

Mar 21, 2020

Generation of SARS-COV-2 RNA transcript standards for qRT-PCR detection assays

 In 1 collection

DOI

<https://dx.doi.org/10.17504/protocols.io.bdv6i69e>

Chantal Vogels¹, Joseph Fauver¹, Isabel Ott¹, Nathan Grubaugh¹

¹Department of Epidemiology of Microbial Diseases, Yale School of Public Health

Coronavirus Method De...



Nathan D Grubaugh

Department of Epidemiology of Microbial Diseases, Yale Schoo...



Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.bdv6i69e>

External link: <https://doi.org/10.1371/journal.pone.0257474>

Protocol Citation: Chantal Vogels, Joseph Fauver, Isabel Ott, Nathan Grubaugh 2020. Generation of SARS-COV-2 RNA transcript standards for qRT-PCR detection assays. protocols.io <https://dx.doi.org/10.17504/protocols.io.bdv6i69e>

Manuscript citation:

Vesga O, Agudelo M, Valencia-Jaramillo AF, Mira-Montoya A, Ossa-Ospina F, Ocampo E, Čiuoderis K, Pérez L, Cardona A, Aguilar Y, Agudelo Y, Hernández-Ortiz JP, Osorio JE (2021) Highly sensitive scent-detection of COVID-19 patients in vivo by trained dogs. PLoS ONE 16(9): e0257474. doi: [10.1371/journal.pone.0257474](https://doi.org/10.1371/journal.pone.0257474)

License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

Has been used to generate PCR-validated transcripts in our group.

Created: March 19, 2020

Last Modified: February 11, 2023

Protocol Integer ID: 34462

Keywords: SARS-CoV-2, COVID-19, qRT-PCR, rna transcript standard, stranded rna transcript standard, virus rna stock, sequences for transcript, us cdc sar, rna, transcript, pcr detection, probe sets for qrt, generation of sar, pcr, china cdc, qrt

Abstract

The protocol describes how to generate high-quality single-stranded RNA transcript standards (starting from a virus RNA stock) targeting the nsp10, RdRp, nsp14, envelope (E), and nucleocapsid (N) coding regions for use with [China CDC](#), [Hong Kong University \(HKU\)](#), [Corman et al. \(Berlin\)](#), and [US CDC SARS-CoV-2 primer and probe sets for qRT-PCR](#). (Sequences for transcripts generated, along with their corresponding assays, are provided under 'Guidelines.')

Guidelines

RNA transcript sequences

	Transcript name	Length (nt)	Genome position	Assays	Sequence
	SARS-COV-2 nsp10 RNA	704	13,122 - 13,825	China CDC-ORF1	GUG GGG GAC AAC CAA UCA CUA AUU GUG UUA AGA UGU UGU GUA CAC ACA CUG GUA CUG GUC AGG CAA UAA CAG UUA CAC CGG AAG CCA AUA UGG AUC AAG AAU CCU UUG GUG GUG CAU CGU GUU GUC UGU ACU GCC GUU GCC ACA UAG AUC AUC



CAA
AUC
CUA
AAG
GAU
UUU
GUG
ACU
UAA
AAG
GUA
AGU
AUG
UAC
AAA
UAC
CUA
CAA
CUU
GUG
CUA
AUG
ACC
CUG
UGG
GUU
UUA
CAC
UUA
AAA
ACA
CAG
UCU
GUA
CCG
UCU
GCG
GUA
UGU
GGA
AAG
GUU
AUG
GCU
GUA
GUU
GUG
AUC
AAC
UCC
GCG
AAC
CCA
UGC
UUC
AGU
CAG
CUG
AUG
CAC
AAU
CGU
UUU
UAA



					ACG
					GGU
					UUG
					CGG
					UGU
					AAG
					UGC
					AGC
					CCG
					UCU
					UAC
					ACC
					GUG
					CGG
					CAC
					AGG
					CAC
					UAG
					UAC
					UGA
					UGU
					CGU
					AUA
					CAG
					GGC
					UUU
					UGA
					CAU
					CUA
					CAA
					UGA
					UAA
					AGU
					AGC
					UGG
					UUU
					UGC
					UAA
					AUU
					CCU
					AAA
					AAC
					UAA
					UUG
					UUG
					UCG
					CUU
					CCA
					AGA
					AAA
					GGA
					CGA
					AGA
					UGA
					CAA
					UUU
					AAU
					UGA
					UUC
					UUA
					CUU
					UGU
					AGU
					UAA



					GAG ACA CAC UUU CUC UAA CUA CCA ACA UGA AGA AAC AAU UUA UAA UUU ACU UAA GGA UUG UCC AGC UGU UGC UAA ACA UGA CUU CUU UAA GUU UAG AAU AGA CGG UGA CAU GGU ACC ACA UAU AUC ACG UCA ACG UCU UAC UAA AUA CAC AAU GGC AGA CCU CGU CU
	SARS-COV-2 RdRp RNA	883	15,094 - 15,976	Berlin-RdRp	AAU AGA GCU CGC ACC GUA GCU



						GGU
						GUC
						UCU
						AUC
						UGU
						AGU
						ACU
						AUG
						ACC
						AAU
						AGA
						CAG
						UUU
						CAU
						CAA
						AAA
						UUA
						UUG
						AAA
						UCA
						AUA
						GCC
						GCC
						ACU
						AGA
						GGA
						GCU
						ACU
						GUA
						GUA
						AUU
						GGA
						ACA
						AGC
						AAA
						UUC
						UAU
						GGU
						GGU
						UGG
						CAC
						AAC
						AUG
						UUA
						AAA
						ACU
						GUU
						UAU
						AGU
						GAU
						GUA
						GAA
						AAC
						CCU
						CAC
						CUU
						AUG
						GGU
						UGG
						GAU
						UAU
						CCU
						AAA
						UGU



					GAU	
					AGA	
					GCC	
					AUG	
					CCU	
					AAC	
					AUG	
					CUU	
					AGA	
					AUU	
					AUG	
					GCC	
					UCA	
					CUU	
					GUU	
					CUU	
					GCU	
					CGC	
					AAA	
					CAU	
					ACA	
					ACG	
					UGU	
					UGU	
					AGC	
					UUG	
					UCA	
					CAC	
					CGU	
					UUC	
					UAU	
					AGA	
					UUA	
					GCU	
					AAU	
					GAG	
					UGU	
					GCU	
					CAA	
					GUA	
					UUG	
					AGU	
					GAA	
					AUG	
					GUC	
					AUG	
					UGU	
					GGC	
					GGU	
					UCA	
					CUA	
					UAU	
					GUU	
					AAA	
					CCA	
					GGU	
					GGA	
					ACC	
					UCA	
					UCA	
					GGA	
					GAU	
					GCC	
					ACA	



					ACU	
					GCU	
					UAU	
					GCU	
					AAU	
					AGU	
					GUU	
					UUU	
					AAC	
					AUU	
					UGU	
					CAA	
					GCU	
					GUC	
					ACG	
					GCC	
					AAU	
					GUU	
					AAU	
					GCA	
					CUU	
					UUA	
					UCU	
					ACU	
					GAU	
					GGU	
					AAC	
					AAA	
					AUU	
					GCC	
					GAU	
					AAG	
					UAU	
					GUC	
					CGC	
					AAU	
					UUA	
					CAA	
					CAC	
					AGA	
					CUU	
					UAU	
					GAG	
					UGU	
					CUC	
					UAU	
					AGA	
					AAU	
					AGA	
					GAU	
					GUU	
					GAC	
					ACA	
					GAC	
					UUU	
					GUG	
					AAU	
					GAG	
					UUU	
					UAC	
					GCA	
					UAU	
					UUG	
					CGU	



					AAA
					CAU
					UUC
					UCA
					AUG
					AUG
					AUA
					CUC
					UCU
					GAC
					GAU
					GCU
					GUU
					GUG
					UGU
					UUC
					AAU
					AGC
					ACU
					UAU
					GCA
					UCU
					CAA
					GGU
					CUA
					GUG
					GCU
					AGC
					AUA
					AAG
					AAC
					UUU
					AAG
					UCA
					GUU
					CUU
					UAU
					UAU
					CAA
					AAC
					AAU
					GUU
					UUU
					AUG
					UCU
					GAA
					GCA
					AAA
					UGU
					UGG
					ACU
					GAG
					ACU
					GAC
					CUU
					ACU
					AAA
					GGA
					CCU
					CAU
					GAA
					UUU
					UGC
					UCU



					CAA CAU ACA AUG CUA GUU AAA CAG GGU GAU GAU UAU GUG UAC CUU CCU UAC CCA GAU CCA UCA AGA AUC CUA GGG GCC GGC UGU UUU GUA GAU G
	SARS-COV-2 nsp14 RNA	848	18,447 - 19,294	HKU- ORF1	UAG UGC UAA ACC ACC GCC UGG AGA UCA AUU UAA ACA CCU CAU ACC ACU UAU GUA CAA AGG ACU UCC UUG GAA UGU AGU GCG UAU AAA GAU UGU



					ACA
					AAU
					GUU
					AAG
					UGA
					CAC
					ACU
					UAA
					AAA
					UCU
					CUC
					UGA
					CAG
					AGU
					CGU
					AUU
					UGU
					CUU
					AUG
					GGC
					ACA
					UGG
					CUU
					UGA
					GUU
					GAC
					AUC
					UAU
					GAA
					GUA
					UUU
					UGU
					GAA
					AAU
					AGG
					ACC
					UGA
					GCG
					CAC
					CUG
					UUG
					UCU
					AUG
					UGA
					UAG
					ACG
					UGC
					CAC
					AUG
					CUU
					UUC
					CAC
					UGC
					UUC
					AGA
					CAC
					UUA
					UGC
					CUG
					UUG
					GCA
					UCA
					UUC
					UAU



					UGG	
					AUU	
					UGA	
					UUA	
					CGU	
					CUA	
					UAA	
					UCC	
					GUU	
					UAU	
					GAU	
					UGA	
					UGU	
					UCA	
					ACA	
					AUG	
					GGG	
					UUU	
					UAC	
					AGG	
					UAA	
					CCU	
					ACA	
					AAG	
					CAA	
					CCA	
					UGA	
					UCU	
					GUA	
					UUG	
					UCA	
					AGU	
					CCA	
					UGG	
					UAA	
					UGC	
					ACA	
					UGU	
					AGC	
					UAG	
					UUG	
					UGA	
					UGC	
					AAU	
					CAU	
					GAC	
					UAG	
					GUG	
					UCU	
					AGC	
					UGU	
					CCA	
					CGA	
					GUG	
					CUU	
					UGU	
					UAA	
					GCG	
					UGU	
					UGA	
					CUG	
					GAC	
					UAU	
					UGA	



					AUA	
					UCC	
					UAU	
					AAU	
					UGG	
					UGA	
					UGA	
					ACU	
					GAA	
					GAU	
					UAA	
					UGC	
					GGC	
					UUG	
					UAG	
					AAA	
					GGU	
					UCA	
					ACA	
					CAU	
					GGU	
					UGU	
					UAA	
					AGC	
					UGC	
					AUU	
					AUU	
					AGC	
					AGA	
					CAA	
					AUU	
					CCC	
					AGU	
					UCU	
					UCA	
					CGA	
					CAU	
					UGG	
					UAA	
					CCC	
					UAA	
					AGC	
					UAU	
					UAA	
					GUG	
					UGU	
					ACC	
					UCA	
					AGC	
					UGA	
					UGU	
					AGA	
					AUG	
					GAA	
					GUU	
					CUA	
					UGA	
					UGC	
					ACA	
					GCC	
					UUG	
					UAG	
					UGA	
					CAA	



					AGC UUA UAA AAU AGA AGA AUU AUU CUA UUC UUA UGC CAC ACA UUC UGA CAA AUU CAC AGA UGG UGU AUG CCU AUU UUG GAA UUG CAA UGU CGA UAG AUA UCC UGC UAA UUC CAU UGU UUG UAG AUU UGA CAC UAG AGU GCU AUC UAA CCU UAA CUU GCC UGG UUG UGA UGG UGG CAG UU
	SARS- COV-2	808	26,207 - 27,116	Berlin-E	GCG UGC CUU



envelope
(E) RNA

UGU
AAG
CAC
AAG
CUG
AUG
AGU
ACG
AAC
UUA
UGU
ACU
CAU
UCG
UUU
CGG
AAG
AGA
CAG
GUA
CGU
UAA
UAG
UUA
AUA
GCG
UAC
UUC
UUU
UUC
UUG
CUU
UCG
UGG
UAU
UCU
UGC
UAG
UUA
CAC
UAG
CCA
UCC
UUA
CUG
CGC
UUC
GAU
UGU
GUG
CGU
ACU
GCU
GCA
AUA
UUG
UUA
ACG
UGA
GUC
UUG
UAA
AAC
CUU



					CUU	
					UUU	
					ACG	
					UUU	
					ACU	
					CUC	
					GUG	
					UUA	
					AAA	
					AUC	
					UGA	
					AUU	
					CUU	
					CUA	
					GAG	
					UUC	
					CUG	
					AUC	
					UUC	
					UGG	
					UCU	
					AAA	
					CGA	
					ACU	
					AAA	
					UAU	
					UAU	
					AUU	
					AGU	
					UUU	
					UCU	
					GUU	
					UGG	
					AAC	
					UUU	
					AAU	
					UUU	
					AGC	
					CAU	
					GGC	
					AGA	
					UUC	
					CAA	
					CGG	
					UAC	
					UAU	
					UAC	
					CGU	
					UGA	
					AGA	
					GCU	
					UAA	
					AAA	
					GCU	
					CCU	
					UGA	
					ACA	
					AUG	
					GAA	
					CCU	
					AGU	
					AAU	
					AGG	
					UUU	



					CCU	
					AUU	
					CCU	
					UAC	
					AUG	
					GAU	
					UUG	
					UCU	
					UCU	
					ACA	
					AUU	
					UGC	
					CUA	
					UGC	
					CAA	
					CAG	
					GAA	
					UAG	
					GUU	
					UUU	
					GUA	
					UAU	
					AAU	
					UAA	
					GUU	
					AAU	
					UUU	
					CCU	
					CUG	
					GCU	
					GUU	
					AUG	
					GCC	
					AGU	
					AAC	
					UUU	
					AGC	
					UUG	
					UUU	
					UGU	
					GCU	
					UGC	
					UGC	
					UGU	
					UUA	
					CAG	
					AAU	
					AAA	
					UUG	
					GAU	
					CAC	
					CGG	
					UGG	
					AAU	
					UGC	
					UAU	
					CGC	
					AAU	
					GGC	
					UUG	
					UCU	
					UGU	
					AGG	
					CUU	



					GAU	
					GUG	
					GCU	
					CAG	
					CUA	
					CUU	
					CAU	
					UGC	
					UUC	
					UUU	
					CAG	
					ACU	
					GUU	
					UGC	
					GCG	
					UAC	
					GCG	
					UUC	
					CAU	
					GUG	
					GUC	
					AUU	
					CAA	
					UCC	
					AGA	
					AAC	
					UAA	
					CAU	
					UCU	
					UCU	
					CAA	
					CGU	
					GCC	
					ACU	
					CCA	
					UGG	
					CAC	
					UAU	
					UCU	
					GAC	
					CAG	
					ACC	
					GCU	
					UCU	
					AGA	
					AAG	
					UGA	
					ACU	
					CGU	
					AAU	
					CGG	
					AGC	
					UGU	
					GAU	
					CCU	
					UCG	
					UGG	
					ACA	
					UCU	
					UCG	
					UAU	
					UGC	
					UGG	
					ACA	



					CCA UCU AGG ACG CUG UGA CAU CAA GGA CCU GCC
	SARS-COV-2 nucleocapsid (N) RNA	1363	28,068 - 29,430	China CDC-N; HKU-N; US CDC-N1; US CDC-N2; US CDC-N3	GAA UUG UGC GUG GAU GAG GCU GGU UCU AAA UCA CCC AUU CAG UAC AUC GAU AUC GGU AAU UAU ACA GUU UCC UGU UUA CCU UUU ACA AUU AAU UGC CAG GAA CCU AAA UUG GGU AGU CUU GUA GUG CGU UGU UCG UUC UAU GAA GAC UUU UUA GAG



					UAU	
					CAU	
					GAC	
					GUU	
					CGU	
					GUU	
					GUU	
					UUA	
					GAU	
					UUC	
					AUC	
					UAA	
					ACG	
					AAC	
					AAA	
					CUA	
					AAA	
					UGU	
					CUG	
					AUA	
					AUG	
					GAC	
					CCC	
					AAA	
					AUC	
					AGC	
					GAA	
					AUG	
					CAC	
					CCC	
					GCA	
					UUA	
					CGU	
					UUG	
					GUG	
					GAC	
					CCU	
					CAG	
					AUU	
					CAA	
					CUG	
					GCA	
					GUA	
					ACC	
					AGA	
					AUG	
					GAG	
					AAC	
					GCA	
					GUG	
					GGG	
					CGC	
					GAU	
					CAA	
					AAC	
					AAC	
					GUC	
					GGC	
					CCC	
					AAG	
					GUU	
					UAC	
					CCA	
					AUA	



					AUA
					CUG
					CGU
					CUU
					GGU
					UCA
					CCG
					CUC
					UCA
					CUC
					AAC
					AUG
					GCA
					AGG
					AAG
					ACC
					UUA
					AAU
					UCC
					CUC
					GAG
					GAC
					AAG
					GCG
					UUC
					CAA
					UUA
					ACA
					CCA
					AUA
					GCA
					GUC
					CAG
					AUG
					ACC
					AAA
					UUG
					GCU
					ACU
					ACC
					GAA
					GAG
					CUA
					CCA
					GAC
					GAA
					UUC
					GUG
					GUG
					GUG
					ACG
					GUA
					AAA
					UGA
					AAG
					AUC
					UCA
					GUC
					CAA
					GAU
					GGU
					AUU
					UCU
					ACU



					ACC
					UAG
					GAA
					CUG
					GGC
					CAG
					AAG
					CUG
					GAC
					UUC
					CCU
					AUG
					GUG
					CUA
					ACA
					AAG
					ACG
					GCA
					UCA
					UAU
					GGG
					UUG
					CAA
					CUG
					AGG
					GAG
					CCU
					UGA
					AUA
					CAC
					CAA
					AAG
					AUC
					ACA
					UUG
					GCA
					CCC
					GCA
					AUC
					CUG
					CUA
					ACA
					AUG
					CUG
					CAA
					UCG
					UGC
					UAC
					AAC
					UUC
					CUC
					AAG
					GAA
					CAA
					CAU
					UGC
					CAA
					AAG
					GCU
					UCU
					ACG
					CAG
					AAG
					GGA



					GCA
					GAG
					GCG
					GCA
					GUC
					AAG
					CCU
					CUU
					CUC
					GUU
					CCU
					CAU
					CAC
					GUA
					GUC
					GCA
					ACA
					GUU
					CAA
					GAA
					AUU
					CAA
					CUC
					CAG
					GCA
					GCA
					GUA
					GGG
					GAA
					CUU
					CUC
					CUG
					CUA
					GAA
					UGG
					CUG
					GCA
					AUG
					GCG
					GUG
					AUG
					CUG
					CUC
					UUG
					CUU
					UGC
					UGC
					UGC
					UUG
					ACA
					GAU
					UGA
					ACC
					AGC
					UUG
					AGA
					GCA
					AAA
					UGU
					CUG
					GUA
					AAG
					GCC
					AAC



					AAC
					AAC
					AAG
					GCC
					AAA
					CUG
					UCA
					CUA
					AGA
					AAU
					CUG
					CUG
					CUG
					AGG
					CUU
					CUA
					AGA
					AGC
					CUC
					GGC
					AAA
					AAC
					GUA
					CUG
					CCA
					CUA
					AAG
					CAU
					ACA
					AUG
					UAA
					CAC
					AAG
					CUU
					UCG
					GCA
					GAC
					GUG
					GUC
					CAG
					AAC
					AAA
					CCC
					AAG
					GAA
					AUU
					UUG
					GGG
					ACC
					AGG
					AAC
					UAA
					UCA
					GAC
					AAG
					GAA
					CUG
					AUU
					ACA
					AAC
					AUU
					GGC
					CGC
					AAA



					UUG
					CAC
					AAU
					UUG
					CCC
					CCA
					GCG
					CUU
					CAG
					CGU
					UCU
					UCG
					GAA
					UGU
					CGC
					GCA
					UUG
					GCA
					UGG
					AAG
					UCA
					CAC
					CUU
					CGG
					GAA
					CGU
					GGU
					UGA
					CCU
					ACA
					CAG
					GUG
					CCA
					UCA
					AAU
					UGG
					AUG
					ACA
					AAG
					AUC
					CAA
					AUU
					UCA
					AAG
					AUC
					AAG
					UCA
					UUU
					UGC
					UGA
					AUA
					AGC
					AUA
					UUG
					ACG
					CAU
					ACA
					AAA
					CAU
					UCC
					CAC
					CAA
					CAG
					AGC

					CUA
					AAA
					AGG
					ACA
					AAA
					AGA
					AGA
					AGG
					CUG
					AUG
					AAA
					CUC
					AAG
					CCU
					UAC
					CGC
					AGA
					GAC
					A

RNA transcripts generated by this protocol, along with their length (in nucleotides), their position on the SARS-COV-2 reference genome, and the assays they serve as positive controls for (since all assays are multiplexed, the target is specified).



Materials

MATERIALS

✕ Q5 High-Fidelity 2X Master Mix - 100 rxns **New England Biolabs Catalog #M0492S**

✕ Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**

✕ Bioanalyzer RNA 6000 Nano Kit **Agilent Technologies Catalog #5067**

✕ SuperScript[®] IV VILO[™] Master Mix **Thermo Fisher Catalog #11756500**

✕ MEGAscript[®] T7 Transcription Kit **Thermo Fisher Catalog #AM1334**

✕ Qubit[®] dsDNA HS Assay Kit **Thermo Fisher Catalog #Q32851**

✕ Qubit[®] RNA HS Assay Kit **Thermo Fisher Catalog #Q32852**

✕ Mag-Bind[®] TotalPure NGS **Omega Biotek Catalog #M1378-01**

STEP MATERIALS

✕ Q5 High-Fidelity 2X Master Mix - 100 rxns **New England Biolabs Catalog #M0492S**

✕ Mag-Bind[®] TotalPure NGS **Omega Biotek Catalog #M1378-01**

✕ Qubit[®] dsDNA HS Assay Kit **Thermo Fisher Catalog #Q32851**

✕ Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**

✕ MEGAscript[®] T7 Transcription Kit **Thermo Fisher Catalog #AM1334**

✕ Qubit[®] RNA HS Assay Kit **Thermo Fisher Catalog #Q32852**

✕ Bioanalyzer RNA 6000 Nano Kit **Agilent Technologies Catalog #5067**

✕ SuperScript[®] IV VILO[™] Master Mix **Thermo Fisher Catalog #11756500**

Protocol materials

✕ Q5 High-Fidelity 2X Master Mix - 100 rxns **New England Biolabs Catalog #M0492S**

✕ MEGAscript[®] T7 Transcription Kit **Thermo Fisher Catalog #AM1334**

✕ Qubit[®] dsDNA HS Assay Kit **Thermo Fisher Catalog #Q32851**

✕ Qubit[®] RNA HS Assay Kit **Thermo Fisher Catalog #Q32852**

✕ Mag-Bind[®] TotalPure NGS **Omega Biotek Catalog #M1378-01**

✕ Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**

✕ Bioanalyzer RNA 6000 Nano Kit **Agilent Technologies Catalog #5067**

✕ SuperScript[®] IV VILO[™] Master Mix **Thermo Fisher Catalog #11756500**



Safety warnings

⚠ Please be aware that full length single-stranded positive sense SARS-CoV-2 RNA is considered infectious and must be handled in BSL-2 or above conditions. Please consult with your institutional biosafety team before handling SARS-CoV-2 RNA.

Before start

Basic outline of steps: cDNA synthesis > PCR > Purification > Quality + Quantity check > T7 RNA transcription > Purification > Quality + Quantity check

Preparation of cDNA

- 1 Isolate viral RNA using Omega Viral DNA/RNA kit, Trizol, or equivalent.
- 2 Many different cDNA synthesis kits can be used, but choose something that is relatively high-fidelity. The current protocol uses SuperScript IV VILO Master Mix because the enzyme has low error rates and the protocol is fast and easy.

 SuperScript® IV VILO™ Master Mix **Thermo Fisher Catalog #11756500**

	Com pon ent	Volu me in 20 µL reac tion
	SSIV VILO Mast er Mix	4 µL
	Nucl ease -free wate r	11 µL
	Viru s RNA	5 µL

- 3 Run the following cycles on a thermocycler:

	Tem pera ture	Tim e
	25° C	10 minu tes
	50° C	10 minu tes

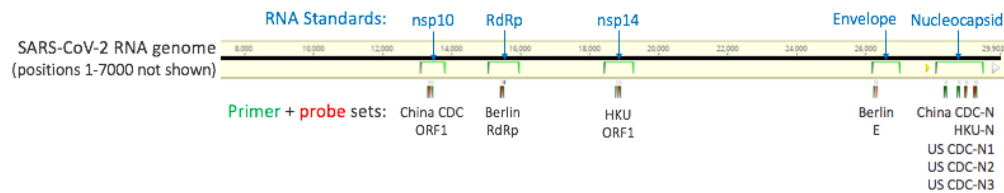


	85° C	5 minu tes
	4°C	∞

- 4 Store samples at 4 °C (same day) or -20 °C (up to a week) until ready for PCR.

PCR

- 5 Prepare the following primers at a concentration of 10 µM, depending on which assay is needed. The nucleocapsid (N) transcript is for the US CDC-N1, N2, and N3 (N3 is not currently in use), HKU-N, and China CDC-N assays. The envelope (E) transcript is for use with the Berlin-E assay, nsp14 transcript is for use with the HKU-ORF1 assay, the RdRp transcript is for use with the Berlin RdRp assay (low sensitivity), and the nsp10 transcript is for use with the China CDC-ORF1 assay. **See here for assay primer sequences and references.**



	Targ et	Prim er	Seq uenc e	Posit ion	Amp licon size
	N	N- Std- T7- Fwd	TAA TAC GAC TCA CTA TAG GGG AAT TGT GCG TGG ATG AGG C	280 68	1363



		N-Std-Rev	TGT CTC TGC GGT AAG GCT TG	2941 1	
	E	E-Std-T7-Fwd	TAA TAC GAC TCA CTA TAG GGG CGT GCC TTT GTA AGC ACA A	262 07	808
		E-Std-Rev	GGC AGG TCC TTG ATG TCA CA	2701 6	
	nsp1 4	nsp1 4-Std-T7-Fwd	TAA TAC GAC TCA CTA TAG GGT AGT GCT AAA CCA CCG CCT G	1844 7	848
		nsp1 4-Std-Rev	AAC TGC CAC CAT CAC AAC CA	1927 5	
	RdRp	RdRp-Std-T7-Fwd	TAA TAC GAC TCA CTA TAG GGA ATA	1509 4	883



			GAG CTC GCA CCG TAG C		
		RdR p- Std- Rev	CAT CTA CAA AAC AGC CGG CC	1595 7	
	nsp1 0	nsp1 0- Std- T7- Fwd	TAA TAC GAC TCA CTA TAG GGG TGG GGG ACA ACC AAT CAC T	1312 2	704
		nsp1 0- Std- Rev	AGA CGA GGT CTG CCA TTG TG	1380 6	

Primers for PCR amplification. Note that forward primers include a T7 promoter sequence.

6 Prepare the following PCR reaction for each sample:



Q5 High-Fidelity 2X Master Mix - 100 rxns **New England**
Biolabs Catalog #M0492S

Com pon ent	Volu me in 25 μL reac tion
Q5 2x Mast	12.5 μL

	er Mix	
	10 μ M Prim er F-T7	1.25 μ L
	10 μ M Prim er R	1.25 μ L
	Nucl ease -free water	8 μ L
	cDN A	2 μ L

7 Run the following cycles on a thermocycler:

Tem perature	Tim e
98° C	30 seco nds
98° C	10 seco nds
55° C	30 seco nds
72°C	90 seco nds
Repeat steps 2-4 for a total of 35 cycles	
72°C	2 minu tes
4°C	∞

Post-PCR cleanup

- 8 Allow Mag-Bind TotalPure NGS beads to equilibrate to room temperature, vortex until homogenous.

 Mag-Bind® TotalPure NGS **Omega Biotek Catalog #M1378-01**

- 9 Bring PCR product volume up to 25 µL with water (if not at volume already).

- 10 Add 20 µL of beads to 25 µL of PCR product, mix well, and incubate at room temperature for 10 minutes.

Note

If your lab has a KingFisher extractor to use for post-PCR cleanup steps, our automated protocols are available here: https://github.com/grubauglab/Kingfisher_protocols (use 'purification.bd' for this cleanup)

- 11 Place tubes on a magnetic stand and incubate until solution appears clear.
- 12 Discard supernatant without disturbing the beads.
- 13 While tubes are on the magnet, add 200 µL of 80% ethanol, incubate for 30 seconds, and discard the ethanol wash.
- 14 Repeat previous 80% ethanol wash and remove as much ethanol as possible.
- 15 Leave tubes on magnet and air dry for 5 minutes.
- 16 Remove tubes from magnet and add 20 µL of nuclease-free water. Mix well by pipetting.
- 17 Place tubes on magnet stand. When solution appears clear, remove supernatant without disturbing the beads and place into new tubes.

Intermediate quantification

- 18 Quantify the DNA concentration using the Qubit High Sensitivity DNA kit (or equivalent) from 1 μL of each product, following manufacturer's protocol.

 Qubit[®]; dsDNA HS Assay Kit **Thermo Fisher Catalog #Q32851**

- 19 Check DNA fragment distributions of the samples using the BioAnalyzer DNA 1000 kit, following manufacturer's protocol.

 Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**

Reverse Transcription (Megascript T7 kit)

- 20 Important: thaw reagents on ice, but keep the 10X reaction buffer at room temperature while assembling the reaction. Add the 10X reaction buffer after water and NTPs have been added to the tube.

 MEGAscript[®]; T7 Transcription Kit **Thermo Fisher Catalog #AM1334**

- 21 Mix equal volumes (2 μL /sample) of the four ribonucleotide solutions together and add 8 μL of the mixture for each sample.

- 22 Use 2 μL of pTRI-Xef in a standard reaction as a transcription control. This should yield a transcript of 1.89 kb.

Com pon ent	Volu me in 20 μL reac tion
Nucl ease -free water	Add to 20 μL
NTP mixture (ATP + CTP + GTP)	8 μL



	+ UTP)	
	10X reac tion buff er	2 μ L
	Purif ied PCR prod uct	0.1- 0.2 μ g PCR prod uct
	Enzy me mix	2 μ L

- 23 Mix by pipetting up and down and microfuge briefly to collect reaction mixture at the bottom of the tube.
- 24 Incubate at  37 °C for 4 hours .
- 25 Add 1 μ L of TURBO DNase, mix well and incubate  37 °C for 15 minutes .

RNA product purification

- 26 Add the following components to a 1.5 mL tube.

	Com pon ent	Volu me in 81 μ L reac tion
	Nucl ease -free water	30 μ L
	Lithi um Chlo ride	30 μ L

T7 tran scrip tion prod uct	21 μL
---	----------

- 27 Mix and chill at  -20 °C for >30 minutes .
- 28 Centrifuge at  4 °C for 15 minutes at maximum speed to pellet the RNA.
- 29 Carefully remove supernatant. Wash pellet with 1 mL of 70% ethanol and re-centrifuge at  4 °C for 15 minutes at maximum speed to maximize removal of unincorporated nucleotides.
- 30 Carefully remove the ethanol and resuspend in 20 μL of nuclease-free water.

Final quantification

- 31 Quantify the RNA concentration using the Qubit High Sensitivity RNA kit from 1 μL of each product, following manufacturer's protocol.
 Qubit[®] RNA HS Assay Kit **Thermo Fisher Catalog #Q32852**
- 32 Check RNA fragment distributions of the samples using the BioAnalyzer RNA 6000 pico kit, following manufacturer's protocol.
 Bioanalyzer RNA 6000 Nano Kit **Agilent Technologies Catalog #5067**
- 33 Use the following [RNA copy number calculator](#) to calculate genome equivalents per uL and prepare standards.