6%
	Reverse primer	98.7%
Influenza B n = 19,000	Forward primer	95.9%
	Probe	98.5%
	Reverse primer	99.1%

Exclusivity (cross-reactivity)

The OPTI SARS-CoV-2/Influenza A/B RT-PCR Test was evaluated for cross-reactivity with a panel of bacteria, viruses, and yeast that represents common respiratory pathogens, and a pool of 30 negative human nasopharyngeal specimens that represents a microbial flora seen in human respiratory specimens. No cross-reactivity was observed, and detection of influenza A, influenza B, and SARS-CoV-2 RNA at ≤3 x LoD was not affected by the presence of the microbial nucleic acid extracts. Potential cross-reactivity of a broader list of bacterial pathogens is highly unlikely as confirmed in our *in silico* analysis.

Coinfection study

Analytical sensitivity of the test in the context of a coinfection scenario was evaluated using negative nasopharyngeal samples spiked with inactivated SARS-CoV-2, live influenza A/H3N2 and live B/Victoria viruses. Two of three targets were spiked at a starting concentration of 3 x LoD in the presence of a third target at high concentration. The test data shows that a high concentration of influenza A did not affect the detection of influenza B and SARS-CoV-2 at 3 x LoD. Similarly, a high concentration of influenza B did not affect the detection of influenza A and SARS-CoV-2 at 3 x LoD. However, a high concentration of SARS-CoV-2 in a clinical sample adversely affected the detection of the influenza A and B viruses at 3 x LoD.

Interfering substances

Twenty-two endogenous substances, including mucin, blood, and nasal sprays, were added into negative NP specimens containing spiked viruses (3x LoD). Samples were tested in the presence of each substance to determine the effect on the detection of the targets. The presence of the endogenous substances in NP samples did not have any effect on the detection of the RNA targets in the OPTI SARS-CoV-2/Influenza A/B RT-PCR Test.

Conclusions

The OPTI SARS-CoV-2/Influenza A/B RT-PCR Test detects and differentiates between SARS-CoV-2, influenza A, and influenza B in a single reaction.

The OPTI SARS-CoV-2/Influenza A/B RT-PCR Test:

- Delivers accurate results with high sensitivity.
- Brings a simple workflow and an optional high-throughput version that allows laboratories to conserve reagents while also processing more COVID-19 and influenza tests.
- Provides an optimized workflow from start to finish by using an internal sample control that targets human RNase P.
- Minimizes the impact of viral mutation by detecting two SARS-CoV-2 regions.
- Has been validated on common PCR instruments.
- Reduces waiting time with results in as little as 2 hours.

The OPTI SARS-CoV-2/Influenza A/B RT-PCR Test has been authorized by the United States Food & Drug Administration (FDA) under an Emergency Use Authorization issued by the FDA on April 21, 2022. This test has not been FDA cleared or approved, and the FDA has not determined that the test is safe or effective for the detection of SARS-CoV-2, influenza A and influenza B. The test has been authorized only for the detection of nucleic acid from SARS-CoV-2, influenza A and influenza B, and not for any other viruses or pathogens. The test is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of in vitro diagnostics for detection and/or diagnosis of COVID-19 under Section 564(b) (1) of the U.S. Federal Food, Drug, and Cosmetic Act, 21 U.S.C. § 360bbb-3(b) (1), unless the authorization is terminated or revoked sooner. Use in the United States is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988, 42 U.S.C. § 263a (CLIA) to perform high complexity tests. The test is not intended for home use.

OPTIMedical