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

Chantal Vogels[1], Joseph Fauver[1], Isabel Ott[2], Nathan Grubaugh[1]

[1]Department of Epidemiology of Microbial Diseases, Yale School of Public Health, [2]Department of Ecology and Evolutionary Biology, Yale University

1 Works for me dx.doi.org/10.17504/protocols.io.bdv6i69e

Coronavirus Method Development Community

Nathan Grubaugh

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	GUGGGGGACA ACCAAUCACUA AUUGUGUUAAG AUGUUGUGUAC ACACACUGGUA CUGGUCAGGCA AUAACAGUUAC ACCGGAAGCCA AUAUGGAUCAA GAAUCCUUUGG UGGUGCAUCGU GUUGUCUGUAC UGCCGUUGCCA CAUAGAUCAUC CAAAUCCUAAA GGAUUUUGUGA CUUAAAAGGUA AGUAUGUACAA AUACCUACAAC UUGUGCUAAUG ACCCUGUGGGU UUUACACUUAA AAACACAGUCU GUACCGUCUGC GGUAUGUGGAA AGGUUAUGGCU GUAGUUGUGAU CAACUCCGCGA

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				ACCCAUGCUUC AGUCAGCUGAU GCACAAUCGUU UUUAAACGGGU UUGCGGUGUAA GUGCAGCCCGU CUUACACCGUG CGGCACAGGCA CUAGUACUGAU GUCGUAUACAG GGCUUUUGACA UCUACAAUGAU AAAGUAGCUGG UUUUGCUAAAU UCCUAAAAACU AAUUGUUGUCG CUUCCAAGAAA AGGACGAAGAU GACAAUUUAAU UGAUUCUUACU UUGUAGUUAAG AGACACACUUU CUCUAACUACC AACAUGAAGAA ACAAUUUAUAA UUUACUUAAGG AUUGUCCAGCU GUUGCUAAACA UGACUUCUUUA AGUUUAGAAUA GACGGUGACAU GGUACCACAUA UAUCACGUCAA CGUCUUACUAA AUACACAAUGG CAGACCUCGUC U
SARS-COV-2 RdRp RNA	883	15,094 - 15,976	Berlin-RdRp	AAUAGAGCUCG CACCGUAGCUG GUGUCUCUAUC UGUAGUACUAU GACCAAUAGAC AGUUUCAUCAA AAAUUAUUGAA AUCAAUAGCCG CCACUAGAGGA GCUACUGUAGU AAUUGGAACAA GCAAAUUCUAU GGUGGUUGGC ACAACAUGUUA AAAACUGUUUA UAGUGAUGUAG AAAACCCUCAC CUUAUGGGUUG GGAUUAUCCUA

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				AAUGUGAUAGA
				GCCAUGCCUAA
				CAUGCUUAGAA
				UUAUGGCCUCA
				CUUGUUCUUGC
				UCGCAAACAUA
				CAACGUGUUGU
				AGCUUGUCACA
				CCGUUUCUAUA
				GAUUAGCUAAU
				GAGUGUGCUCA
				AGUAUUGAGUG
				AAAUGGUCAUG
				UGUGGCGGUUC
				ACUAUAUGUUA
				AACCAGGUGGA
				ACCUCAUCAGG
				AGAUGCCACAA
				CUGCUUAUGCU
				AAUAGUGUUUU
				UAACAUUUGUC
				AAGCUGUCACG
				GCCAAUGUUAA
				UGCACUUUUAU
				CUACUGAUGGU
				AACAAAAUUGC
				CGAUAAGUAUG
				UCCGCAAUUUA
				CAACACAGACU
				UUAUGAGUGUC
				UCUAUAGAAAU
				AGAGAUGUUGA
				CACAGACUUUG
				UGAAUGAGUUU
				UACGCAUAUUU
				GCGUAAACAUU
				UCUCAAUGAUG
				AUACUCUCUGA
				CGAUGCUGUUG
				UGUGUUUCAAU
				AGCACUUAUGC
				AUCUCAAGGUC
				UAGUGGCUAGC
				AUAAAGAACUU
				UAAGUCAGUUC
				UUUAUUAUCAA
				AACAAUGUUUU
				UAUGUCUGAAG
				CAAAAUGUUGG
				ACUGAGACUGA
				CCUUACUAAAG
				GACCUCAUGAA
				UUUUGCUCUCA
				ACAUACAAUGC
				UAGUUAAACAG

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				GGUGAUGAUUA UGUGUACCUUC CUUACCCAGAU CCAUCAAGAAU CCUAGGGGCCG GCUGUUUUGUA CAUC
SARS-COV-2 nsp14 RNA	848	18,447 - 19,294	HKU-ORF1	UAGUGCUAAAC CACCGCCUGGA GAUCAAUUUAA ACACCUCAUAC CACUUAUGUAC AAAGGACUUCC UUGGAAUGUAG UGCGUAUAAAG AUUGUACAAAU GUUAAGUGACA CACUUAAAAAU CUCUCUGACAG AGUCGUAUUUG UCUUAUGGGCA CAUGGCUUUGA GUUGACAUCUA UGAAGUAUUUU GUGAAAAUAGG ACCUGAGCGCA CCUGUUGUCUA UGUGAUAGACG UGCCACAUGCU UUUCCACUGCU UCAGACACUUA UGCCUGUUGGC AUCAUUCUAUU GGAUUUGAUUA CGUCUAUAAUC CGUUUAUGAUU GAUGUUCAACA AUGGGGUUUUA CAGGUAACCUA CAAAGCAACCA UGAUCUGUAUU GUCAAGUCCAU GGUAAUGCACA UGUAGCUAGUU GUGAUGCAAUC AUGACUAGGUG UCUAGCUGUCC ACGAGUGCUUU GUUAAGCGUGU UGACUGGACUA UUGAAUAUCCU AUAAUUGGUGA UGAACUGAAGA UUAAUGCGGCU UGUAGAAAGGU UCAACACAUGG

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				UUGUUAAAGCU GCAUUAUUAGC AGACAAAUUCC CAGUUCUUCAC GACAUUGGUAA CCCUAAAGCUA UUAAGUGUGUA CCUCAAGCUGA UGUAGAAUGGA AGUUCUAUGAU GCACAGCCUUG UAGUGACAAAG CUUAUAAAAUA GAAGAAUUAUU CUAUUCUUAUG CCACACAUUCU GACAAAUUCAC AGAUGGUGUAU GCCUAUUUUGG AAUUGCAAUGU CGAUAGAUAUC CUGCUAAUUCC AUUGUUUGUAG AUUUGACACUA GAGUGCUAUCU AACCUUAACUU GCCUGGUUGUG AUGGUGGCAGU U
SARS-COV-2 envelope (E) RNA	808	26,207 - 27,116	Berlin-E	GCGUGCCUUUG UAAGCACAAGC UGAUGAGUACG AACUUAUGUAC UCAUUCGUUUC GGAAGAGACAG GUACGUUAAUA GUUAAUAGCGU ACUUCUUUUUC UUGCUUUCGUG GUAUUCUUGCU AGUUACACUAG CCAUCCUUACU GCGCUUCGAUU GUGUGCGUACU GCUGCAAUAUU GUUAACGUGAG UCUUGUAAAAC CUUCUUUUUAC GUUUACUCUCG UGUUAAAAAUC UGAAUUCUUCU AGAGUUCCUGA UCUUCUGGUCU AAACGAACUAA AUAUUAUAUUA GUUUUUCUGUU

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				UGGAACUUUAA UUUUAGCCAUG GCAGAUUCCAA CGGUACUAUUA CCGUUGAAGAG CUUAAAAAGCU CCUUGAACAAU GGAACCUAGUA AUAGGUUUCCU AUUCCUUACAU GGAUUUGUCUU CUACAAUUUGC CUAUGCCAACA GGAAUAGGUUU UUGUAUAUAAU UAAGUUAAUUU UCCUCUGGCUG UUAUGGCCAGU AACUUUAGCUU GUUUUGUGCUU GCUGCUGUUUA CAGAAUAAAUU GGAUCACCGGU GGAAUUGCUAU CGCAAUGGCUU GUCUUGUAGGC UUGAUGUGGCU CAGCUACUUCA UUGCUUCUUUC AGACUGUUUGC GCGUACGCGUU CCAUGUGGUCA UUCAAUCCAGA AACUAACAUUC UUCUCAACGUG CCACUCCAUGG CACUAUUCUGA CCAGACCGCUU CUAGAAAGUGA ACUCGUAAUCG GAGCUGUGAUC CUUCGUGGACA UCUUCGUAUUG CUGGACACCAU CUAGGACGCUG UGACAUCAAGG ACCUGCC
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	GAAUUGUGCGU GGAUGAGGCU GGUUCUAAAUC ACCCAUUCAGU ACAUCGAUAUC GGUAAUUAUAC AGUUUCCUGUU UACCUUUUACA

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				AUUAAUUGCCA
				GGAACCUAAAU
				UGGGUAGUCUU
				GUAGUGCGUUG
				UUCGUUCUAUG
				AAGACUUUUUA
				GAGUAUCAUGA
				CGUUCGUGUUG
				UUUUAGAUUUC
				AUCUAAACGAA
				CAAACUAAAAU
				GUCUGAUAAUG
				GACCCCAAAAU
				CAGCGAAAUGC
				ACCCCGCAUUA
				CGUUUGGUGGA
				CCCUCAGAUUC
				AACUGGCAGUA
				ACCAGAAUGGA
				GAACGCAGUGG
				GGCGCGAUCAA
				AACAACGUCGG
				CCCCAAGGUUU
				ACCCAAUAAUA
				CUGCGUCUUGG
				UUCACCGCUCU
				CACUCAACAUG
				GCAAGGAAGAC
				CUUAAAUUCCC
				UCGAGGACAAG
				GCGUUCCAAUU
				AACACCAAUAG
				CAGUCCAGAUG
				ACCAAAUUGGC
				UACUACCGAAG
				AGCUACCAGAC
				GAAUUCGUGGU
				GGUGACGGUAA
				AAUGAAAGAUC
				UCAGUCCAAGA
				UGGUAUUUCUA
				CUACCUAGGAA
				CUGGGCCAGAA
				GCUGGACUUCC
				CUAUGGUGCUA
				ACAAAGACGGC
				AUCAUAUGGGU
				UGCAACUGAGG
				GAGCCUUGAAU
				ACACCAAAAGA
				UCACAUUGGCA
				CCCGCAAUCCU
				GCUAACAAUGC
				UGCAAUCGUGC
				UACAACUUCCU
				CAAGGAACAAC

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				AUUGCCAAAAG
				GCUUCUACGCA
				GAAGGGAGCA
				GAGGCGGCAG
				UCAAGCCUCUU
				CUCGUUCCUCA
				UCACGUAGUCG
				CAACAGUUCAA
				GAAAUUCAACU
				CCAGGCAGCAG
				UAGGGGAACUU
				CUCCUGCUAGA
				AUGGCUGGCAA
				UGGCGGUGAU
				GCUGCUCUUGC
				UUUGCUGCUGC
				UUGACAGAUUG
				AACCAGCUUGA
				GAGCAAAAUGU
				CUGGUAAAGGC
				CAACAACAACA
				AGGCCAAACUG
				UCACUAAGAAA
				UCUGCUGCUGA
				GGCUUCUAAGA
				AGCCUCGGCAA
				AAACGUACUGC
				CACUAAAGCAU
				ACAAUGUAACA
				CAAGCUUUCGG
				CAGACGUGGUC
				CAGAACAAACC
				CAAGGAAAUUU
				UGGGGACCAG
				GAACUAAUCAG
				ACAAGGAACUG
				AUUACAAACAU
				UGGCCGCAAAU
				UGCACAAUUUG
				CCCCCAGCGCU
				UCAGCGUUCUU
				CGGAAUGUCGC
				GCAUUGGCAUG
				GAAGUCACACC
				UUCGGGAACGU
				GGUUGACCUAC
				ACAGGUGCCAU
				CAAAUUGGAUG
				ACAAAGAUCCA
				AAUUUCAAAGA
				UCAAGUCAUUU
				UGCUGAAUAAG
				CAUAUUGACGC
				AUACAAAACAU
				UCCCACCAACA
				GAGCCUAAAAA

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				GGACAAAAAGA AGAAGGCUGAU GAAACUCAAGC CUUACCGCAGA GACA

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

NAME	CATALOG #	VENDOR
Q5 High-Fidelity 2X Master Mix - 100 rxns	M0492S	New England Biolabs
Agilent High Sensitivity DNA Kit	5067-4626	Agilent Technologies
Bioanalyzer RNA 6000 Nano Kit	5067	Agilent Technologies
SuperScript™ IV VILO™ Master Mix	11756500	Thermo Fisher
MEGAscript™ T7 Transcription Kit	AM1334	Thermo Fisher
Qubit™ dsDNA HS Assay Kit	Q32851	Thermo Fisher
Qubit™ RNA HS Assay Kit	Q32852	Thermo Fisher
Mag-Bind® TotalPure NGS	M1378-01	Omega Biotek

STEPS MATERIALS

NAME	CATALOG #	VENDOR
SuperScript™ IV VILO™ Master Mix	11756500	Thermo Fisher
Q5 High-Fidelity 2X Master Mix - 100 rxns	M0492S	New England Biolabs
Mag-Bind® TotalPure NGS	M1378-01	Omega Biotek
Qubit™ dsDNA HS Assay Kit	Q32851	Thermo Fisher
Agilent High Sensitivity DNA Kit	5067-4626	Agilent Technologies
MEGAscript™ T7 Transcription Kit	AM1334	Thermo Fisher
Qubit™ RNA HS Assay Kit	Q32852	Thermo Fisher
Bioanalyzer RNA 6000 Nano Kit	5067	Agilent Technologies

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 STARTING

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

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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
by Thermo Fisher
Catalog #: 11756500

Component	Volume in 20 µL reaction
SSIV VILO Master Mix	4 µL
Nuclease-free water	11 µL
Virus RNA	5 µL

3 Run the following cycles on a thermocycler:

Temperature	Time
25°C	10 minutes
50°C	10 minutes
85°C	5 minutes
4°C	∞

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

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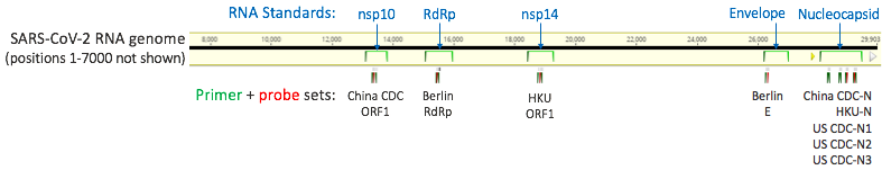
PCR

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.



Target	Primer	Sequence	Position	Amplicon size
N	N-Std-T7-Fwd	TAATACGACTCACTATAGGGGAATTGTGCGTGGATGAGGC	28068	1363
	N-Std-Rev	TGTCTCTGCGGTAAGGCTTG	29411	
E	E-Std-T7-Fwd	TAATACGACTCACTATAGGGGCGTGCCTTTGTAAGCACAA	26207	808
	E-Std-Rev	GGCAGGTCCTTGATGTCACA	27016	
nsp14	nsp14-Std-T7-Fwd	TAATACGACTCACTATAGGGTAGTGCTAAACCACCGCCTG	18447	848
	nsp14-Std-Rev	AACTGCCACCATCACAACCA	19275	
RdRp	RdRp-Std-T7-Fwd	TAATACGACTCACTATAGGGAATAGAGCTCGCACCGTAGC	15094	883
	RdRp-Std-Rev	CATCTACAAAACAGCCGGCC	15957	
nsp10	nsp10-Std-T7-Fwd	TAATACGACTCACTATAGGGGTGGGGGACAACCAATCACT	13122	704
	nsp10-Std-Rev	AGACGAGGTCTGCCATTGTG	13806	

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

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6 Prepare the following PCR reaction for each sample:

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

Component	Volume in 25 µL reaction
Q5 2x Master Mix	12.5 µL
10 µM Primer F-T7	1.25 µL
10 µM Primer R	1.25 µL
Nuclease-free water	8 µL
cDNA	2 µL

7 Run the following cycles on a thermocycler:

Temperature	Time
98°C	30 seconds
98°C	10 seconds
55°C	30 seconds
72°C	90 seconds
Repeat steps 2-4 for a total of 35 cycles	
72°C	2 minutes
4°C	∞

Post-PCR cleanup

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

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

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

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

If your lab has a KingFisher extractor to use for post-PCR cleanup steps, our automated protocols are available here: https://github.com/grubaughlab/Kingfisher_protocols (use 'purification.bdz' 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
by 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
by Agilent Technologies
Catalog #: 5067-4626

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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
by 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.

Component	Volume in 20 µL reaction
Nuclease-free water	Add to 20 µL
NTP mixture (ATP + CTP + GTP + UTP)	8 µL
10X reaction buffer	2 µL
Purified PCR product	0.1-0.2 µg PCR product
Enzyme mix	2 µL

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 µL 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.

Component	Volume in 81 µL reaction
Nuclease-free water	30 µL
Lithium Chloride	30 µL
T7 transcription product	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.

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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
by 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
by Agilent Technologies
Catalog #: 5067

33 Use the following RNA copy number calculator to calculate genome equivalents per uL and prepare standards.

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