Direct RT-qPCR Kit

EXTRACTION-FREE NUCLEIC ACID AMPLIFICATION TESTING (NAAT)

The Direct RT-qPCR Kit* is an extraction-free chemistry designed for use in one-step real-time RT-PCR (RT-qPCR) for the detection of viral RNA, including SARS-CoV-2, using probe detection using the 5′-nuclease method. The kit provides fast, accurate and sensitive detection of viral RNA directly from unpurified biological nasopharyngeal, nose or throat samples in universal transfer medium (UTM™). We call this "direct RT qPCR" because it does not require a separate extraction step prior to amplification. The kit includes our proprietary Anti-Inhibitor Mix (AIC Mix), RT-qPCR master mix and reconstitution buffer.

*For research use only

Features

- Proprietary anti-inhibitor mix (AIC) enables the direct detection of low-copy viral pathogen targets.
- Convenient RT-qPCR enzyme master mix for detection of low-copy viral pathogen targets.
- High specificity and sensitivity across a wide range of sample sources.

Limit of detection: reliable direct detection of SARS-CoV-2 viral RNA down to 1.25 copies/µL

We used SARS-CoV-2 heat inactivated viral particles (ATCC VR-1986HK) to assess the performance and define the approximate limit of detection of the Direct RT-qPCR Kit in an experimental setting relevant to COVID-19 detection from human swab samples. We spiked viral particles at between 0.625 to 5 copies/µL into a diluent consisting of a suspension of human cells (~ 3 × 10^5/mL) and viral transport medium (VTM)† to mimic a clinical sample (1). Unspiked cell suspension (no viral RNA) served as the assay control to eliminate the possibility of false positives. We processed the unpurified samples prior to direct RT-qPCR amplification using the Direct RT-qPCR Kit protocol. Briefly, 5 µL of each sample was added to 6 µL AIC Mix, heated at 70°C for 5 minutes, then added to 9 µL RT-qPCR reaction master mix, providing 20 µL reactions.

†VTM produced in support of the Centers for Disease Control and Prevention (CDC) Coronavirus Disease 2019 (COVID-19) outbreak response, containing 2% FBS, 100 µg/mL Gentamicin, 0.5 µg/mL Amphotericin B in 1 × HBSS with Ca2+ and Mg2+, no phenol red, based on the following protocol https://www.cdc.gov/coronavirus/2019-ncov/downloads/Viral-Transport-Medium.pdf

Fig 1. The Direct RT-qPCR Kit includes the Anti-Inhibitor Complex (AIC Mix), RT-qPCR enzyme master mix and reconstitution buffer.

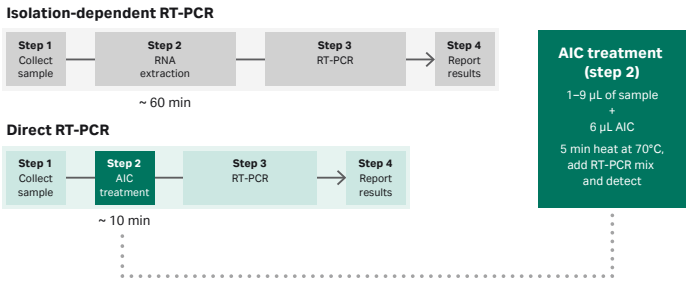


Fig 2. Cytiva's RT-qPCR anti-inhibitor complex (AIC) allows direct RT-qPCR detection of nucleic acid material from unprocessed samples. The proprietary RT-qPCR anti-inhibitor complex eliminates the need for nucleic acid isolation, requiring only a short 5 minutes heating treatment at 70°C prior the RT-qPCR reaction.

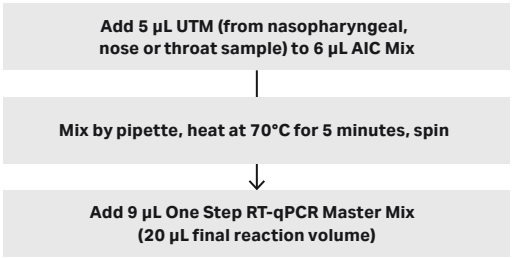


Fig 3. Direct RT-qPCR amplification workflow using the Direct RT-qPCR Kit.

One-step reverse transcription and amplification reactions were carried out following these thermal cycling conditions:

1. 50°C for 10 minutes
2. 95°C for 3 minutes
3. 45 cycles of 95°C for 10 seconds
4. 60°C for 30 seconds.

We tested the Direct RT-qPCR Kit using Centers for Disease Control and Prevention (CDC) Novel Coronavirus (2019-n-CoV) Diagnostic Panel primers (N1 and N2 targeting two regions of the SARS-CoV-2 nucleocapsid gene, Integrated DNA Technologies) in simplex format.

Samples were run in technical triplicate in three independent experiments. As per CDC guidelines, only samples for which both markers (N1 and N2) crossed the threshold at Ct < 40 were considered positive. No amplification in any of the SARS-CoV-2 specific primer sets were observed in unspiked controls (data not shown). The results presented in Figure 4 confirm the Direct RT-qPCR Kit is robust and sensitive enough for reproducible detection of low viral titer down to 1.25 copes/µL directly from unpurified samples (100% hit rate). Quantification down to 0.625 copes/µL is also achievable (89% hit rate).

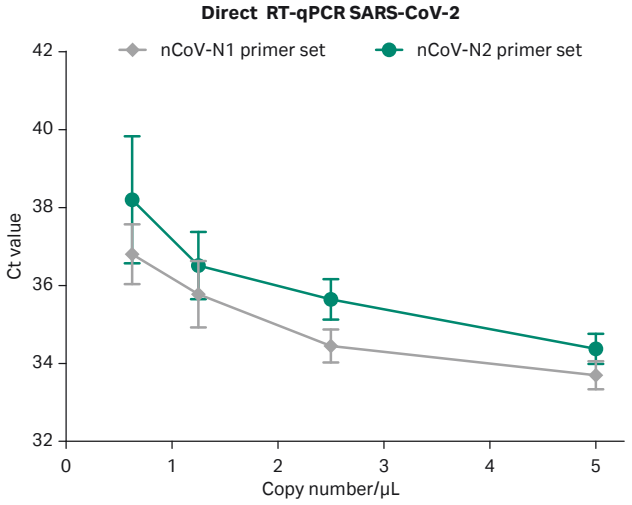


Fig 4. Limit of Detection (LOD) data for the Direct RT-qPCR Kit following direct RT-qPCR amplification of varying amounts of heat inactivated viral particles spiked at 0.625, 1.25, 2.5, and 5 copies/µL in the input sample. Ct values averaged from three independent experiments, n = 3 samples per experiment. Error bars represent standard deviation.

Comparative performance of the Direct RT-qPCR Kit

Performance of the Direct RT-qPCR Kit was compared with QIAprep&amp™ Viral RNA UM Kit (Qiagen) using SARS-CoV-2 heat inactivated viral particles (ATCC VR-1986HK).

SARS-CoV-2 heat inactivated viral particles were spiked at 0.625, 1.25, 2.5, and 25 copies/µL into a diluent consisting of a suspension of human cells (~ 3 × 10^5/mL) and VTM to mimic a clinical sample. Unspiked cell suspension (no viral RNA) served as the assay control to eliminate the possibility of false positives. We then processed the unpurified samples prior to direct RT-qPCR amplification using the Direct RT-qPCR Kit and QIAprep&amp™ Viral RNA UM Kit following the manufacturers' protocols. Briefly, sample processing involved the following steps:

- For the Cytiva kit, 5 µL of each sample was added to 6 µL AIC Mix, heated at 70°C for 5 minutes then added to 9 µL RT-qPCR reaction master mix.
- For the Qiagen kit, 5 µL of each sample plus 3 µL VTM were heated at 70°C for 10 minutes, added to 2 µL Viral RNA UM Prep Buffer, incubated at room temperature for 2 minutes then added to 10 µL of RT-qPCR reaction master mix.

We tested the Direct RT-qPCR Kit using CDC Novel Coronavirus (2019-n-CoV) Diagnostic Panel primers (N1 and N2 targeting two regions of the SARS-CoV-2 nucleocapsid gene) in simplex format.

The QIAprep&amp™ Viral RNA UM Kit was tested using the SARS-CoV-2 N1+N2 Assay Kit (Qiagen), a reagent containing both N1 and N2 primer sets with sequences based on the CDC primer design: this reagent is recommended for use with the QIAprep&amp™ Viral RNA UM Kit. We also tested the Direct RT-qPCR Kit using the SARS-CoV-2 N1+N2 Assay Kit (Qiagen) for a direct comparison. All samples tested were treated equally (with the number of replicates being the same) and according to manufacturers' protocol and recommendations.

Data from three independent experiments presented in Figure 5 shows comparable results from both kits in detecting the presence of viral RNA down to 0.625 copies/µL in the input sample.

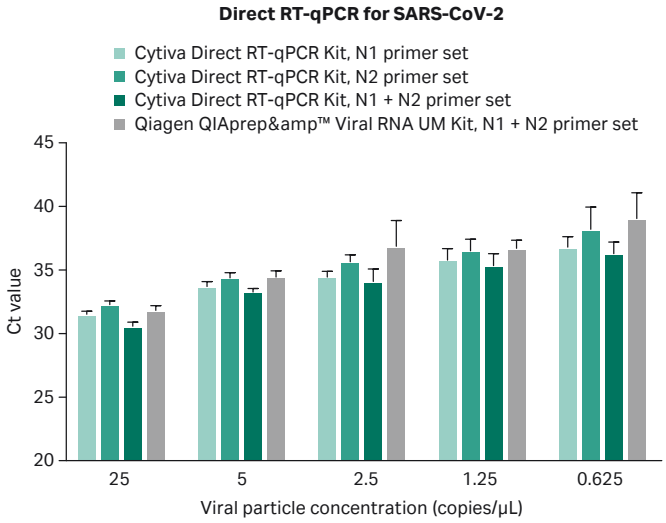


Fig 5. Ct values obtained for the Direct RT-qPCR Kit and QIAprep&amp™ Viral RNA UM Kit for varying amounts of the heat inactivated viral particles in the input sample as described in the graph. Values averaged from three independent experiments; error bars presented as standard deviation.

This data is based on a minimum of three independent experiments/replicate trials with an equal number of replicates in each experiment. All samples tested were treated equally (with the number of replicates being the same for all products tested in the comparison) and according to manufacturers' protocol and recommendations. Data was collected at Cytiva, Maynard Centre, Cardiff, UK (R&D Laboratory) during February 2021 and is held at this location.

Table 1. The percentage hit rate for samples represented in Figure 4 for varying amounts of heat inactivated viral particles in the input

Sample details	Hit rate (%)				
	25 copies/µL	5 copies/µL	2.5 copies/µL	1.25 copies/µL	0.625 copies/µL
Direct RT-qPCR Kit, N1 primer set	100	100	100	100	89
Direct RT-qPCR Kit, N2 primer set	100	100	100	100	89
Direct RT-qPCR Kit, N1 + N2 primer set	100	100	100	100	89
Qiagen QIAprep&amp™ Viral RNA UM Kit, N1 + N2 primer set	100	100	100	67	89

Performance of the Direct RT-qPCR Kit with other transport media

Nasopharyngeal swabs are the recommended sample type for COVID-19 nucleic acid amplification tests (NAATs). These swabs are commonly stored in universal transport medium (UTM™). One of the challenges encountered by the public health organizations and diagnostic labs during the COVID-19 pandemic was the shortage of transport media for collecting samples. The Centers for Disease Control and Prevention (CDC) recommend alternative collection media that can be prepared locally, including phosphate buffered saline (PBS), saline, or more widely used standard universal viral transport media suitable for cell culture (1, 2).

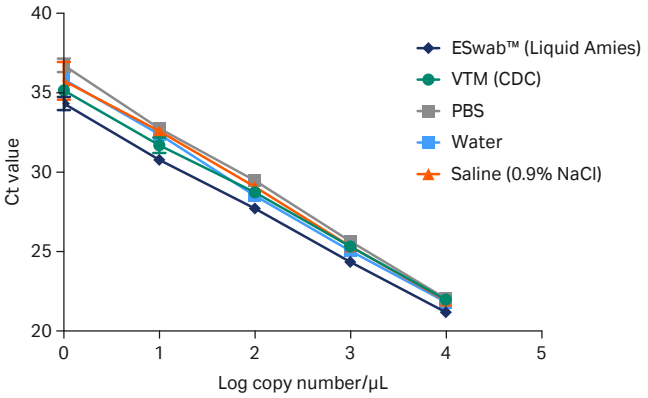
The Direct RT-qPCR Kit has been optimized for direct amplification of SARS-COV-2 viral RNA from unpurified nasopharyngeal swab samples contained in five different types of transport media. For the experiments below, direct amplification of SARS-COV-2 viral RNA from five alternative sample transport media was demonstrated using CDC 2019 Novel Coronavirus (2019-nCoV) Diagnostic Panel primers (N1 and N2) as previously described. SARS-CoV-2 heat inactivated viral particles were spiked at between one and 10 000 copies/µL into a suspension of human cells (~ 3 × 10^5/mL) diluted in the following sample transport media:

- ESwab™ 480C,Liquid Amies Medium (Copan)
- VTM (prepared according to CDC guidelines)
- PBS
- Saline (0.9% NaCl)
- Water

Samples were subjected to Direct RT-qPCR amplification using the protocol described previously.

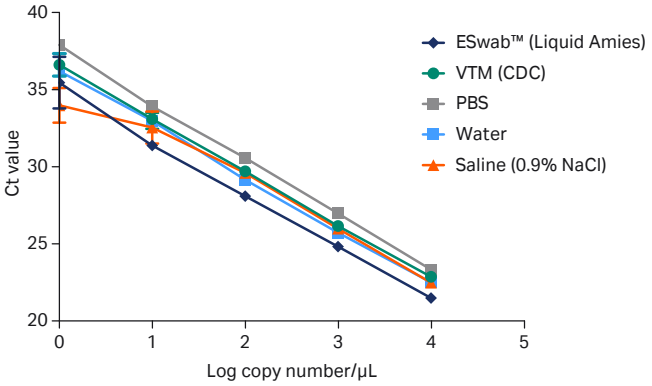
Data presented in Figure 6 confirms successful detection of viral RNA load down to 1 copy/µL in the input sample when using alternative transport media.

(A) **Direct RT-qPCR SARS-CoV-2 N1 primer set**



Transport media	Primer	Slope	R^2	Efficiency
Liquid Amies (ESwab™)	N1	-3.27	0.997	104.4
VTM (CDC)	N1	-3.30	0.998	96.4
PBS	N1	-3.58	0.997	89.2
Water	N1	-3.49	0.997	97.2
Saline (0.9%NaCl)	N1	-3.58	0.998	92.0

(B) **Direct RT-qPCR SARS-CoV-2 N2 primer set**



Transport media	Primer	Slope	R^2	Efficiency
Liquid Amies (ESwab™)	N2	-3.22	0.998	102.2
VTM (CDC)	N2	-3.41	0.993	101.0
PBS	N2	-3.61	0.998	90.2
Water	N2	-3.39	0.999	93.4
*Saline (0.9% NaCl)	N2	-3.53	0.999	90.2

*1 copy/µL data not used to calculate slope or R^2 value

Fig 6. Ct values obtained for the Direct RT-qPCR Kit for viral particles spiked at 1, 10, 100, 1000, and 10 000 copies/µL in alternative transport media as described in the graph. Values averaged from three technical replicates; error bars represent standard deviation.

Multiplexing

The Direct RT-qPCR Kit is compatible with multiplexing reactions, allowing simultaneous detection of exogenous or endogenous targets or controls for quality control or increased efficiency.

Performance of the kit was assessed over four orders of magnitude using heat inactivated SARS-CoV-2 viral particles spiked at between 10 and 10 000 copies/µL into a diluent consisting of a suspension of human cells (~ 3 × 10^5/mL) in VTM. Samples were processed and subjected to direct RT-qPCR as described previously using CDC Novel Coronavirus (2019-n-CoV) Diagnostic Panel primers (N1 and N2 targeting two regions of SARS-CoV-2 nucleocapsid gene and RNase P primers targeting human RNase P gene) in triplex format, using TaqMan™ probes (ThermoFisher Scientific) labeled with Fluorescein amidites (FAM), Tetramethylrhodamine (TAMRA) and Cy™5 (Cytiva) reporter dyes respectively to provide detection of three individual targets in a single reaction (3).

The Direct RT-qPCR Kit allows for reproducible multiplex detection of viral RNA down to 10 copies/µL in the input sample. The results presented in Figure 7 demonstrate simultaneous linear and sensitive detection of an exogenous control (RNase P) and two viral RNA targets (N1 and N2) across a wide range of viral loads.

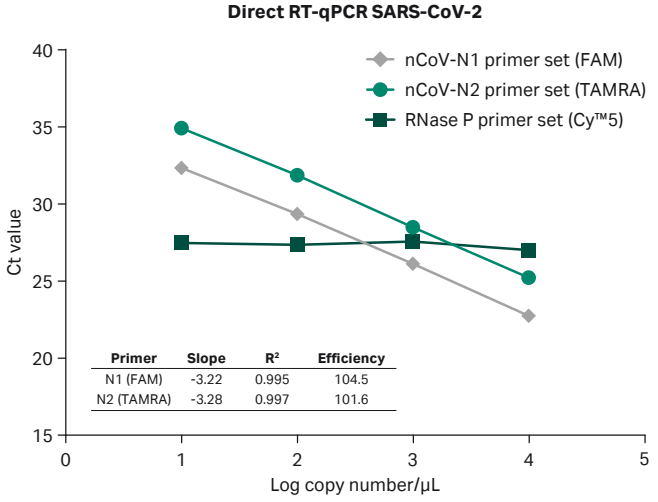


Fig 7. Ct values obtained for a triplex reaction using RNase P and two SARS-CoV-2 specific primer sets with varying amounts of heat inactivated viral particles in the input sample as described on the graph. Values averaged from ten technical replicates; error bars presented as standard deviation.

Reproducible, sensitive detection of low copy inputs of viral RNA

Consistency of Ct values for three unique lots of the RT-qPCR Master Mix

Cytiva's RT-qPCR Kit generates significant and reproducible Ct values for detection of ≤ 10 copy inputs of target RNA. We carried out experiments to test RT-qPCR amplification of synthetic viral RNA (Microbiologics 9009) covering the nucleocapsid gene of SARS-CoV-2 to assess performance and lot to lot variability of the RT-qPCR Master Mix.

Briefly, SARS-CoV-2 viral RNA samples (5 µL per reaction) at 1 and 10 copies/µL were run in technical replicates (n = 10) using three unique lots of the RT-qPCR Kit and CDC 2019 Novel Coronavirus (2019-nCoV) Diagnostic Panel primers (N1, N2, and N3 targeting three regions of SARS-CoV-2 nucleocapsid gene). As per CDC guidelines, only samples for which all three markers (N1, N2, and N3) crossed the threshold at Ct < 40 were considered positive. No amplification in any of the SARS-CoV-2 specific primer sets were observed in un-spiked controls (data not shown). Please note that N3 primer set has been recently removed from the CDC diagnostic panel but it is still included in research use only (RUO).

Data presented in Figure 8 demonstrates three distinct lots of the RT-qPCR Master Mix provide consistent, reproducible detection of target RNA present at ≤ 10 copies/µL in the input sample.

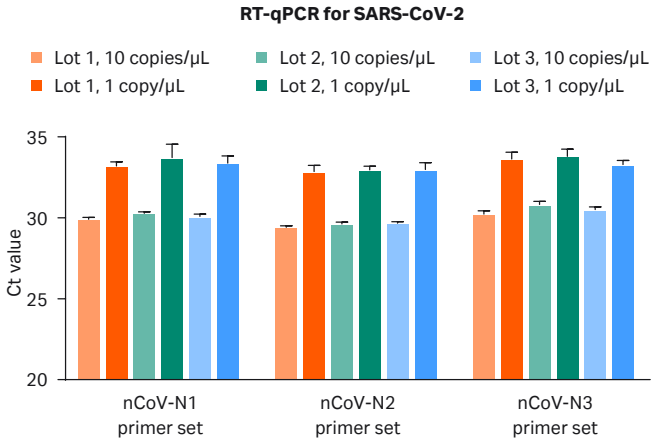


Fig 8. Ct values obtained for RT-qPCR Kit for varying amounts of viral synthetic RNA in the input sample, amplified using three unique lots of RT-qPCR Master Mix as described in the graph. Values averaged from 10 technical replicates; error bars presented as standard deviation.

Consistency of Ct values for five unique lots of AIC Mix

The AIC Mix provides robust, sensitive and accurate detection of COVID-19 viral particles from unpurified nasopharyngeal samples spiked in with as little as 1.25 copies of viral particles per µL. Performance of five unique lots of the AIC Mix were compared using heat inactivated SARS-CoV-2 viral particles spiked at 1.25, 2.5, and 5 copies/µL into a diluent consisting of a suspension of human cells (~ 3 × 10^5/mL) in VTM. Samples were processed and subjected to direct RT-qPCR as described previously using CDC Novel Coronavirus (2019-n-CoV) Diagnostic Panel primers targeting the nucleocapsid gene (N1).

Data presented in Figure 9 demonstrates five distinct lots of the AIC Mix provide reliable, reproducible detection of viral RNA down to 1.25 copies/µL in the input sample.

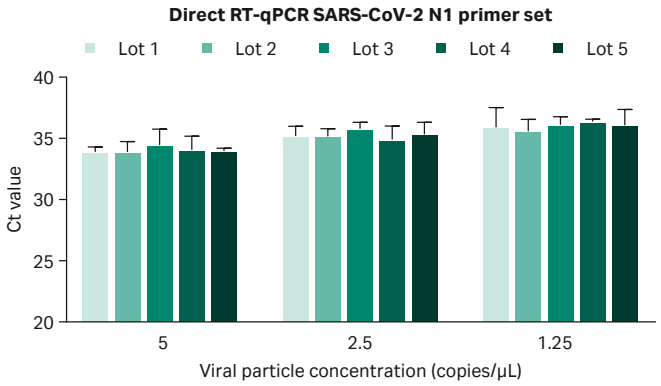


Fig 9. Ct values obtained for Direct RT-qPCR Kit for varying amounts of heat inactivated viral particles in the input sample, amplified using five unique lots of AIC Mix, as described in the graph. Values averaged from three technical replicates; error bars presented as standard deviation.

Tolerance to high volume of universal transport media

The Direct RT-qPCR Kit is highly tolerant of high volumes of transport media, allowing up to 8 µL input of patient sample per reaction. This is beneficial when working with low viral load samples, such as those observed in some COVID-19 patients where viral loads vary with the progression of the disease (4, 5). Hence, a wider range of tolerance to transport media may be advantageous for successful detection of low viral loads when testing borderline positive samples.

Performance of the kit was assessed using heat inactivated SARS-CoV-2 viral particles spiked at 25 copies/µL into a diluent consisting of a suspension of human cells (~ 3 × 10^5/mL) in UTM™ 330C (Copan or Becton Dickinson). 3, 5, and 8 µL sample volumes were processed and subjected to direct RT-qPCR as described previously.

Data presented in Figure 10 demonstrates the Direct RT-qPCR Kit is robust and exhibits high tolerance to sample transport media, enabling detection of low viral loads when the input volume reaches 40% of the final reaction volume.

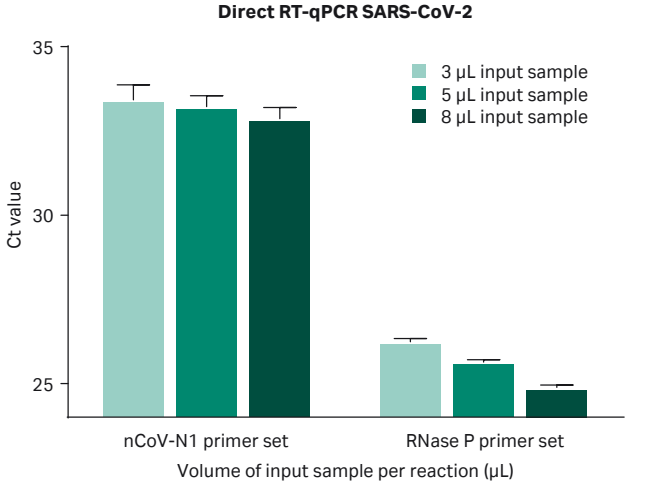


Fig 10. Ct values obtained for N1 specific SARS-CoV-2 primer set and human-specific RNase P primer set with varying volumes of the input sample, as described on the graph. Values averaged from three technical replicates. Error bars represent standard deviation.

Conclusions

Cytiva's Direct RT-PCR Kit is an extraction-free nucleic acid amplification test (NAAT). The kit provides fast, accurate and sensitive detection of viral RNA directly from unpurified nasopharyngeal patient samples, reducing both the cost of the assay and the time to result in comparison to the traditional extraction method.

The Direct RT-PCR Kit allows for reproducible detection of the viral load down to 1 copy/µL in the input sample when diluted in five different types of transport media. The chemistry is versatile and robust, with increased RNA stability in the sample and high tolerance to larger volumes (up to 9 µL per reaction) of transport media.

Cytiva's Direct RT-PCR kit offers a convenient alternative to current NAAT protocols affected by bottlenecks resulting from shortages of nucleic acid purification (NAP) kits and consumables. It benefits testing labs looking to streamline nucleic acid testing workflows, accelerate high throughput testing, decrease risk of cross contamination and reduce costs associated with NAP, including reagents, consumables and equipment.

References

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Ordering information

Product	Pack size	Product code
Direct RT-qPCR Kit	100 reactions	29656615

Related products	Pack size	Product code
AIC Mix	100 reactions	29639678
RT-qPCR Kit	100 reactions	29639679
Sera-Xtracta™ Virus/Pathogen Kit	96 extractions 1000 extractions	29506009 29514201
Sera-Xtracta™ Cell-Free DNA Kit	96 purifications (2 mL input)	29437807
Sera-Xtracta™ Genomic DNA Kit	96 purifications	29429140
RNAspin Mini Kit	20 preps 50 preps 250 preps	25050070 25050071 25050072
RNAspin 96 Kit	4 × 96 preps	25050075
Sera-Mag™ Select	5 mL 60 mL 450 mL	29343045 29343052 29343057
PuReTaq Ready-To-Go™ PCR beads	Multiwell plate, 96 reactions Multiwell plate, 5 × 96 reactions 0.5 mL tubes, 100 reactions 0.2 mL hinged tube with cap, 96 reactions	27955701 27955702 27955801 27955901
GenomiPhi™ V2 DNA Amplification Kit	100 reactions 500 reactions	25660031 25660032
GenomiPhi™ V3 Ready To Go™ DNA Amplification Kit	10 purifications 100 purifications 200 purifications	28903466 28903470 28903471
GFX™ 96 PCR Purification Kit	96 purifications	28903445
Blood genomicPrep Mini Spin Kit	10 purifications 50 purifications 250 purifications	28904263 28904264 28904265
Tissue and Cells genomicPrep Mini Spin Kit	50 purifications 250 purifications	28904275 28904276
MagRack Maxi	15 mL/50 mL tubes	28986441
MagRack 6	1.5 mL/2.0 mL microtubes	28948964

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