# Instructions for Use

# LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0

# LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0

For detection of SARS-CoV-2, Influenza A, Influenza B and Respiratory Syncytial Virus from human specimens

For use with

CFX96™ (BioRad)
CFX Opus 96 (BioRad)

IVD For *in vitro* diagnostic use

REF Product No.: 881203

96 reactions

Please refer to Storage and Shelf Life in this IFU

Ver. 02 / June 2025

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## Content

## 1. Intended Use

The LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 is intended for the specific detection of SARS-CoV-2, Influenza A, Influenza B and Respiratory Syncytial Virus (RSV) RNA in human respiratory specimens (bronchoalveolar lavage, tracheal aspirate, sputum, nasopharyngeal and oropharyngeal swabs placed in VTM, nasopharyngeal wash/aspirate, and nasal wash/aspirate). The LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 is a four-target assay comprising of detection and differentiation assays targeting the SARS-CoV-2 specific RNA-dependent RNA polymerase (*RdRP*) gene, the Influenza A specific Matrix (*M*) gene, the Influenza B specific Haemagglutinin (*HA*) gene and the RSV specific Matrix (*M*) gene.

Besides compatible extraction systems, the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 has been validated for use with a direct PCR protocol, without prior nucleic acid extraction. **Refer to 12.1.2 Sample Preparation via Direct PCR Protocol.** The direct PCR protocol has been validated only for Viral Transport Media (VTM) that do NOT contain Guanidinium Thiocyanate. **Refer to 9. Product Description for validated VTM.**

The LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 is for professional use only.

**NOTE**

⚠ ***Viral Transport Media (VTM) containing Guanidinium Thiocyanate are NOT suitable for direct PCR protocols.***

## 2. Kit Components

| Catalog no. | 881203 |
|---|---|
| Master A | 2 x 300 µl |
| Master B | 4 x 330 µl |
| Internal Control (IC) | 800 µl |
| Positive Control (PC) | 200 µl |
| PCR grade water | 500 µl |

## 3. Storage and Shelf Life

- The LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 has a shelf life of 12 months from the manufacturing date.
- Store all reagents at -15°C to -25°C upon arrival.
- Repeated thawing and freezing should be avoided, as this might affect the performance of the assay. Master B should be frozen in aliquots, if they are to be used intermittently.
- Mix Master A thoroughly by vortex mixing, and centrifuge briefly before use.
- Protect Master B from light.
- All frozen reagents should be completely thawed to room temperature before use. Immediately return unused portions to the freezer for storage.

## 4. Quality Control

In compliance with AstronDX Technologies' EN ISO 13485 certified Quality Management System, each lot of the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 is tested against pre-determined specifications to ensure consistent product quality.

## 5. Product Use Limitations and Precautions

- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR procedures.
- Specimens should always be treated as if infectious and/or biohazardous in accordance with safe laboratory procedures.
- Wear protective disposable powder-free gloves, a laboratory coat and eye protection when handling specimens.
- Avoid microbial and nuclease (DNAse/RNAse) contamination of the specimen and the components of the kit.
- Always use DNAse/RNAse-free disposable pipette tips with aerosol barriers.
- Always wear protective disposable powder-free gloves when handling kit components.
- Use separated and segregated working areas for (i) specimen preparation, (ii) reaction set-up and (iii) amplification/detection activities.

- Workflow in the laboratory should proceed in unidirectional manner.
- Always wear disposable gloves in each area and change them before entering different areas.
- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.
- Store positive and/or potentially positive material separated from all other components of the kit.
- Do not open the reaction tubes/plates post amplification, to avoid contamination with amplicons.
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.
- Do not use components of the kit that have passed their expiration date.
- Discard sample and assay waste according to your local safety regulations.
- Wash hands thoroughly after handling specimens and test reagents.
- Do not use kits from different lots together.
- Do not use an expired kit.
- In case of damage to the packaging and leaking vials, do not use the kit (possible contamination or deterioration that can cause false interpretation).
- Direct PCR protocol is NOT recommended if patient has recently used Difflam Forte throat spray. Ethanol present in this anti-inflammatory throat spray may cause PCR interference.

## 6. Product Warranty

AstronDX Technologies guarantees the performance of the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 for applications as described in the manual. The user must determine the suitability of the product for the particular intended use. Should the product fail to perform satisfactorily in the described applications, please contact AstronDX Technologies Technical Support (**16. Technical Support**) for troubleshooting.

AstronDX Technologies reserves the right to change, alter, or modify any product to enhance its performance and design.

## 7. Product Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles/face masks. For more information, please consult the appropriate material safety data sheets (MSDSs).

## 8. Introduction

Coronaviruses are a group of enveloped viruses with a positive-sense, single stranded RNA genome. The novel coronavirus SARS-CoV-2, which caused the outbreak of Coronavirus disease 2019 (COVID-19) belongs to the genus *Betacoronavirus* and is closely related to bat-SARS-like Coronavirus, but genetically distinct / divergent from SARS-CoV and MERS-CoV. Thus, SARS-CoV-2 is thought to be originated from bats and spread by animal-to-human transmission, via yet unknown intermediate animal host/s [1]. Reports of infection among healthcare workers and family members who are in close contact with SARS-CoV-2-infected patients, also indicated human-to-human transmission. COVID-19 has had a catastrophic effect on the world's demographics resulting in more than 6 million deaths worldwide, emerging as the most consequential global health crisis [2].

Influenza virus is comprised of 8 single-stranded, negative-sense viral RNA segments and belongs to the orthomyxovirus family. There are four types of influenza viruses: A, B, C and D. Humans can be infected with avian, swine and other zoonotic Influenza A viruses, such as avian influenza virus subtypes A(H5N1), A(H7N9), and A(H9N2) and swine influenza virus subtypes A(H1N1), A(H1N2) and A(H3N2). On the other hand, Influenza B is divided into two lineages namely Yamagata and Victoria lineages. However, Influenza B can only be spread from human to human. Influenza C is detected less frequently with mild infections while Influenza D viruses are known to primarily infect cattle. Thus, both Influenza C and D do not present public health importance. Human influenza A and B viruses are responsible for the seasonal epidemics of disease, also known as flu season [3]. It is estimated that during an annual seasonal epidemic, 10–20% of the global population experiences symptomatic influenza, which includes 3–5 million cases of severe illness and 290,000–650,000 deaths from influenza-related respiratory complications [4].

Respiratory Syncytial Virus (RSV) is a single-stranded, negative-strand, RNA virus that is classified into two main groups, RSV-A and RSV-B. RSV was first isolated in 1956 from a captive chimpanzee. Initially named Chimpanzee Coryza Agent, RSV was soon recovered from infants with lower respiratory illness and identified as a human pathogen [5]. RSV has since been one of the most common viruses to infect

children worldwide. Each year RSV causes an estimated 3.2 million hospital admissions and 118,200 deaths in children under five years of age, predominantly in low- and middle-income countries. RSV is also responsible for substantial morbidity and mortality in the elderly and in severely immunocompromised individuals. RSV causes seasonal epidemics in both tropical and temperate regions of the world [6].

As SARS-CoV-2, Influenza and RSV are respiratory viruses that share similar clinical symptoms and transmission mechanisms, it is very difficult to distinguish them using only general practice. Therefore, a highly sensitive and specific differential test helps to improve the laboratory diagnosis of SARS-CoV-2, Influenza and RSV, and at the same time, assists the management of emerging co-infections of SARS-CoV-2 with Influenza or RSV.

Various real-time RT-PCR assays have been published that simultaneously detect SARS-CoV-2, Influenza and RSV. The LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 is a four-target assay comprising of detection and differentiation assays targeting the SARS-CoV-2 specific RNA-dependent RNA polymerase (*RdRP*) gene, the Influenza A specific Matrix (*M*) gene, the Influenza B specific Haemagglutinin (*HA*) gene and the RSV specific Matrix (*M*) gene.

[1] Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., Tan, W. *et al.,* (2020). Genomic characterization and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *The Lancet*, 395(10224), 565-574.

[2] Rajnik, M., Cascella, M., Cuomo, A., Dulebohn, S. C., and Di Napoli, R. (2021). Features, evaluation, and treatment of coronavirus (COVID-19). Uniformed Services University of The Health Sciences.

[3] Centers for Disease Control and Prevention, National Center for Immunization and Respiratory Diseases (NCIRD) 2022. Available online https://www.cdc.gov/flu/about/viruses/types.html

[4] WHO Influenza (seasonal) Geneva: World Health Organization 2023. Available online https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal)

[5] Walsh, E. E. and Hall, C. B. (2015). Respiratory Syncytial Virus (RSV). Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 1948-1960. e3.

[6] Eden, J.S., Sikazwe, C., Xie, R., Deng, Y.M., Sullivan, S.G., Michie, A., Levy,

A., Cutmore, E., Blyth, C.C., Britton, P.N. and Crawford, N. (2022) Off-season RSV epidemics in Australia after easing of COVID-19 restrictions. *Nature Communications,* 13(1), 2884.

## 9. Product Description

The LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 is an *in vitro* diagnostic test system, based on real-time PCR technology, for the qualitative detection of novel coronavirus (SARS-CoV-2) specific RNA, as well as Influenza A, Influenza B, and Respiratory Syncytial Virus (RSV) specific RNA. The LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 consists of a single tube assay targeting four genes; RNA-dependent RNA polymerase (*RdRP*) gene of the SARS-CoV-2 genome, the Matrix (*M*) gene specific for Influenza A, the Haemagglutinin (*HA*) gene specific for Influenza B and Matrix (*M*) gene specific for RSV. The LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 includes a heterologous amplification system (Internal Control) to identify possible RT-PCR inhibition and to confirm the integrity of the reagents of the kit. The Internal Control template used in the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 is an *in vitro* transcribed RNA of an artificial sequence with no homology to any known genomes.

Real-time RT-PCR technology utilizes reverse-transcriptase (RT) reaction to convert RNA into complementary DNA (cDNA), polymerase chain reaction (PCR) for the amplification of specific target sequences, and target-specific probes for the detection of the amplified DNA. The probes are labelled with a fluorescent reporter and quencher dyes. The probes used for specific amplification of SARS-CoV-2, Influenza A, Influenza B and RSV specific RNA are labelled with the fluorophore Cy5™, Tye705, Tex615, and FAM, respectively. The *RdRP* gene probe is specific to SARS-CoV-2 only, *M* gene of Influenza A probe detects all strains of Influenza A of the *Alphainfluenzavirus* genus and *HA* gene probe is specific to Influenza B of the *Betainfluenzavirus* genus. The *M* gene of the RSV probe is specific for Respiratory Syncytial Virus A and B of the genus *Orthopneumovirus*. The probe specific to the target of the Internal Control (IC) is labelled with the fluorophore HEX. Using probes linked to distinguishable dyes enables the parallel detection of SARS-CoV-2, Influenza A, Influenza B, RSV specific RNA and the Internal Control in the corresponding detector channels of the real-time PCR instrument.

The oligonucleotides included in the four assays were designed, modified and based on the sequences/target regions published in the articles listed below:

| Target | Publication |
|---|---|
| SARS-CoV-2 | Chan JF-W *et al*., (2020) |
| Influenza A | World Health Organization (2020) |
| Influenza B | Van Elden *et al*., (2001) |
| RSV | Lee *et al*., (2021) |
| Internal Control | Deer *et al*., (2010) |

The test consists of three processes in a single tube assay:

- Reverse transcription of target and Internal Control RNA to cDNA
- PCR amplification of target and Internal Control cDNA
- Simultaneous detection of PCR amplicons by fluorescent dye labelled probes

The LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 consists of:

- Two Master reagents (Master A and Master B)
- The template of the Internal Control (IC)
- The template of the Positive Control (PC)
- PCR grade water (for setting up of “No Template Control”, NTC)

Master A and Master B reagents contain all components (buffer, enzymes, primers and probes) to allow PCR mediated reverse transcription, amplification and target detection of *RdRP* gene of SARS-CoV-2 specific RNA, *M* gene of Influenza A specific RNA, *HA* gene of Influenza B specific RNA, *M* gene of RSV specific RNA, and Internal Control in one reaction setup.

The Positive Control (PC) contains *in vitro* transcripts of synthesized target genes of SARS-CoV-2, Influenza A, Influenza B and RSV.

The Internal Control used in the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 is an *in vitro* transcribed RNA of an artificial sequence with no homology to any known genomes.

The LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 was developed and validated to be used with the following real-time PCR instruments:

- CFX96™ (BioRad)
- CFX Opus 96 (BioRad)

The LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 is compatible for use with a direct PCR protocol, without prior nucleic acid extraction. The direct PCR protocol to be used with the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 has been validated to be used with the following viral transport media:

- LyteStar™ Direct PCR VTM:
  Transport media with nasal swab, 3 ml inactivated medium, 10 ml tube
  (AstronDX Technologies, Cat. No. 8011930G-VSM02G)

- Inactivating Viral Transport Medium:
  PD VTM with nasal & throat swab, 3 ml inactivated medium, 10 ml tube
  (Premier Diagnostics, Cat. No. 8011900)

- Non-Inactivating Viral Transport Medium:
  PD VTM with nasal & throat swab, 3 ml non-inactivated medium, 10 ml tube
  (Premier Diagnostics, Cat. No. 8011903)

**NOTE**

⚠ ***Viral Transport Media (VTM) containing Guanidinium Thiocyanate are NOT suitable for direct PCR protocols.***

The direct PCR protocol to be used with the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 was developed and validated to be used with the following real-time PCR instruments:

- CFX96™ (BioRad)
- CFX Opus 96 (BioRad)

## 10. Material and Devices required but Not Provided

- Appropriate real-time PCR instrument
- Appropriate nucleic acid extraction system or kit
- 1.5 ml microcentrifuge tubes (with safe-lock or screw cap)
- Microcentrifuge (with speed ≥ 13,000 rpm)
- Pipettes, adjustable (range: 10 µl, 100 µl, 200 µl, 1000 µl)
- Pipette tips (with aerosol barriers)
- Disposable gloves (powder-free)

- Heating block for lysis of specimens during extraction
- Vortex mixer
- Appropriate 96-well reaction plates or reaction tubes with corresponding (optical) closing material **(Clear tubes are recommended. Do not use white tubes)**

## 11. Specimen Storage

- Suitable specimens include bronchoalveolar lavage, tracheal aspirate, sputum, nasopharyngeal and oropharyngeal swabs placed in VTM, nasopharyngeal wash/aspirate and nasal wash/ aspirate.

- Follow specimen transport and storage conditions outlined in the following guidelines:

  - World Health Organization (2000). Guidelines for the collection of clinical specimens during field investigation of outbreaks. https://apps.who.int/iris/handle/10665/66348
  - World Health Organization (2020). Interim Guidance on Laboratory testing for coronavirus disease 2019 (COVID-19) in suspected human cases. https://apps.who.int/iris/handle/10665/331501
  - Centers for Disease Control and Prevention (2022). Interim Guidelines for Collecting and Handling of Clinical Specimens for COVID-19 Testing. https://www.cdc.gov/coronavirus/2019-nCoV/lab/guidelines-clinical-specimens.html
  - World Health Organization (2011). Global Influenza Surveillance Network. Manual for the laboratory diagnosis and virological surveillance of influenza (https://apps.who/int.iris/handle/10665/44518)
  - Centers for Disease Control and Prevention (2018). Influenze Specimen Collection. https://www.cdc.gov/flu/pdf/freeresources/healthcare/flu-specimen-collection-guide.pdf
  - World Health Organization (2023). Respiratory Syncytial Viral Surveillance. Collection, transport, and storage of clinical specimens https://www.who.int/teams/global-influenza-programme/global-respiratory-syncytial-virus-surveillance/collection-transport-and-storage

# 12. Instructions for Use

## 12.1. Sample Preparation

### 12.1.1. Sample Preparation via Nucleic Acid Extraction System

Extracted RNA is the starting material for the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0. The quality of the extracted RNA has a profound impact on the performance of the whole test system. It has to be ensured that the nucleic acid extraction system used is compatible with real-time PCR technology.

The following nucleic acid extraction kits / systems are suitable for use with the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0:

- SpinStar™ Viral Nucleic Acid Kit (AstronDX Technologies)
- MagCore® Plus II Automated Nucleic Acid Extractor (RBC Bioscience)
- QIAamp® MinElute Virus Spin Kit (Qiagen)
- QIAamp® Viral RNA Mini Kit (Qiagen)
- QIAsymphony® (Qiagen)

Alternative nucleic acid extraction systems and kits might also be appropriate. The suitability of the nucleic acid extraction procedure for use with LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 has to be validated by the user.

If using a spin column-based sample preparation procedure including washing buffers containing ethanol, an additional centrifugation step for 10 min at approximately 17000 × g (~ 13000 rpm), using a new collection tube, prior to the elution of the nucleic acid is highly recommended.

**NOTE**

***Ethanol is a strong inhibitor in real-time PCR. If your sample preparation system is using washing buffers containing ethanol, you need to make sure to eliminate any traces of ethanol prior to elution of the nucleic acid.***

### 12.1.2. Sample Preparation via Direct PCR Protocol

Swab samples collected in VTM is the starting material for the direct PCR protocol to be used with LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0.

The following direct PCR protocols are suitable for use with the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0

**Protocol 1**: For LyteStar™ Direct PCR VTM

1) Mix the specimen collected in the LyteStar™ Direct PCR VTM by tapping, vortexing or pipetting.

2) Use 5 µl of the sample as PCR template and continue with Master Mix Setup.

**Protocol 2**: For Viral Transport Medium (VTM)

1) Mix the specimen collected in the Viral Transport Medium (VTM) by vortexing or pipetting.

2) Dilute the sample in a 1 : 2 ratio with nuclease free-water (200 µl specimen diluted in 400 µl nuclease-free water) in a 1.5ml Eppendorf tube.

3) Pulse vortex the tube for 15 seconds, and briefly centrifuge.

4) Transfer the tube into a dry bath (heat-block), pre-heated to 95°C, for 4 minutes. (This step may be omitted when using Inactivating VTMs.)

5) Mix the tube gently by tapping or vortexing, and centrifuge at 10,000 rpm for 1 minute.

6) Use 5 µl of the sample as PCR template and continue with Master Mix Setup.

**NOTE**

⚠ ***The heat inactivation step (Step 4) may be omitted when using Inactivating Viral Transport Medium***

## 12.2. Master Mix Setup

1. All reagents and samples should be thawed completely, mixed (by gentle vortex mixing) and centrifuged briefly before use. Prepare a marginal excess (additional 0.5 reaction) of the required Master Mix volume.

2. The LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 contains a heterologous Internal Control (IC), which can either be used as (i) a PCR inhibition control or as (ii) a control of the sample preparation procedure (nucleic acid extraction) and PCR inhibition control.

**NOTE**

⚠ ***For the direct PCR protocol, the Internal Control is used as a PCR inhibition control ONLY and is added to the Master Mix***

(i) If the IC is used as a PCR inhibition control, but not as a control for the sample preparation procedure, the Master Mix is set up according to the following pipetting scheme:

| Number of Reactions | 1 | 12 |
|---|---|---|
| Master A | 6.25 µl | 75 µl |
| Master B | 13.75 µl | 165 µl |
| IC | 0.5 µl | 6 µl |
| **Volume Master Mix** | **20.5 µl** | **246 µl** |

(ii) If the IC is used as a control for the sample preparation procedure and as a PCR inhibition control, the IC has to be added during the nucleic acid extraction procedure.

No matter which method/system is used for nucleic acid extraction, the IC **must not** be added directly to the specimen. The IC should always be added to the specimen/lysis buffer mixture.

The volume of the IC which has to be added depends always and only on the elution volume. It represents **10% of the elution volume**. For instance, if the nucleic acid is going to be eluted in 60 µl of elution buffer or water, 6 µl of IC must be added into the specimen/lysis buffer mixture.

If the IC was added during the sample preparation procedure, the Master Mix is set up according to the following pipetting scheme:

| Number of Reactions | 1 | 12 |
|---|---|---|
| Master A | 6.25 µl | 75 µl |
| Master B | 13.75 µl | 165 µl |
| **Volume Master Mix** | **20 µl** | **240 µl** |

**NOTE**

⚠ ***Never add the Internal Control directly to the specimen.***

## 12.3. Reaction Setup

1. Pipette 20 µl Master Mix into each required well of an appropriate optical 96-well reaction plate or an appropriate optical reaction tube.

2. Add 5 µl of the sample (eluate from the nucleic acid extraction) or 5 µl of the controls (Positive Control; or water as No Template Control, NTC).

3. Make sure at least one Positive Control and one NTC are used per run.

4. Thoroughly mix the samples and controls with the Master Mix by pipetting up and down.

5. Close the 96-well reaction plate with an appropriate optical adhesive film and the reaction tubes with appropriate caps.

6. Centrifuge the 96-well reaction plate at 1,000 x g (~3,000 rpm) for 30s.

| Reaction Setup | |
|---|---|
| Master Mix | 20 µl |
| Sample or Control | 5 µl |
| **Total Volume** | **25 µl** |

# 13. Programming the Real-Time PCR Instrument

## 13.1 Settings

• Define the following settings:

| Settings | |
|---|---|
| Reaction Volume | 25 µl |
| Ramp Rate | Default |

## 13.2 Fluorescent Detectors (Dyes)

• Define the following fluorescent detectors:

| Detection | Detector Name | Reporter | Quencher |
|---|---|---|---|
| SARS-CoV-2 (*RdRP* gene) specific RNA | SARS-CoV-2 | Cy5 | IBRQ |
| Influenza A (*M* gene) specific RNA | Flu A | Quasar705 | BHQ1 |
| Influenza B (*HA* gene) specific RNA | Flu B | Tex615 | IBRQ |
| RSV (*M* gene) specific RNA | RSV | FAM | IBFQ |
| Internal Control | IC | HEX | IBFQ |

## 13.3 Temperature Profile and Dye Acquisition

• Define the temperature profile and dye acquisition:

| | Stage | Cycle Repeats | Acquisition | Temperature | Time |
|---|---|---|---|---|---|
| Reverse-transcription | Hold | 1 | - | 50 °C | 10:00 min |
| Denaturation | Hold | 1 | - | 95 °C | 2:00 min |
| Amplification | Cycling | 45 | - | 95 °C | 5 sec |
| | | | √ | 55 °C | 30 sec |

√ Signal acquisition: activate Cy5, Quasar705, Tex615, FAM, and HEX channels in all runs.

## 14. Data Analysis

For basic information regarding data analysis on specific real-time PCR instruments, please refer to the manual of the respective instrument. For detailed instructions regarding data analysis of the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 on different real-time PCR instruments please contact our Technical Support (**16.Technical Support**).

### 14.1. Validity of Diagnostic Test Runs

#### 14.1.1 Valid Diagnostic Test Runs (Qualitative)

For a **valid** diagnostic test run (qualitative), the following control conditions must be met:

| Control ID | Cy5/Quasar705/Tex615/FAM Detection Channel | HEX Detection Channel |
|---|---|---|
| Positive Control | POSITIVE | POSITIVE |
| Negative Control | NEGATIVE | POSITIVE |

#### 14.1.2 Target CT values of PC and IC

| | Positive Control *(SARS-CoV-2)* | Positive Control *(Flu A)* | Positive Control *(Flu B)* | Positive Control *(RSV)* | Internal Control |
|---|---|---|---|---|---|
| Target CT value | < 35 cycles | < 35 cycles | < 35 cycles | < 35 cycles | ≤ 40 cycles* |

*Required for unknown samples that do not amplify in Cy5, Quasar705, Tex615, and FAM channels

**Note:** The above CT target values are exclusively given for **monitoring the integrity of the product and validated assay conditions** and should be achieved **ONLY for the provided Positive Control (PC) and Internal Control (IC)** when used as per the instructions given under section 12.3. Reaction set up. **The target CT values for PC MUST NOT be misinterpreted as the diagnostic cut-off values for clinical samples.**

### 14.1.3 Invalid Diagnostic Test Runs (Qualitative)

A **qualitative** diagnostic test run is **invalid**, (i) if the run has not been completed or (ii) if any of the control conditions for a **valid** diagnostic test run are not met.

In case of an **invalid** diagnostic test run, **repeat testing by using the remaining purified nucleic acids** or start from the original samples again.

## 14.2 Interpretation of Results

| **Cy5** *SARS-CoV-2* | **Quasar 705** *Flu A* | **Tex615** *Flu B* | **FAM** *RSV* | **HEX** *Internal Control* | **Result Interpretation** |
|---|---|---|---|---|---|
| + | + | + | + | +* | SARS-CoV-2, Influenza A, Influenza B and RSV specific RNA detected.<br>*Positive for SARS-CoV-2, Influenza A, Influenza B and RSV* |
| + | + | + | - | +* | SARS-CoV-2, Influenza A and Influenza B specific RNA detected.<br>*Positive for SARS-CoV-2, Influenza A and Influenza B* |
| + | + | - | + | +* | SARS-CoV-2, Influenza A and RSV specific RNA detected.<br>*Positive for SARS-CoV-2, Influenza A and RSV* |
| + | - | + | + | +* | SARS-CoV-2, Influenza B and RSV specific RNA detected.<br>*Positive for SARS-CoV-2, Influenza B and RSV* |
| - | + | + | + | +* | Influenza A, Influenza B and RSV specific RNA detected.<br>*Positive for Influenza A, Influenza B and RSV* |

| | | | | | |
|---|---|---|---|---|---|
| + | + | - | - | +* | SARS-CoV-2 and Influenza A specific RNA detected.<br>*Positive for SARS-CoV-2 and Influenza A* |
| + | - | + | - | +* | SARS-CoV-2 and Influenza B specific RNA detected.<br>*Positive for SARS-CoV-2 and Influenza B* |
| + | - | - | + | +* | SARS-CoV-2 and RSV specific RNA detected.<br>*Positive for SARS-CoV-2 and RSV* |
| - | + | + | - | +* | Influenza A and Influenza B specific RNA detected.<br>*Positive for Influenza A and Influenza B* |
| - | + | - | + | +* | Influenza A and RSV specific RNA detected.<br>*Positive for Influenza A and RSV* |
| - | - | + | + | +* | Influenza B and RSV specific RNA detected.<br>*Positive for Influenza B and RSV* |
| + | - | - | - | +* | SARS-CoV-2 specific RNA detected.<br>*Positive for SARS-CoV-2* |
| - | + | - | - | +* | Influenza A specific RNA detected.<br>*Positive for Influenza A* |
| - | - | + | - | +* | Influenza B specific RNA detected.<br>*Positive for Influenza B* |
| - | - | - | + | +* | RSV specific RNA detected.<br>*Positive for RSV* |

| | | | | | |
|---|---|---|---|---|---|
| - | - | - | - | + | SARS-CoV-2, Influenza A, Influenza B and RSV specific RNA not detected. The samples do not contain detectable amounts of SARS-CoV-2, Influenza A, Influenza B and/or RSV specific RNA. |
| - | - | - | - | - | PCR inhibition or reagent failure. Repeat testing from original sample or collect and test a new sample. |

**Note: For SARS-CoV-2 *RdRP* gene (Cy5 channel), Influenza A *M* gene (Quasar705 channel), Influenza B *HA* gene (Tex615 channel) and RSV *M* gene (FAM channel) "+" refers to amplification curve detected at CT ≤ 45 cycles. "-" refers to no amplification / no CT obtained.**

* Detection of the Internal Control in the HEX channel is not required for positive results in the Cy5/Quasar705/Tex615/FAM detection channels. A high SARS-CoV-2 Influenza A, Influenza B and/or RSV viral load in the sample can lead to reduced or absent Internal Control signals.

### 14.2.1 Threshold Settings for Cycler Software

| Cycler | Threshold | | | | |
|---|---|---|---|---|---|
| | **Cy5** SARS-CoV-2 **Channel** | **Quasar705** Flu A **Channel** | **Tex615** Flu B **Channel** | **FAM** RSV **Channel** | **HEX** IC **Channel** |
| CFX96™ | 100 RFU | 100 RFU | 100 RFU | 100 RFU | 100 RFU |
| CFX Opus 96 | 100 RFU | 100 RFU | 100 RFU | 100 RFU | 100 RFU |

### 14.2.2 CT Cut-Off Values for Clinical Samples

| | **Cy5** SARS-CoV-2 **Channel** | **Quasar705** Flu A **Channel** | **Tex615** Flu B **Channel** | **FAM** RSV **Channel** |
|---|---|---|---|---|
| CT Cut-Off Value | < 45 cycles | < 45 cycles | < 45 cycles | < 45 cycles |

## 15. Performance Evaluation

The analytical performance evaluation of the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 was accomplished using quantified SARS-CoV-2/ Influenza A/ Influenza B/ RSV specific RNA.

### 15.1 Analytical Sensitivity

The analytical sensitivity (limit of detection: LoD) of LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 is defined as the concentration of SARS-CoV-2, Influenza A, Influenza B and RSV RNA molecules that can be detected with a positivity rate of ≥ 95%. The analytical sensitivity was determined in consideration of a selected nucleic acid extraction method and the direct PCR protocol, by analyzing samples with known concentration.

#### 15.1.1. In consideration of a Nucleic Acid Extraction Procedure

A dilution series of the AmpliRun® Total SARS-CoV-2/FluA/FluB/RSV Control (swab) was prepared by spiking into viral transport medium (VTM) and extracted with the SpinStar™ Viral Nucleic Acid Extraction Kit 1.0. Extracted RNA was analyzed with LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0. Results were analyzed by Probit analysis (Table 1, Table 2, Table 3, and Table 4).

Nucleic Acid Extraction Procedure:

SpinStar™ Viral Nucleic Acid Extraction Kit 1.0 (AstronDX Technologies)
Sample volume: 200 µl
Elution volume: 60 µl

The analytical sensitivity of the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 in consideration of SpinStar™ nucleic acid extraction method was determined at 0.31 copies/µl for SARS-CoV-2 *RdRP* gene target, 0.82 copies/µl for Influenza A *M* gene target, 0.85 copies/µl for Influenza B *HA* gene target and 1.53 copies/µl for RSV *M* target (p≤ 0.05).

Table 1. PCR results used for the calculation of the analytical sensitivity of SARS-CoV-2 *RdRP* gene target for the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 in consideration of a particular extraction method and in combination with the CFX96™ platform (BioRad).

| Concentration (copies/µl) | No. of Samples Tested | No. of Samples Positive | Hit rate (%) |
|---|---|---|---|
| 14.0 | 12 | 12 | 100 |
| 7.0 | 12 | 12 | 100 |
| 4.5 | 12 | 12 | 100 |
| 1.4 | 12 | 12 | 100 |
| 0.45 | 12 | 12 | 100 |
| 0.14 | 12 | 8 | 66.7 |
| 0.045 | 12 | 4 | 33.3 |
| 0.014 | 12 | 0 | 0 |
| 0.0045 | 12 | 0 | 0 |

Table 2. PCR results used for the calculation of the analytical sensitivity of Influenza A *M* gene target for the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 in consideration of a particular extraction method and in combination with the CFX96™ platform (BioRad).

| Concentration (copies/µl) | No. of Samples Tested | No. of Samples Positive | Hit rate (%) |
|---|---|---|---|
| 21.0 | 12 | 12 | 100 |
| 10.0 | 12 | 12 | 100 |
| 6.5 | 12 | 12 | 100 |
| 2.1 | 12 | 12 | 100 |
| 0.65 | 12 | 5 | 41.7 |
| 0.21 | 12 | 0 | 0 |
| 0.065 | 12 | 0 | 0 |
| 0.021 | 12 | 0 | 0 |
| 0.0065 | 12 | 0 | 0 |

Table 3. PCR results used for the calculation of the analytical sensitivity of Influenza B *HA* gene target for the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 in consideration of a particular extraction method and in combination with the CFX96™ platform (BioRad).

| Concentration (copies/µl) | No. of Samples Tested | No. of Samples Positive | Hit rate (%) |
|---|---|---|---|
| 20.0 | 12 | 12 | 100 |
| 10.0 | 12 | 12 | 100 |
| 6.3 | 12 | 12 | 100 |
| 2.0 | 12 | 12 | 100 |
| 0.63 | 12 | 11 | 91.7 |
| 0.20 | 12 | 7 | 58.3 |
| 0.063 | 12 | 6 | 50.0 |
| 0.020 | 12 | 1 | 8.3 |
| 0.0063 | 12 | 0 | 0 |

Table 4. PCR results used for the calculation of the analytical sensitivity of RSV *M* gene target for the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 in consideration of a particular extraction method and in combination with the CFX96™ platform (BioRad).

| Concentration (copies/µl) | No. of Samples Tested | No. of Samples Positive | Hit rate (%) |
|---|---|---|---|
| 25.0 | 12 | 12 | 100 |
| 12.4 | 12 | 12 | 100 |
| 7.8 | 12 | 12 | 100 |
| 2.5 | 12 | 12 | 100 |
| 0.78 | 12 | 8 | 66.7 |
| 0.25 | 12 | 1 | 8.3 |
| 0.078 | 12 | 0 | 0 |
| 0.025 | 12 | 0 | 0 |
| 0.0078 | 12 | 0 | 0 |

### 15.1.2. In consideration of a Direct PCR Protocol

A dilution series of the AmpliRun® Total SARS-CoV-2/FluA/FluB/RSV Control (swab) was prepared by spiking into the LyteStar™ Direct PCR VTM and undergoing the sample preparation via the direct PCR protocol (**12.1.2 Sample Preparation via Direct PCR Protocol**). SARS-CoV-2, Influenza A, Influenza B and RSV RNA was analyzed with LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0. Results were analyzed by Probit analysis. (Table 5, Table 6, Table 7 and Table 8).

The analytical sensitivity of the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0, when used with the direct PCR protocol was determined at 0.65 copies/µl for SARS-CoV-2 *RdRP* gene target, 4.96 copies/µl for Influenza A *M* gene target, 0.44 copies/µl for Influenza B *HA* gene target and 2.30 copies/µl for RSV *M* gene target ($p \leq 0.05$).

Table 5. PCR results used for the calculation of the analytical sensitivity of SARS-CoV-2 *RdRP* gene target for the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 using LyteStar™ Direct PCR VTM in combination with the CFX96™ platform (BioRad).

| Concentration (copies/µl) | No. of Samples Tested | No. of Samples Positive | Hit rate (%) |
|---|---|---|---|
| 23.0 | 12 | 12 | 100 |
| 7.2 | 12 | 12 | 100 |
| 3.6 | 12 | 12 | 100 |
| 2.3 | 12 | 12 | 100 |
| 0.72 | 12 | 11 | 91.7 |
| 0.23 | 12 | 8 | 66.7 |
| 0.072 | 12 | 0 | 0 |
| 0.023 | 12 | 0 | 0 |
| 0.0072 | 12 | 0 | 0 |

Table 6. PCR results used for the calculation of the analytical sensitivity of Influenza A *M* gene target for the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 using LyteStar™ Direct PCR VTM in combination with the CFX96™ platform (BioRad).

| Concentration (copies/µl) | No. of Samples Tested | No. of Samples Positive | Hit rate (%) |
|---|---|---|---|
| 33.0 | 12 | 12 | 100 |
| 10.4 | 12 | 12 | 100 |
| 5.2 | 12 | 12 | 100 |
| 3.3 | 12 | 9 | 75.0 |
| 1.1 | 12 | 3 | 25.0 |
| 033 | 12 | 0 | 0 |
| 0.11 | 12 | 0 | 0 |
| 0.033 | 12 | 0 | 0 |
| 0.011 | 12 | 0 | 0 |

Table 7. PCR results used for the calculation of the analytical sensitivity of Influenza B *HA* gene target for the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 using LyteStar™ Direct PCR VTM in combination with the CFX96™ platform (BioRad).

| Concentration (copies/µl) | No. of Samples Tested | No. of Samples Positive | Hit rate (%) |
|---|---|---|---|
| 32.0 | 12 | 12 | 100 |
| 10.1 | 12 | 12 | 100 |
| 5.1 | 12 | 12 | 100 |
| 3.2 | 12 | 12 | 100 |
| 1.0 | 12 | 12 | 100 |
| 032 | 12 | 6 | 50.0 |
| 0.10 | 12 | 0 | 0 |
| 0.032 | 12 | 0 | 0 |
| 0.010 | 12 | 0 | 0 |

Table 8. PCR results used for the calculation of the analytical sensitivity of RSV *M* gene target for the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 using LyteStar™ Direct PCR VTM in combination with the CFX96™ platform (BioRad).

| Concentration (copies/µl) | No. of Samples Tested | No. of Samples Positive | Hit rate (%) |
|---|---|---|---|
| 40.0 | 12 | 12 | 100 |
| 12.5 | 12 | 12 | 100 |
| 6.3 | 12 | 12 | 100 |
| 4.0 | 12 | 12 | 100 |
| 1.3 | 12 | 9 | 75.0 |
| 0.40 | 12 | 2 | 16.7 |
| 0.13 | 12 | 0 | 0 |
| 0.04 | 12 | 0 | 0 |
| 0.013 | 12 | 0 | 0 |

## 15.2 Analytical Specificity

The analytical specificity of the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 is ensured by the thorough selection of the oligonucleotides (primers and probes). The oligonucleotides were checked by sequence comparison analysis against publicly available sequences to ensure that the applied primer/probes in LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 specifically detect SARS-CoV-2, Influenza A, Influenza B and RSV.

The specificity of the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 was evaluated by testing genomic RNA/DNA extracted from other pathogens likely to be present in the same sample material as SARS-CoV-2, Influenza A, Influenza B and RSV virus, or that cause similar symptoms to these viruses (Table 9).

Table 9. Microorganisms tested to demonstrate the analytical specificity of the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR 1.0 Kit.

| LyteStar™ COVID/Flu/ RSV Differentiation RT-PCR Kit 1.0 | | | | | |
|---|---|---|---|---|---|
| **Organisms** | ***SARS-CoV-2 (Cy5 channel)*** | ***Flu A (Quasar 705 channel)*** | ***Flu B (Tex615 channel)*** | ***RSV (FAM channel)*** | ***Internal Control (HEX channel)*** |
| Enterovirus 68 | negative | negative | negative | negative | valid |
| Enterovirus 71 | negative | negative | negative | negative | valid |
| Enterovirus (Coxsackie A6) | negative | negative | negative | negative | valid |
| Human rhinovirus 77 | negative | negative | negative | negative | valid |
| Human rhinovirus 16 | negative | negative | negative | negative | valid |
| Human adenovirus 1 | negative | negative | negative | negative | valid |
| Human metapneumovirus | negative | negative | negative | negative | valid |
| Human parainfluenza virus 1 | negative | negative | negative | negative | valid |
| Human parainfluenza virus 2 | negative | negative | negative | negative | valid |
| Human parainfluenza virus 3 | negative | negative | negative | negative | valid |
| *Bordetella parapertussis* | negative | negative | negative | negative | valid |
| *Bordetella pertussis* | negative | negative | negative | negative | valid |
| *Chlamydophila pneumoniae* | negative | negative | negative | negative | valid |
| *Haemophilus influenza* | negative | negative | negative | negative | valid |
| *Legionella pneumophila* subsp. *Pneumophila* | negative | negative | negative | negative | valid |
| *Moraxella catarrhalis* | negative | negative | negative | negative | valid |
| *Streptococcus pneumoniae* | negative | negative | negative | negative | valid |
| *Mycobacterium tuberculosis* | negative | negative | negative | negative | valid |
| *Streptococcus pyogenes* | negative | negative | negative | negative | valid |
| *Mycoplasma pneumoniae* | negative | negative | negative | negative | valid |
| Human coronavirus OC43 | negative | negative | negative | negative | valid |
| Human coronavirus 229E | negative | negative | negative | negative | valid |
| Human coronavirus HKU1 | negative | negative | negative | negative | valid |
| Human coronavirus NL63 | negative | negative | negative | negative | valid |

| | | | | | |
|---|---|---|---|---|---|
| MERS coronavirus | negative | negative | negative | negative | valid |
| Human bocavirus | negative | negative | negative | negative | valid |
| Human respiratory syncytial virus | negative | negative | negative | positive | valid |
| SARS-CoV-2 Delta variant | positive | negative | negative | negative | valid |
| SARS-CoV-2 Omicron variant | positive | negative | negative | negative | valid |
| Influenza A virus (H1N1) | negative | positive | negative | negative | valid |
| Influenza A virus (H3N2) | negative | positive | negative | negative | valid |
| Influenza A virus (H5N1) | negative | positive | negative | negative | valid |
| Influenza A virus (H7N9) | negative | positive | negative | negative | valid |
| Influenza A virus (H9N2) | negative | positive | negative | negative | valid |
| Influenza B virus (Victoria lineage) | negative | negative | positive | negative | valid |
| Influenza B virus (Yamagata lineage) | negative | negative | positive | negative | valid |

The LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 did not cross-react with any pathogen or genotype/subtype other than its own target.

## 15.3 Diagnostic Sensitivity and Specificity

The clinical performance of the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0, in regards to diagnostic sensitivity and specificity, was evaluated through retrospective studies performed at various hospitals and public health laboratories. The diagnostic sensitivity and specificity of the LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 was compared to the clinical site's reference method.

The LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 achieved a diagnostic sensitivity of 100% for the detection of SARS-CoV-2, Influenza A, Influenza B and RSV, and a diagnostic specificity of 100% for the detection of SARS-CoV-2, 99.2% for the detection of Influenza A, 100% for the detection of Influenza B and 98.2% for the detection of RSV.

| | | Reference Method | | | |
|---|---|---|---|---|---|
| | | SARS-CoV-2 (n=102) | Influenza A (n=28) | Influenza B (n=35) | RSV (n=29) |
| | | Positive | | | |
| LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 | Detected | 102 | 28 | 35 | 29 |
| | Not Detected | 0 | 0 | 0 | 0 |
| | Total | **102** | **28** | **35** | **29** |
| Overall Concordance | | **100 %** 102 / 102 Sensitivity | **100 %** 28 / 28 Sensitivity | **100 %** 35 / 35 Sensitivity | **100 %** 29 / 29 Specificity |

| | | Reference Method | | | |
|---|---|---|---|---|---|
| | | SARS-CoV-2 (n=105) | Influenza A (n=119) | Influenza B (n=119) | RSV (n=113) |
| | | Negative | | | |
| LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 | Detected | 0 | 1 | 0 | 2 |
| | Not Detected | 105 | 118 | 119 | 111 |
| | Total | **105** | **119** | **119** | **113** |
| Overall Concordance | | **100 %** 105 / 105 Specificity | **99.2 %** 118 / 119 Specificity | **100 %** 119 / 119 Specificity | **98.2 %** 111 / 113 Specificity |

To calculate the diagnostic sensitivity of LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0:

$$\text{Diagnostic Sensitivity [\%]} = \frac{\text{“correct positive”}}{\text{(“correct positive” + “false positive”)}} \times 100$$

To calculate the diagnostic specificity of LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0:

$$\text{Diagnostic Specificity [\%]} = \frac{\text{“correct negative”}}{\text{(“correct negative” + “false negative”)}} \times 100$$

## 16. Technical Support

For customer support, please contact our Technical Support:

**e-mail:** **techsupport@astrondx.com**
**phone:** **+603 7931 6760**

## 17. Appendix

**Explanation of Symbols**

| | |
|---|---|
|  | *In vitro* diagnostic medical device |
|  | Product Number |
|  | Batch Code |
|  | Manufacturer |
|  | Date of Manufacture |
|  | Contains sufficient for "n" tests/rxns |
|  | Temperature limitation |
|  | Version |
|  | Use-By Date |

## 18. Ordering Information

| Products | Packing (reactions) | Order No. |
|---|---|---|
| LyteStar™ COVID/ Flu/ RSV Differentiation RT-PCR Kit 1.0 | 96 | 881203 |
| SpinStar™ Viral Nucleic Acid Extraction Kit 1.0 | 100 | 811803 |
| MagCore® Viral Nucleic Acid Extraction Kit, High Sensitivity (200µl/400µl), CART CODE 203 | 96 | MVN400-06 |

## NOTES

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