ZYMO RESEARCH

SARS-CoV-2 Quantification Standards

Quickly Quantify COVID-19 and Emerging Strains via RT-qPCR

Highlights

- Ready-to-plate SARS-CoV-2 N-Gene RNA ladder for quantification of samples with unknown concentrations of SARS-CoV-2 RNA
- Guidelines for analyzing resulting RT-qPCR data, generating standard curves, and calculating sample concentrations
- Allows cross comparison of data between different PCR plates

Catalog Number:
R3016

Scan with your smart-phone camera to view the online protocol/video.

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Product Contents

Materials Provided

SARS-CoV-2 Quantification Standards	Catalog No.	Amount	Storage Temp.[1]
SARS-CoV-2 RNA Standard 1 – 5000 copies/µL	R3016-1-200	200 µL	-80°C
SARS-CoV-2 RNA Standard 2 – 1000 copies/µL	R3016-2-200	200 µL	-80°C
SARS-CoV-2 RNA Standard 3 – 200 copies/µL	R3016-3-200	200 µL	-80°C
SARS-CoV-2 RNA Standard 4 – 40 copies/µL	R3016-4-200	200 µL	-80°C
SARS-CoV-2 RNA Standard 5 – 8 copies/µL	R3016-5-200	200 µL	-80°C
No Template Control (NTC)	R3011-4-1	1000 µL	-80°C

Reagent Storage and Handling. The **SARS-CoV-2 Quantification Standards** is to be shipped on dry ice. If received in a condition other than the label indicates, or if damaged, contact Zymo Research Corp. directly. Upon receipt, all components of the kit should be stored at ≤ -80°C. Reagents are stable for up to three (3) freeze-thaw cycles.

[1] Storage Temperature - Store all kit components (i.e., buffers, columns) at ≤ -80°C. Before use: Make sure ice or cooling block is prepared to store reagents.

Specifications

- **SARS-CoV-2 RNA Quantification –** Detection range from 80 copies to 50,000 copies per reaction.

- **Sample Source –** Quantify SARS-CoV-2 RNA in purified RNA samples such as wastewater, sputum and saliva.

- **Compatibility –** Purified, synthetic SARS-CoV-2 RNA ladder is validated for use with Bio-Rad's **CFX96 Touch™ Real—Time PCR Detection System** with corresponding **CFX Maestro** software and the ***Quick* SARS-CoV-2 Multiplex Kit** (R3013). However, it can be used in any RT-qPCR system and RT-qPCR master mix that targets the **N-gene** in SARS-CoV-2.

Equipment and Reagents Needed (User Provided)

Item	Catalog No.	Manufacturer
Quick SARS-CoV-2 Multiplex Kit	R3013	Zymo Research
CFX96 Touch™ Real-Time PCR Detection System (with optics capable of detecting HEX and Quasar 670) with CFX Maestro software	1855195	Bio-Rad
Microcentrifuge	Non-specific	Non-specific
Mini Plate Spinner	Non-specific	Non-specific
Hard Shell PCR Plate, 96-well, thin wall	HSP9601	Bio-Rad
Microseal 'B' seal	MSB1001	Bio-Rad
Aerosol barrier pipette tips (Nuclease-Free)	Non-specific	Non-specific
Micropipettes (2, 10, 200, 1000 µl)	Non-specific	Non-specific
Disposable gloves, powder-free	Non-specific	Non-specific
Vortex mixer	Non-specific	Non-specific
Freezer (≤ -80°C)	Non-specific	Non-specific

Product Description

The **SARS-CoV-2 Quantification Standards** consists of a set of synthetic SARS-CoV-2 **nucleocapsid (N) gene** RNA at varying concentrations. These standards are ready to plate, allowing users to easily quantify levels of SARS-CoV-2 present in their samples (Figs 1 and 2).

These standards are recommended to be used with the ***Quick* SARS-CoV-2 Multiplex Kit** (R3013, not provided), which can target both the **N-gene** in SARS-CoV-2 and **RNase P** in human cells.

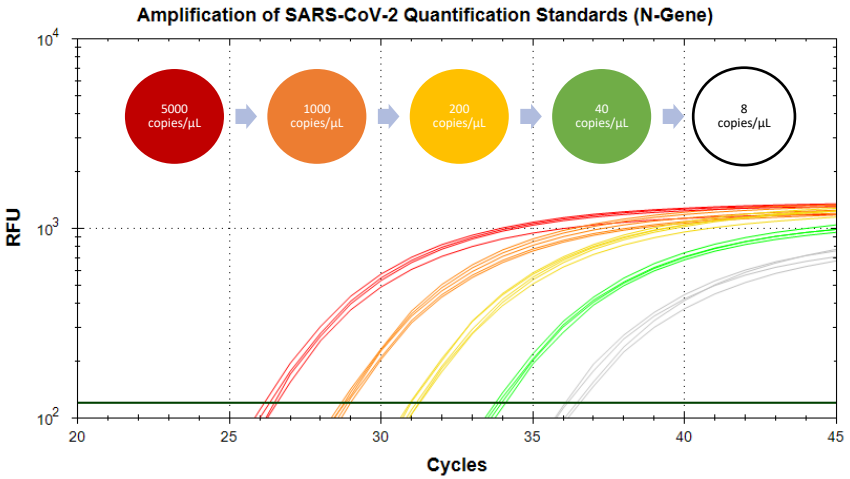

*Figure 1: Resulting amplification curves of **SARS-CoV-2 Quantification Standards** using recommended **Quick-SARS-CoV-2 Multiplex Kit** master mix.*

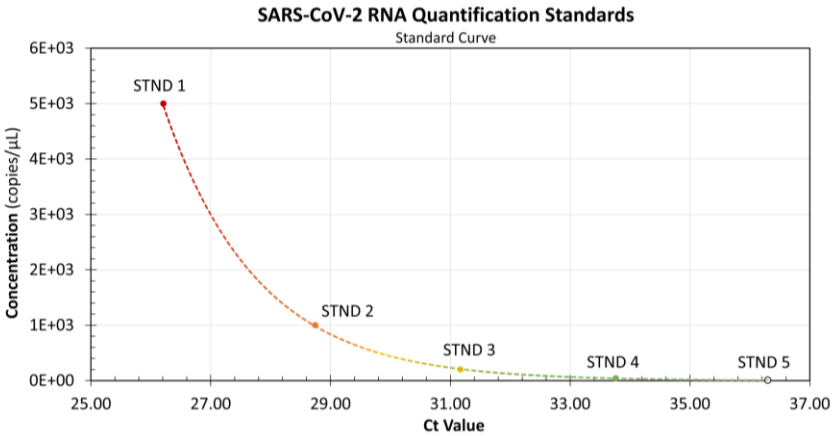

*Figure 2: Example standard curve generated from the average Ct readings for a set of the **SARS-CoV-2 Quantification Standards***

Each standard in this kit is also spiked with a small amount of human HeLa RNA for stability. This spike-in can be used to gauge standard integrity. As each standard, regardless of the concentration of SARS-CoV-2 RNA, was spiked with equal amounts of HeLa RNA, the resulting Ct values should be near identical (Figure 3). Deviation from the expected average Ct of 27.5±2 would indicate loss or degradation of SARS-CoV-2 RNA template.

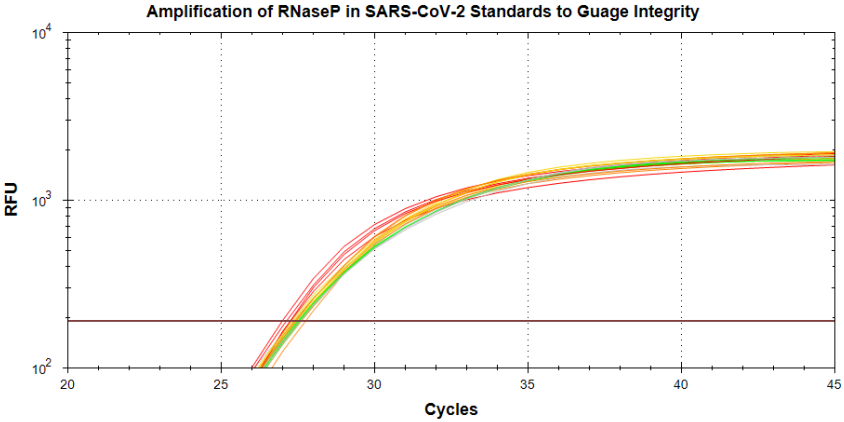

*Figure 3: Resulting amplification of HeLa RNA in the **SARS-CoV-2 Quantification Standards.***

Protocol: RT-PCR Set-Up

- ✓ Before starting, thaw frozen **2X CV Master Mix** (not provided) on ice, mix 10 times by inversion, centrifuge briefly, and place back on ice, covered, until ready to use.
- ✓ Thaw frozen **SARS-CoV-2 Standards** on ice until ready to use.
- ✓ **If this is your first time thawing these standards, aliquot each standard in 25µL volumes and store at ≤ -80°C for future use to avoid excessive freeze-thaw cycles.**
- ✓ Avoid exposing the **2X CV Mix** and subsequent reactions to direct light and keep the RT-PCR plate on ice during preparation.
- ✓ To prevent contamination, handle all reagents carefully and aliquot the necessary master mixes in the RT-PCR plate before handling any test samples, standards, or controls.

Plate Set Up

1. Pipette 10µL of the **2X CV Master Mix** into each well following the template plate shown below.

2. Each standard curve control (**STND1-STND5**) will be plated in duplicate; add 10µL of RNA sample or standard to each well according to the template[1].

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND1	STND2	STND3	STND4	STND5	NTC						
B	STND1	STND2	STND3	STND4	STND5	NTC						
C												
D												
E												
F												
G												
H												

3. Firmly seal the 96-well PCR plate with an optically transparent sealing film.

4. Briefly vortex the 96-well PCR plate and centrifuge to eliminate bubbles and bring any droplets to the bottom of the well.

[1] The exact layout is dependent on the number of samples being processed. It is strongly recommended to plate standards in technical duplicates; plating samples in technical replicates is up to user discretion.

Real-Time PCR Set Up

✓ Ideally, have the PCR program and plate layout prepared prior to plating. Otherwise, avoid exposing the prepared plate to light and keep on ice until ready.

1. Using the Real-Time PCR software (e.g., Bio-Rad CFX Maestro for the CFX96), create a PCR program using the following parameters.

Step	Temperature	Time (min:sec)	
1	55°C	15:00	
2	95°C	10:00	
3	95°C	00:05	45 cycles
4	72°C	00:30	
5	57°C	00:30	
6	**PLATE READ**		
7	**END**		

2. Assign each well the corresponding sample names, targets, and fluorophores.

Targets	Fluorophores	Excitation	Emission
N1	HEX	535 nm	556 nm
RP	Quasar® 670	647 nm	666 nm

3. Enable all the light filters required to detect both fluorophores.

4. Load the RT-PCR plate into the Real-Time PCR machine and start the program. When finished, the program will return Ct values for both the HEX channel and the Quasar 670® channel.

5. Proceed to **Standard Curve Analysis** (page 8) to generate a standard curve from the HEX channel Ct values.

Protocol: Standard Curve Analysis

Example Data

This section will cover generating a Standard Curve from the Ct values obtained from your RT-PCR. It will also cover how to calculate the original sample concentration.

The following sample data will be provided as reference for data analysis and standard curve generation.

Table 1. Concentration (ng/µL) of SARS-CoV-2 Quantification Standards

HEX (N1) Readings	Standard 1 (Red)	Standard 2 (Orange)	Standard 3 (Yellow)	Standard 4 (Green)	Standard 5 (Clear)
Concentration	5000	1000	200	40	8

Table 2. HEX channel Ct values for standards plated in duplicate.

HEX (N1) Readings	Standard 1 (Red)	Standard 2 (Orange)	Standard 3 (Yellow)	Standard 4 (Green)	Standard 5 (Clear)
Raw Ct	25.40	27.60	30.24	33.01	35.08
	25.17	27.93	30.19	32.88	35.68
Average Ct	**25.29**	**27.77**	**30.52**	**32.95**	**35.38**

Table 3. Quasar® 670 channel Ct values for standards plated in duplicate.

Quasar 670 Readings	Standard 1 (Red)	Standard 2 (Orange)	Standard 3 (Yellow)	Standard 4 (Green)	Standard 5 (Clear)
Raw Ct	26.99	27.13	27.26	27.39	27.48
	26.83	27.32	27.38	27.40	27.34
Average Ct	**26.91**	**27.23**	**27.32**	**27.40**	**27.41**

Standard Curve Generation

✓ Before starting, ensure the Quasar® 670 Ct values for each standard falls within 27.5 ± 2 Ct. As can be seen in the sample data (Table 3), all values fall within this range.

To generate a standard curve (Figure 4), plot **standard concentrations** (Table 1) as an exponential function (Equation 1) of the resulting **average Ct for each standard** in the HEX channel (Table 2). To better visualize the standard curve, the concentrations can be plotted on a logarithmic (base 10) scale.

Equation 1. Standard curve relating **Ct** to **template concentration ($C_{template}$)** with exponential coefficients a and b.

$$C_{template} = a \times e^{b(Ct)}$$

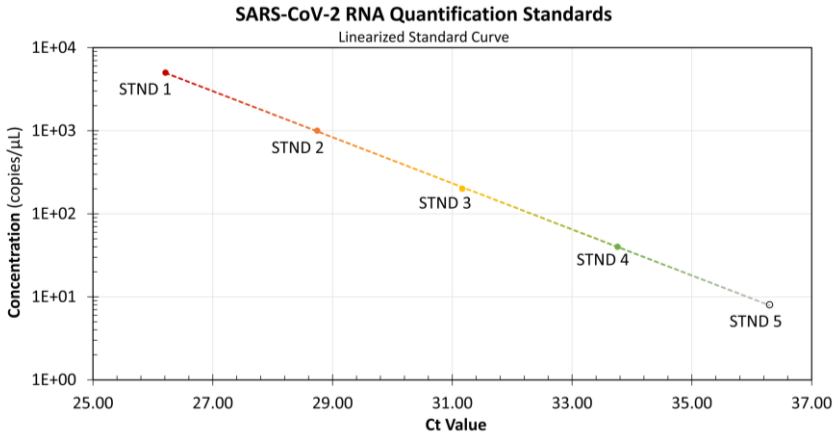

Figure 4: An example standard curve generated using the above formula.

In the case of the sample data (Table 2), the resulting regression curve is:

$$C_{template} = (4.48 \times 10^{10}) \times e^{-0.634(Ct)}$$

This standard curve has a coefficient of determination of $R^2 = 1.000$, indicating a very strong correlation between template concentration ($C_{template}$) and Ct. This allows accurate predictions of the concentrations of unknown samples based on resulting Ct values.

Once the regression curve for this standard curve has been generated, samples can then be quantified. The next section will provide guidelines on how to account for any dilution factors to accurately calculate the original sample concentration.

Calculating Sample Concentrations

- ✓ Before starting, please have the following information available to quantify samples:
  - The **sample input volume** V_{sample} (µL) that was initially processed
  - The **elution volume** $V_{elution}$ (µL) of the final extracted RNA
  - The standard curve calculated in the previous section.

- ✓ The above information will be used to calculate:
  - The **original sample concentration** C_{sample} (genomic copies per µL)
  - The **template concentration** $C_{template}$ (genomic copies per µL) which is directly generated from our standard curve and is the concentration of the input template directly added to the PCR reaction.

1. The previously generated regression curve can be used to predict the **template concentration** $\boldsymbol{C_{template}}$ (copies/µL) by inputting the sample Ct into the resulting exponential equation (Equation 1).

   Example: A sample of unknown SARS-CoV-2 concentration with a Ct value of **30** would yield a template concentration as follows:

$$\boldsymbol{C_{template}} = (4.48 \times 10^{10})e^{-0.634(\boldsymbol{Ct})}$$
$$\boldsymbol{C_{template}} = \boldsymbol{245\ copies\ per\ \mu L}$$

   However, this concentration represents the **concentration of the template** ($C_{template}$), not the sample concentration (C_{sample}).

2. The **template concentration** ($C_{template}$) can be converted to the **sample concentration** (C_{sample}) using **dilution factor *D***, which relates **input sample volume** (V_{sample}) to **elution volume** ($V_{elution}$):

$$D = \frac{V_{elution}}{V_{sample}}$$

   Example: If a total volume of 500µL of our original sample was processed, but eluted in 35µL, the dilution factor would be:

$$D = \frac{35\ \mu L}{500\ \mu L} = 0.07$$

3. Using this **dilution factor**, the original **sample concentration** (C_{sample}) can be related to the **template concentration** ($C_{template}$):

$$C_{sample} = C_{template} \times D$$
$$C_{sample} = (245\ copies\ per\ \mu L) \times (0.07)$$
$$C_{sample} = 17.15\ copies\ per\ \mu L$$

Ordering Information

Product Description	Catalog No.	Size
SARS-CoV-2 Quantification Standards	R3016	20 rxns
***Quick* SARS-CoV-2 Multiplex Kit**	R3013 R3013-1K R3013-10K	100 rxns 1,000 rxns 10,000 rxns

Complete Your Workflow

DNA/RNA Shield™

Cat. Nos. R1100-50, R1100-250,
R1200-25 R1200-125

- ✓ Abides by Center for Disease Control's (CDC) guidelines for pathogen inactivation
- ✓ Stabilizes DNA/RNA

Zymo Environ Water RNA Kit
Cat. No. R2042

- ✓ Inhibitor-free RNA extraction
- ✓ Safely inactivates SARS-CoV-2
- ✓ Enhanced viral enrichment and low elution volume for low concentration samples

Quick SARS-CoV-2 Multiplex Kit
Cat. Nos. R3013, R3013-1K, R3013-10K

- ✓ High sensitivity for low concentrations
- ✓ Ready to use PCR Master Mix

CE IVD

Online qPCR Concentration Calculator
Coming soon!

- ✓ Calculates concentration of samples directly from Ct values
- ✓ Generates standard curve graph and equation

Troubleshooting Guide

Problem	Possible Causes and Suggested Solutions
Background Contamination	Workspace contamination: - Clean workspace, microcentrifuge, and pipettes with 10% bleach routinely to avoid contamination. - Use of kit in exposed environment without proper filtration can lead to background contamination. Check pipettes, pipette tips, microcentrifuge tubes, workspace, etc. for contamination - Make sure all reagent tubes and bottles are properly sealed for storage. Use of these outside a clean room or hood can result in contamination.
Loss of Volume during PCR	Adhesive seal: - A loosened adhesive seal on the PCR plate can lead to sample evaporation. Ensure that the plate seal is secure on every well during targeted sequence amplification. Lid pressure: - Inconsistent lid pressure. Ensure that the lid pressure on the real-time quantitative PCR instrument is consistent over the PCR plate according to the manufacturer's recommendation.
Low Coefficient of Determination	Degradation of standards: - Standard degradation can be checked by viewing Ct readings under the Quasar® 670 channel. Each standard should give a Quasar® 670 Ct reading of approximately 27.5±2 Ct. A significantly higher Ct indicates loss of viable standard. - Ensure that kit arrived on dry ice and that standards were stored at -80°C upon arrival. - Do not excessively freeze-thaw the standards. Standards are stable for up to 3 freeze-thaw cycles. Plating contamination: - If Quasar® 670 readings appear normal, check plating techniques to prevent well to well contamination. Standards can be transferred from original tubes to strip tubes for use with multichannel pipettors.
High Standard Deviation/No Signal for Standard 5	Limits of detection - Standard 5 (2 copies/µL) approaches the limits of detection when used with the recommended CFX96 Touch™ Real—Time PCR Detection System with corresponding CFX Maestro software and the Quick SARS-CoV-2 Multiplex Kit (R3013). It is not abnormal to have very low or no signals with high standard deviation at this concentration. Using a different RT-PCR detection system - These standards have been verified for use with the above system. Other systems may have different limits of detection, leading to loss of signal at the lower concentrations.

For technical assistance, please contact 1-888-882-9682 or email tech@zymoresearch.com

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This product is for research use only and should only be used by trained professionals. It is not for use in diagnostic procedures. Some reagents included with this kit are irritants. Wear protective gloves and eye protection. Follow the safety guidelines and rules enacted by your research institution or facility.

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