07/21/2021: Lab Alert: Changes to CDC RT-PCR for SARS-CoV-2 Testing

Audience: Individuals Performing COVID-19 Testing

Level: Laboratory Alert

After December 31, 2021, CDC will withdraw the request to the U.S. Food and Drug Administration (FDA) for Emergency Use Authorization (EUA) of the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel, the assay first introduced in February 2020 for detection of SARS-CoV-2 only. CDC is providing this advance notice for clinical laboratories to have adequate time to select and implement one of the many FDA-authorized alternatives.

Visit the FDA website for a list of authorized COVID-19 diagnostic methods. For a summary of the performance of FDA-authorized molecular methods with an FDA reference panel, visit this page.

In preparation for this change, CDC recommends clinical laboratories and testing sites that have been using the CDC 2019-nCoV RT-PCR assay select and begin their transition to another FDA-authorized COVID-19 test. CDC encourages laboratories

https://www.rijksoverheid.nl/documenten/publicaties/2021/03/10/ontheffingen-antigeentesten

Van een paar testen is de ontheffing vervallen, omdat zij inmiddels een CE-markering voor zelfgebruik hebben. Deze testen zijn eerder onder de ontheffing in de handel gebracht en kregen kort daarna een CE-markering. Dus hiervan zijn beide varianten in omloop: zonder 4 cijfers en met 4 cijfers (beiden met CE-logo). Het gaat om de volgende testen:

- Roche SD Biosensor SARS-CoV-2 Rapid Antigen Nasal Test
- BIOSYNEX COVID-19 Ag BSS rapid test
- Acon Biotech Flowflex SARS-CoV-2 Antigen Rapid Test
- MP Biomedicals Rapid SARS-CoV-2 Antigen Sante-Group
- Beijing Lepu Medical Technology Co., Ltd. SARSCoV-2 Antigen Rapid Test Kit (Colloidal Gold Immunochromatography) Orange Swallow

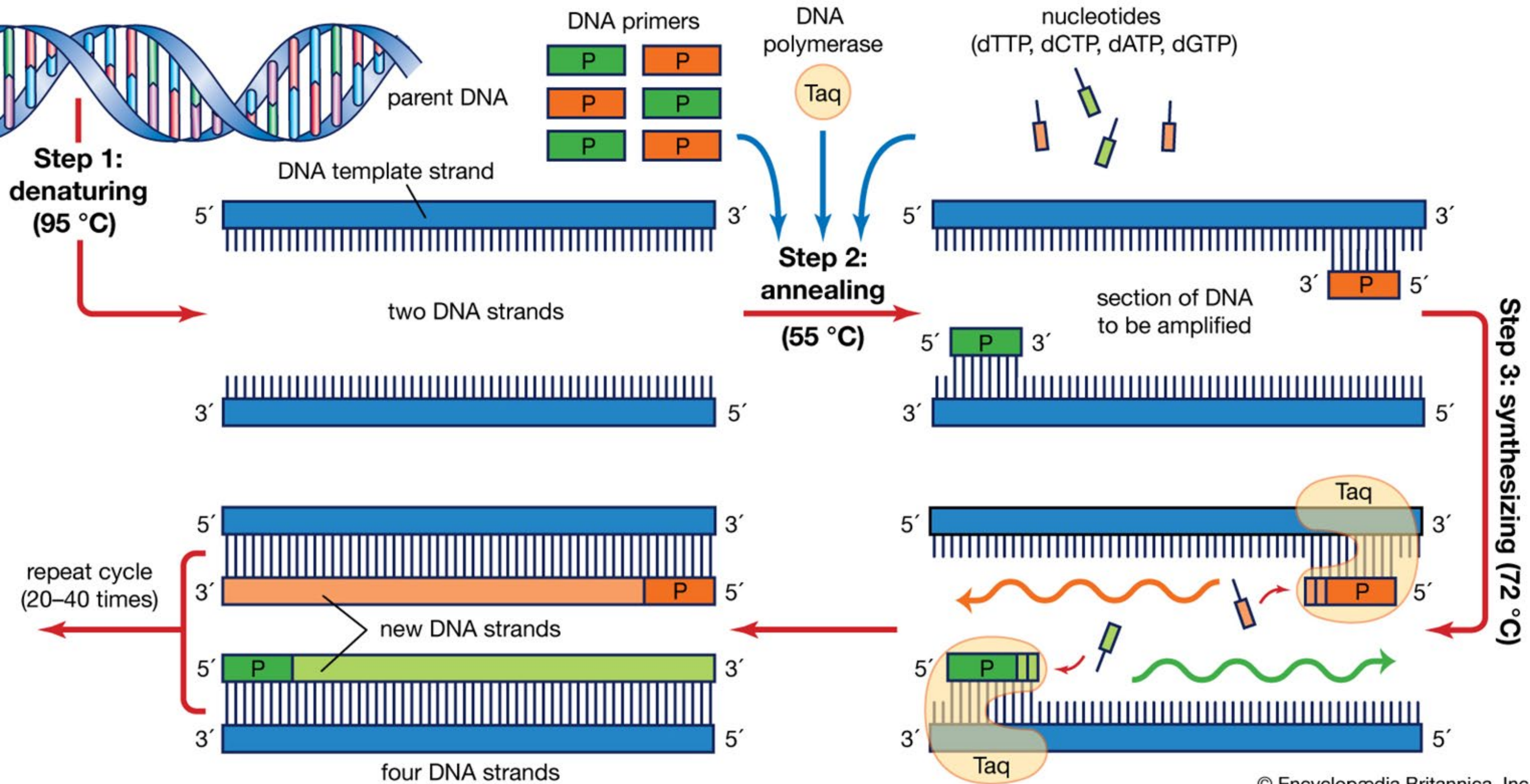
Tijdelijke ontheffingen geldig tot en met 31 december 2021

De ontheffingen zijn geldig tot en met 31 december 2021. De ontheffingen zijn bedoeld om op een veilige en verantwoorde manier de (versnelde) beschikbaarheid van coronatesten voor zelfgebruik mogelijk te maken. Verlenen van een ontheffing betekent dat het ministerie van VWS toestaat dat de test nog niet aan alle wettelijke eisen voldoet op het gebied van diagnostische testen.

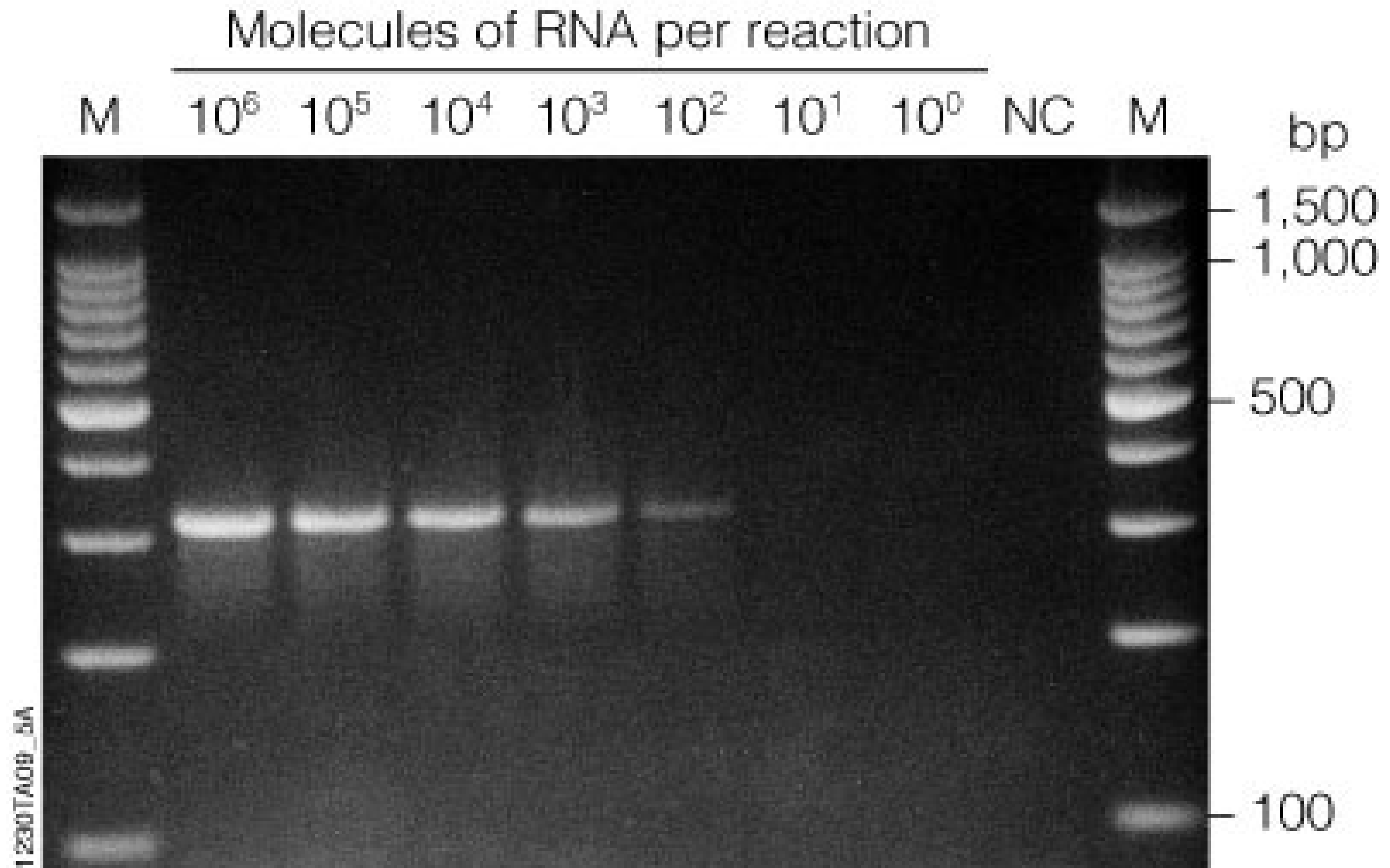
Zie ook

› Coronavirus COVID-19

PCR



Controle



PCR Terminology

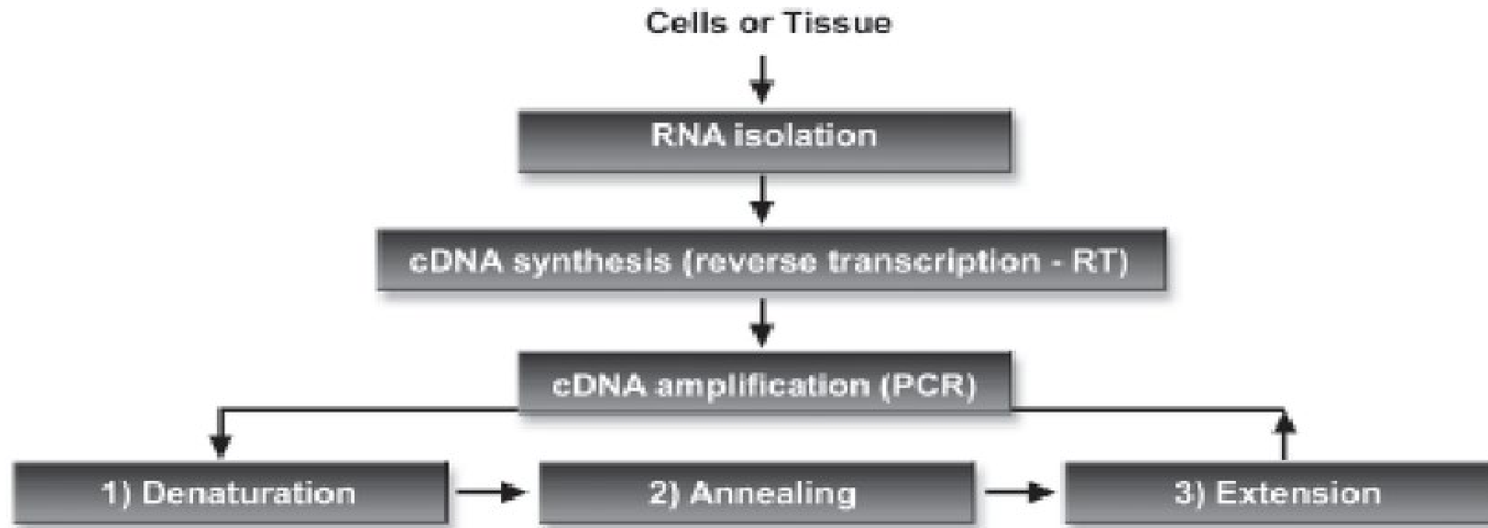
Polymerase chain reaction	PCR
Reverse transcription-polymerase chain reaction	RT-PCR
Real-time polymerase chain reaction	qPCR
RT-PCR / qPCR combined technique	qRT-PCR

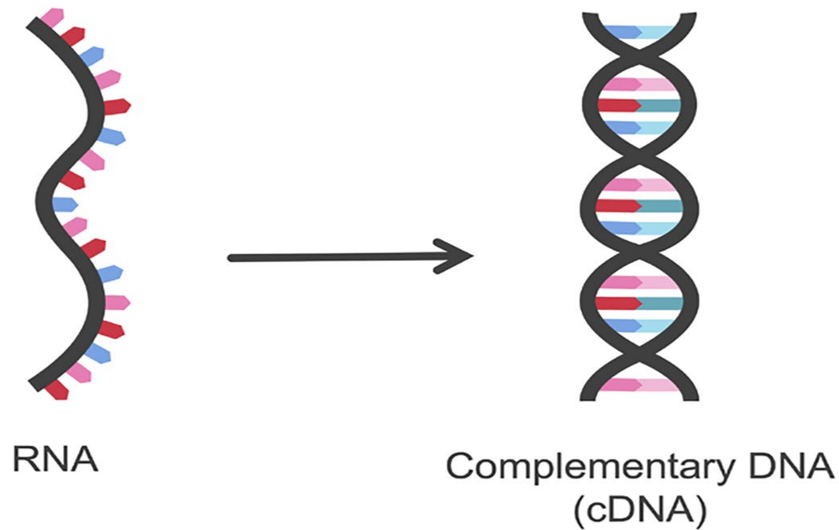
Principle of Real Time PCR

This same principle of amplification of PCR is employed in real-time PCR. But instead of looking at bands on a gel at the end of the reaction, the process is monitored in "real-time". The reaction is placed into a real-time PCR machine that watches the reaction occur with a camera or detector.

Although many different techniques are used to monitor the progress of a PCR reaction, all have one thing in common. They all link the amplification of DNA to the generation of fluorescence which can simply be detected with a camera during each PCR cycle. Hence, as the number of gene copies increases during the reaction, so does the fluorescence, indicating the progress of the reaction.

RT-PCR





Nested primers

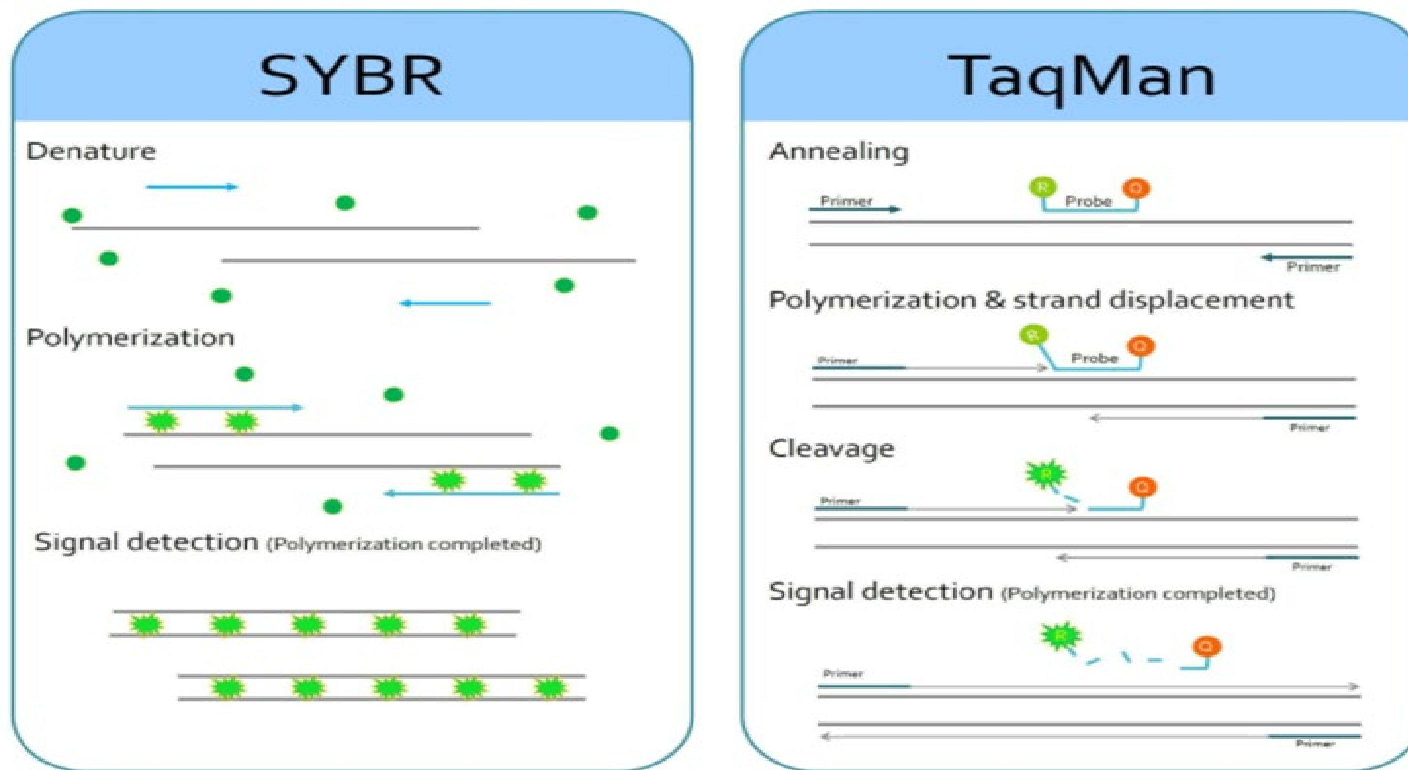
Overview of Molecular Diagnostics Principles

Audrey Wanger, ... Amitava Dasgupta, in Microbiology and Molecular Diagnosis in Pathology, 2017

Nested Polymerase Chain Reaction

Nested PCR is a technique that reduces nonspecific amplification of the DNA template. It is performed by two successive PCRs. The first reaction is performed with primers that cover the target sequence and some additional sequence flanking both ends of the target sequence. After the first reaction, a second reaction is performed on the products of the first PCR with primers that bind to the target sequence and are within the amplified sequence of the first PCR. This reduces the amount of nonspecific binding because in the second reaction, most of the amplicons of the first reaction only contain the target sequence and its surrounding sequences.

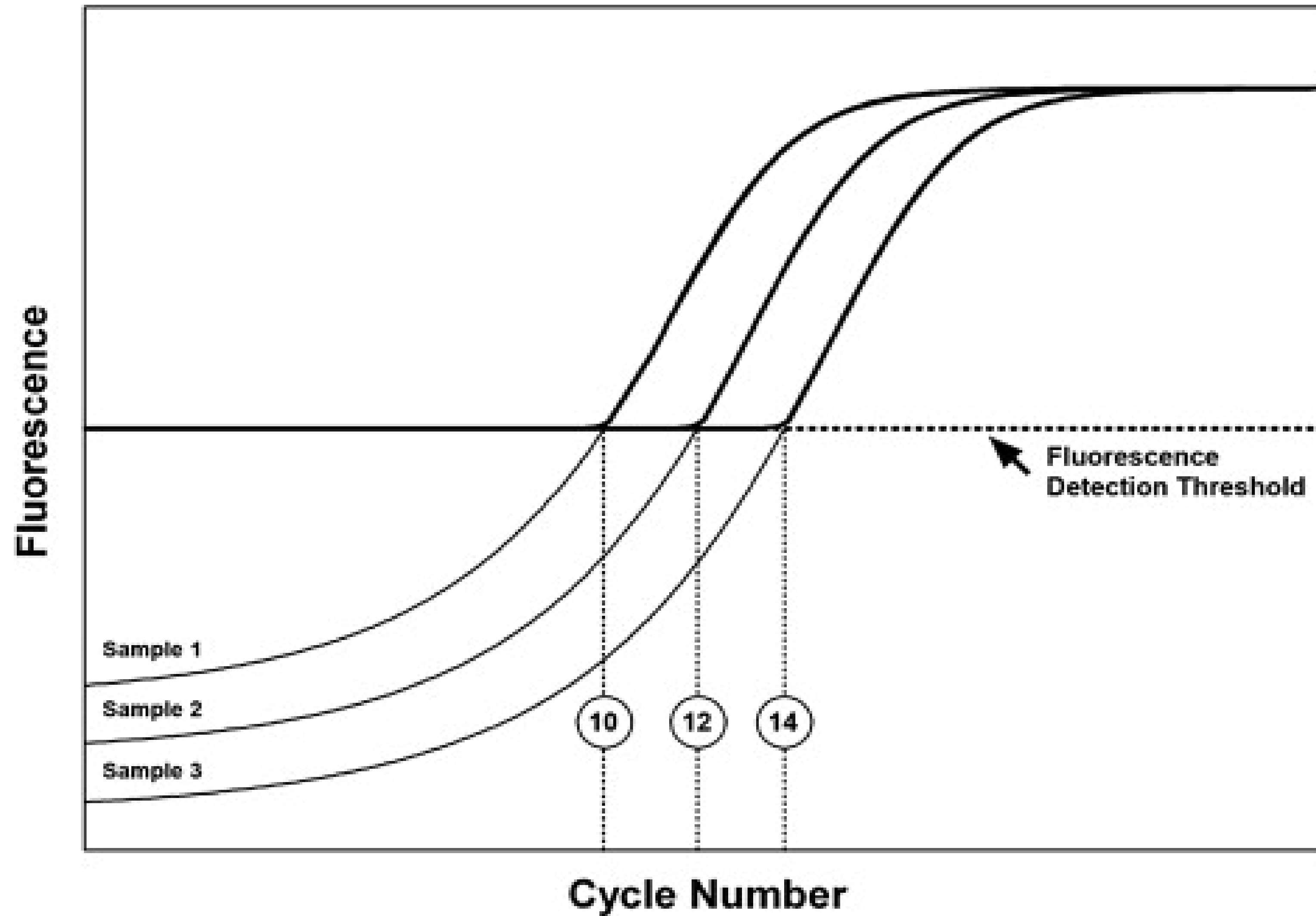
RT-qPCR



SYBR Green

- This is a dye that emits prominent fluorescent signal when it binds at the minor groove of DNA, nonspecifically.
- Other fluorescent dyes like Ethidium Bromide or Acridine Orange can also be used but SYBR Green is better used for its higher signal intensity.
- SYBR Green is more preferred than the Taqman Probe as it can provide information about each cycle of amplification as well as about the melting temperature which is not obtained from the Taqman probe.
- However, its disadvantage is the lack of specificity as compared to Taqman Probe.

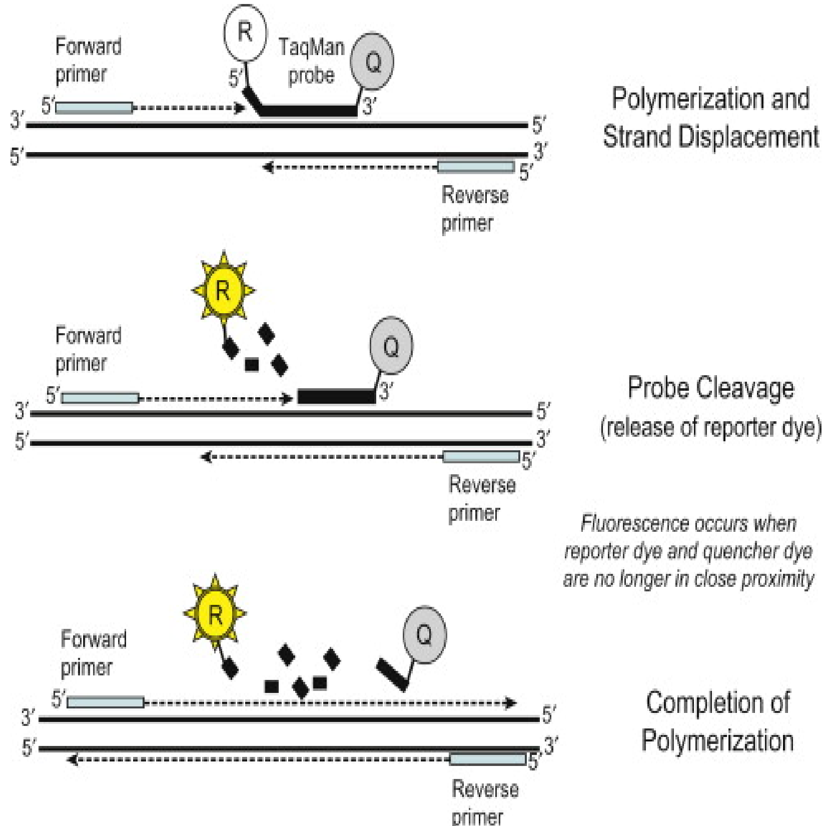
Indirecte detectie

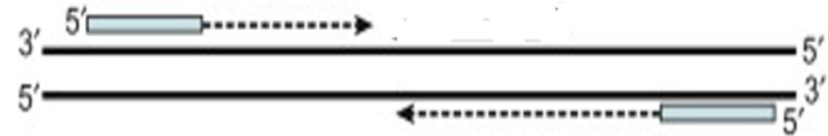


TagMan qPCR (RT-qPCR) is inherently flawed in nucleic acid sequence determination

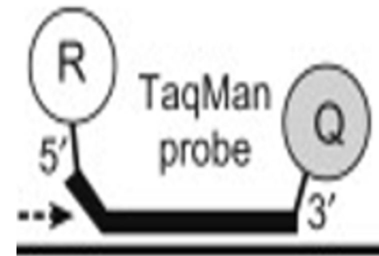
Left panel was from Laboratory Methods in Cell Biology, Yibing Jia, in Methods in Cell Biology, 2012

RT-qPCR is not PCR – It is a PCR amplification-dependent probe hydrolysis process





This is PCR-to produce a mass of DNA molecules for sequence analysis



This is hybridization- the probe must be short and does not always fully match the target DNA sequence-leading to false positives

13 jan

Berlin, 13.01.2020

Diagnostic detection of Wuhan coronavirus 2019 by real-time RT-PCR

-Protocol and preliminary evaluation as of Jan 13, 2020-

Victor Corman, Tobias Bleicker, Sebastian Brünink, Christian Drosten
Charité Virology, Berlin, Germany

Olfert Landt, Tib-Molbiol, Berlin, Germany

Marion Koopmans
Erasmus MC, Rotterdam, The Netherlands

Maria Zambon
Public Health England, London

Additional advice by Malik Peiris, University of Hong Kong

All assays can use SARS-CoV genomic RNA as positive control. Synthetic control RNA for Wuhan virus will be provided shortly.

First line screening assay: E gene assay
Confirmatory assay: RdRp gene assay
Additional confirmatory assay: N gene assay

Figure 1 relative positions of amplicon targets on SARS-CoV ad Wuhan-CoV genome. N: nucleocapsid; ORF: open reading frame; RdRp: RNA-dependent RNA polymerase. Numbers below amplicon are genome positions according to SARS-CoV, NC_004718.

Assay/ Use	Oligonucleotide ID	Sequence (5'–3')	Comment
RdRP gene	RdRP_SARSr-F2	GTGARATGGTCATGTGTGGCGG	use 600 nM per reaction
	RdRP_SARSr-R1	CARATGTTAAASACACTATTAGCATA	use 800 nM per reaction
	RdRP_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ	Specific for Wuhan-CoV, will not detect SARS-CoV use 100 nM per reaction and mix with P1
	RdRP_SARSr-P1	FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ	Pan Sarbeco-Probe, will detect Wuhan virus, SARS-CoV and bat-SARS-related CoVs use 100 nM per reaction and mix with P2
E gene	E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT	use 400 nM per reaction
	E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA	use 400 nM per reaction
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	use 200 nM per reaction
N gene	N_Sarbeco_F1	CACATTGGCACCCGCAATC	use 600 nM per reaction
	N_Sarbeco_R1	GAGGAACGAGAAGAGGCTTG	use 800 nM per reaction
	N_Sarbeco_P1	FAM-ACTTCCTCAAGGAACAACATTGCCA-BBQ	use 200 nM per reaction

16 jan

HKU Med LKS Faculty of Medicine School of Public Health 香港大學公共衛生學院

Detection of 2019 novel coronavirus (2019-nCoV) in suspected human cases by RT-PCR

This protocol is designed to detect 2019-nCoV in human clinical specimens. The two monoplex assays described here are reactive with coronaviruses under the subgenus *Sarbecovirus* that includes 2019-nCoV, SARS-CoV and bat SARS-like coronaviruses. The rationales for using this detection approach are: 1) the genetic diversity of 2019-nCoV in humans and animals is yet to be fully determined and 2) many laboratories lack positive controls for 2019-nCoV. ***Viral RNA extracted from SARS-CoV can be used a positive control in the assays below.*** As SARS was eliminated in humans, suspected cases that are positive in these RT-PCR assays should be considered to be infected by the 2019-nCoV. The N gene RT-PCR is recommended as a screening assay and the Orf1b assay as a confirmatory one. In the event of a positive PCR result,

Primer and probe sequences

Assay 1 (Target: ORF1b-nsp14)

Forward primer (HKU-ORF1b-nsp14F): 5'-TGGGGYTTTACRGGTAACCT-3'

Reverse primer (HKU- ORF1b-nsp14R): 5'-AACRCGCTTAACAAAGCACTC-3'

Probe (HKU-ORF1b-nsp141P): 5'-FAM-TAGTTGTGATGCWATCATGACTAG-TAMRA-3'

Assay 2 (Target: N)

Forward primer (HKU-NF): 5'-TAATCAGACAAGGAACTGATTA-3'

Reverse primer (HKU-NR): 5'-CGAAGGTGTGACTTCCATG-3'

Probe (HKU-NP): 5'-FAM-GCAAATTGTGCAATTTGCGG-TAMRA-3'

17 jan

Berlin, Jan 17th, 2020

Diagnostic detection of 2019-nCoV by real-time RT-PCR

-Protocol and preliminary evaluation as of Jan 17, 2020-

Victor Corman, Tobias Bleicker, Sebastian Brünink, Christian Drosten
Charité Virology, Berlin, Germany

Olfert Landt, Tib-Molbiol, Berlin, Germany

Marion Koopmans
Erasmus MC, Rotterdam, The Netherlands

Maria Zambon
Public Health England, London

Additional advice by Malik Peiris, University of Hong Kong

All assays can use SARS-CoV genomic RNA as positive control. Synthetic control RNA for 2019-nCoV E gene assay is available via EVAg. Synthetic control for 2019-nCoV RdRp is expected to be available via EVAg from Jan 21st onward.

First line screening assay: E gene assay
Confirmatory assay: RdRp gene assay

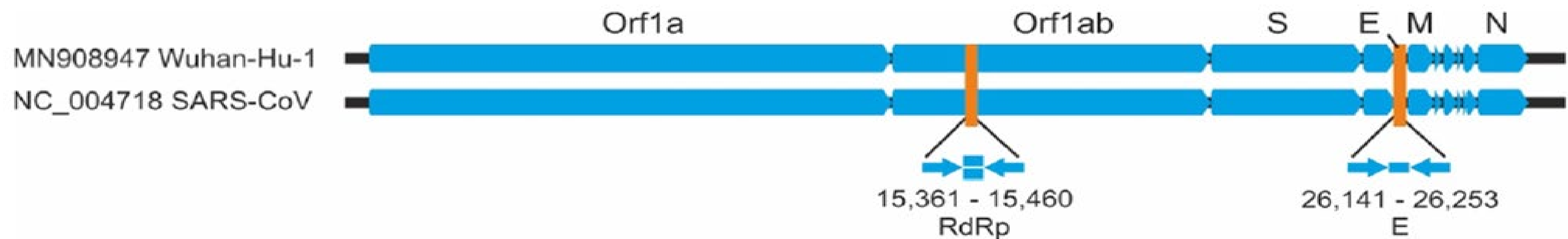


Figure 1 relative positions of amplicon targets on SARS-CoV an 2019-nCoV genome. ORF: open reading frame; RdRp: RNA-dependent RNA polymerase. Numbers below amplicon are genome positions according to SARS-CoV, NC_004718.

Materials and assay formulation

Assay/ Use	Oligonucleotide ID	Sequence (5'–3')	Comment
RdRP gene	RdRP_SARSr-F2	GTGARATGGTCATGTGTGGCGG	use 600 nM per reaction
	RdRP_SARSr-R1	CARATGTTAAASACACTATTAGCATA	use 800 nM per reaction
	RdRP_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ	Specific for 2019-nCoV, will not detect SARS-CoV use 100 nM per reaction and mix with P1
	RdRP_SARSr-P1	FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ	Pan Sarbeco-Probe, will detect 2019-nCoV, SARS-CoV and bat-SARS-related CoVs use 100 nM per reaction and mix with P2
E gene	E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT	use 400 nM per reaction
	E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA	use 400 nM per reaction
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	use 200 nM per reaction

W is A/T; R is G/A; M is A/C ; FAM, 6-carboxyfluorescein; BBQ, blackberry quencher

Pasteur

Primers and probes

Name	Sequences (5'-3')	Length (bases)	PCR product size	Ref.
RdRp gene / nCoV_IP2				
nCoV_IP2-12669Fw	ATGAGCTTAGTCCTGTTG	17	108 bp	1
nCoV_IP2-12759Rv	CTCCCTTTGTTGTGTTGT	18		
nCoV_IP2-12696bProbe(+)	AGATGTCTTGTGCTGCCGGTA [5']Hex [3']BHQ-1	21		
RdRp gene / nCoV_IP4				
nCoV_IP4-14059Fw	GGTAACTGGTATGATTTCG	19	107 bp	1
nCoV_IP4-14146Rv	CTGGTCAAGGTTAATATAGG	20		
nCoV_IP4-14084Probe(+)	TCATACAAACCACGCCAGG [5']Fam [3']BHQ-1	19		
E gene / E_Sarbeco				
E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT	18	125 bp	2
E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA	20		
E_Sarbeco_P1	ACACTAGCCATCCTTACTGCGCTTCG [5']Fam [3']BHQ-1	20		

1/ National Reference Center for Respiratory Viruses, Institut Pasteur, Paris.
2/ Corman et al. Eurosurveillance[1]

SPECIFICITY

Cross-reactivity with other respiratory viruses was tested with specimens known to be positive for a panel of respiratory viruses (influenza A(H1N1)pdm09, A(H3N2), B-Victoria, B-Yamagata; influenza C; RSV A, B; hBoV; hPIV; hMPV; HRV/enterovirus; adenovirus; hCoV (HKU1, OC43, 229E and NL63); MERS-CoV. None of the tested viruses showed reactivity with PCR2 and PCR4.

POSITIVE CONTROL FOR SARS-CoV-2 REAL-TIME RT-PCR

One specific control has been designated.

Positive control for real-time RT-PCR is an *in vitro* transcribed RNA derived from strain BetaCoV_Wuhan_WIV04_2019 (EPI_ISL_402124). The transcript contains the amplification regions of the ***RdRp*** *and* ***E gene*** as positive strand. Each microtube contains 10^{11} copies of target sequences diluted in yeast tRNA, and lyophilised.

Reconstitution of transcribed RNA

Add 100 µl of RNase/DNAse-free H2O to obtain a solution at a concentration of 10^9 copies/µl. Store at -80°C. Dilute to prepare a master bank at $2x10^6$ copies/µl. Store at -80°C.
From this prepare a working bank of reagent at $2x10^4$ copies/µl in order to avoid freeze/thaw cycles. Working tubes may be stored at -20°C for less than one week.

Positive controls are available upon request (grippe@pasteur.fr)

SARS

The NEW ENGLAND JOURNAL of MEDICINE

ORIGINAL ARTICLE

Identification of a Novel Coronavirus in Patients with Severe Acute Respiratory Syndrome

Christian Drosten, M.D., Stephan Günther, M.D., Wolfgang Preiser, M.D., Sylvie van der Werf, Ph.D., Hans-Reinhard Brodt, M.D., Stephan Becker, Ph.D., Holger Rabenau, Ph.D., Marcus Panning, M.D., Larissa Kolesnikova, Ph.D., Ron A.M. Fouchier, Ph.D., Annemarie Berger, Ph.D., Ana-Maria Burguière, Ph.D., Jindrich Cinatl, Ph.D., Markus Eickmann, Ph.D., Nicolas Escriou, Ph.D., Klaus Grywna, M.Sc., Stefanie Kramme, M.D., Jean-Claude Manuguerra, Ph.D., Stefanie Müller, M.Sc., Volker Rickerts, M.D., Martin Stürmer, Ph.D., Simon Vieth, Hans-Dieter Klenk, M.D., Albert D.M.E. Osterhaus, Ph.D., Herbert Schmitz, M.D., and Hans Wilhelm Doerr, M.D.

ABSTRACT

BACKGROUND

The severe acute respiratory syndrome (SARS) has recently been identified as a new clinical entity. SARS is thought to be caused by an unknown infectious agent.

METHODS

Clinical specimens from patients with SARS were searched for unknown viruses with the use of cell cultures and molecular techniques.

From the Bernhard Nocht In:
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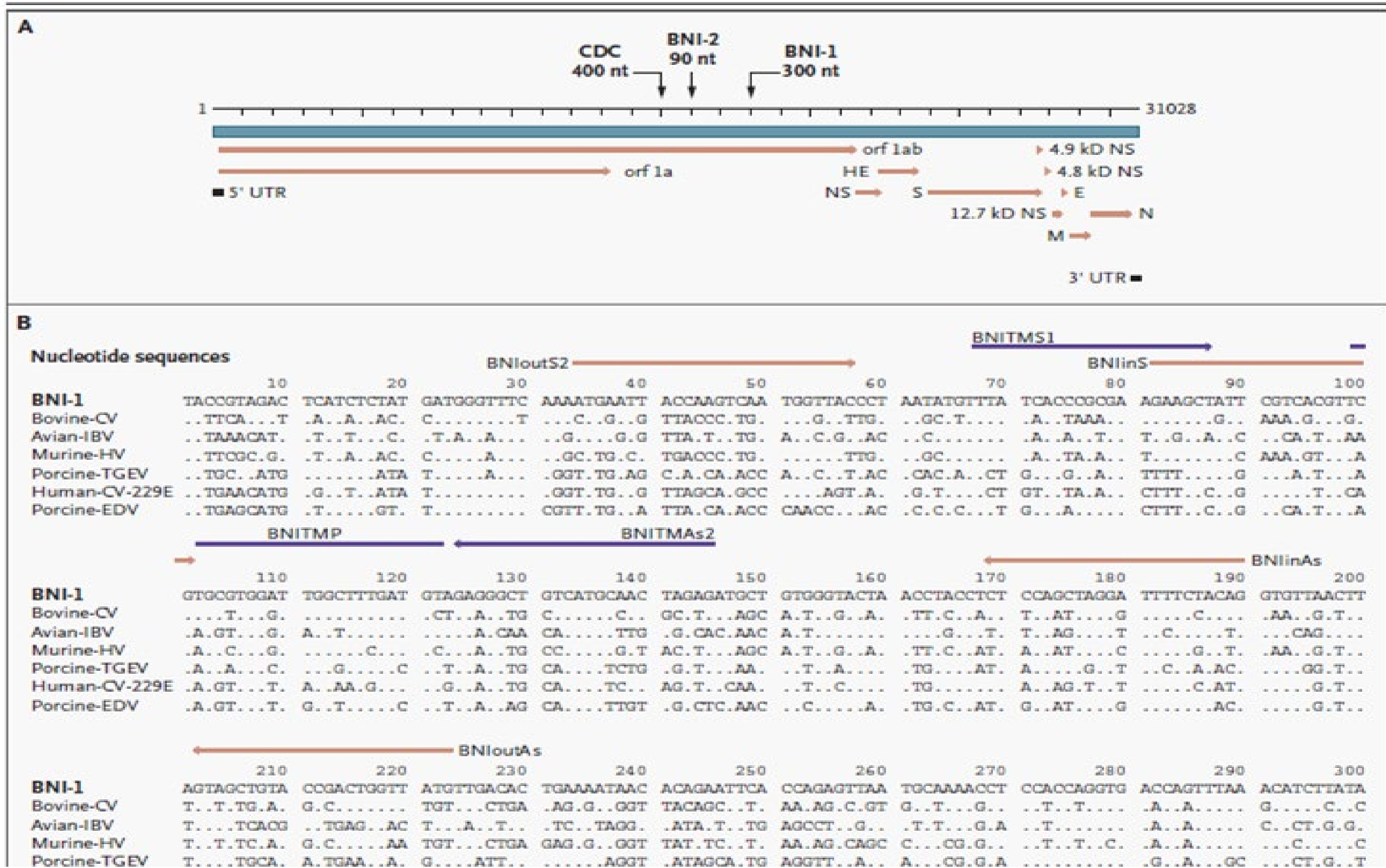
for Tropical Infectious Disea
(C.D., S.G., M.P., K.G., S.
H.S.); the Institute of Me
(W.P., H.R., A.B., J.C., M.S., H
Medical Clinic III (H.-R.B.,
Wolfgang Goethe University
Main; and the Institute of

Table 1. Primers and Protocols for Diagnostic Polymerase-Chain-Reaction Assays.

Protocol No.	Oligonucleotides	Target and Fragment Length	Reagent Formulation	Thermal Cycling Profile
1	**IN-2*** ggg TTg ggA CTA TCC TAA gTg TgA **IN-4*** TAA CAC ACA AAC ACC ATC ATC A	CDC fragment 452 bp	10 µl 2× reaction buffer† 2.45 mM magnesium sulfate† 500 nM each primer 0.4 µl reverse-transcriptase/*Taq* DNA polymerase mixture† 2 µl RNA 20 µl total volume	45°C, 30 min 95°C, 3 min 10 cycles of 95°C, 10 sec 60°C, 10 sec (decrease by 1°C per cycle) 72°C, 30 sec 40 cycles of 95°C, 10 sec 56°C, 10 sec 72°C, 30 sec
2	**IN-6*** ggT Tgg gAC TAT CCT AAg TgT gA **IN-7*** CCA TCA TCA gAT AgA ATC ATC ATA	CDC fragment 440 bp		
3	**BNIoutS2** ATg AAT TAC CAA gTC AAT ggT TAC **BNIoutAs** CAT AAC CAg TCg gTA CAg CTA	BNI-1 fragment 189 bp		
4	**SAR1S** CCT CTC TTg TTC TTg CTC gCA **SAR1As** TAT AgT gAg CCg CCA CAC Atg	CDC fragment nested PCR for protocols 1 and 2 121 bp	5 µl 10× reaction buffer‡ 200 µM dNTP 2.5 mM magnesium chloride‡ 200 nM each primer 1.25 units platinum *Taq* polymerase‡ 1 µl PCR product from previous round 50 µl total volume	95°C, 3 min 10 cycles of 95°C, 10 sec 60°C, 10 sec (decrease by 1°C per cycle) 72°C, 20 sec 20 cycles of 95°C, 10 sec 56°C, 10 sec 72°C, 20 sec
5	**BNIinS** gAA gCT ATT CgT CAC gTT Cg **BNIinAs** CTg TAg AAA ATC CTA gCT ggA g	BNI-1 fragment nested PCR for protocol 3 108 bp		
6	**BNITMSARS1** TTA TCA CCC gCg AAg AAg CT **BNITMSARAs2** CTC TAg TTg CAT gAC AgC CCT C **BNITMSARP** 6-carboxyfluorescein-	BNI-1 fragment 5′-nuclease real-time 77 bp	12.5 µl 2× reaction buffer† 3.6 mM magnesium sulfate† 1 µg bovine serum albumin§ 240 nM probe 200 nM each primer 0.6 µl reverse-transcriptase/*Taq* DNA polymerase mixture†	45°C, 15 min 95°C, 3 min 40 cycles of 95°C, 10 sec 58°C, 30 sec Fluorescence measured at 58°C¶


A
CDC
400 nt
BNI-2
90 nt
BNI-1
300 nt
1
31028
orf 1ab
4.9 kD NS
orf 1a
HE
4.8 kD NS
5' UTR
NS
S
E
12.7 kD NS
N
M
3' UTR
B
Nucleotide sequences
BNIoutS2
BNITMS1
BNIinS
BNI-1
Bovine-CV
Avian-IBV
Murine-HV
Porcine-TGEV
Human-CV-229E
Porcine-EDV
TACCGTAGAC TCATCTCTAT GATGGGTTTC AAAATGAATT ACCAAGTCAA TGGTTACCCT AATATGTTTA TCACCCGCGA AGAAGCTATT CGTCACGTTC
BNITMP
BNITMAs2
BNIinAs
GTGCGTGGAT TGGCTTTGAT GTAGAGGGCT GTCATGCAAC TAGAGATGCT GTGGGTACTA ACCTACCTCT CCAGCTAGGA TTTTCTACAG GTGTTAACTT
BNIoutAs
AGTAGCTGTA CCGACTGGTT ATGTTGACAC TGAAAATAAC ACAGAATTCA CCAGAGTTAA TGCAAAACCT CCACCAGGTG ACCAGTTTAA ACATCTTATA

1

A Pancoronavirus RT-PCR Assay for Detection of All Known Coronaviruses

Leen Vijgen, Elien Moës, Els Keyaerts, Sandra Li, and Marc Van Ranst

Abstract

The recent discoveries of novel human coronaviruses, including the coronavirus causing SARS, and the previously unrecognized human coronaviruses HCoV-NL63 and HCoV-HKU1, indicate that the family *Coronaviridae* harbors more members than was previously assumed. All human coronaviruses characterized at present are associated with respiratory illnesses, ranging from mild common colds to more severe lower respiratory tract infections. Since the etiology of a relatively large percentage of respiratory tract diseases remains unidentified, it is possible that for a certain number of these illnesses, a yet unknown viral causative agent may be found. Screening for the presence of novel coronaviruses requires the use of a method that can detect all coronaviruses known at present. In this chapter, we describe a pancoronavirus degenerate primer-based method that allows

1. Introduction

At present, viral culture is the "gold standard" for laboratory diagnosis of respiratory infections. Since coronaviruses are very difficult to grow in cell culture, accurate and sensitive diagnoses are not feasible by this technique. To overcome the lack of sensitivity and to obtain rapid diagnostic results, more sensitive molecular methods for the detection of human coronaviruses (HCoVs) have been developed, including reverse-transcriptase polymerase chain reaction

From: *Methods in Molecular Biology, vol. 454: SARS- and Other Coronaviruses,*
Edited by: D. Cavanagh, DOI: 10.1007/978-1-59745-181-9_1, © Humana Press, New York, NY

(RT-PCR), nested RT-PCR, and recently real-time RT-PCR ***(1)***. Nevertheless, HCoVs are not often diagnosed in clinical laboratories, although the SARS epi-

	Forward primer region	Reverse primer region
HCoV-NL63 :	ACACAGCTGAATCTTAAGTATGC	TGGGATTATCCCAAATGTGA
HCoV-229E :	ACTCAGTTAAATCTTAAATACGC	TGGGACTATCCTAAGTGTGA
FIPV :	ACTCAAATGAATTTGAAATATGC	TGGGACTATCCTAAGTGTGA
TGEV :	ACTCAGTTAAATCTTAAATACGC	TGGGACTATCCTAAGTGTGA
PEDV :	ACACAGCTCAACCTTAAATACGC	TGGGATTACCCAAAGTGCGA
HCoV-OC43 :	ACTCAAATGAATTTGAAATATGC	TGGGATTATCCTAAGTGTGA
BCoV :	ACTCAAATGAATTTGAAATATGC	TGGGATTATCCTAAGTGTGA
PHEV :	ACTCAAATGAATTTGAAATATGC	TGGGATTATCCAAAGTGTGA
CRCV :	ACTCAGATGAATTTGAAATATGC	TGGGATTATCCTAAGTGTGA
MHV :	ACTCAAATGAATCTTAAATATGC	TGGGACTATCCTAAATGTGA
SDAV :	ACTCAAATGAATCTTAAATATGC	TGGGACTATCCTAAGTGTGA
SARS-CoV :	ACTCAAATGAATCTTAAGTATGC	TGGGATTATCCAAAATGTGA
IBV :	ACTCAAATGAATTTAAAATATGC	TGGGATTATCCTAAGTGTGA
TCoV :	ACTCAAATGAATTTAAAATATGC	TGGGATTATCCTAAGTGTGA
	ACWCARHTVAAYYTNAARTAYGC	TGGGAYTAYCCHAARTGYGA

Fig. 1. Selection of primers for the novel pancoronavirus RT-PCR. Shown is the alignment of 14 coronaviral sequences of a conserved region of the polymerase gene. The forward (Cor-FW) and reverse (Cor-RV) primer sequences are shown at the bottom (Y=C/T, W=A/T, V=A/C/G, R=A/G, H=A/T/C, N=A/C/T/G).

MERS

RAPID COMMUNICATIONS

Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections

V M Corman[1,2], M A Müller[1,2], U Costabel[3], J Timm[4], T Binger[1], B Meyer[1], P Kreher[5], E Lattwein[6], M Eschbach-Bludau[1], A Nitsche[5], T Bleicker[1], O Landt[7], B Schweiger[5], J F Drexler[1], A D Osterhaus[8], B L Haagmans[8], U Dittmer[4], F Bonin[3], T Wolff[5], C Drosten (drosten@virology-bonn.de)[1]

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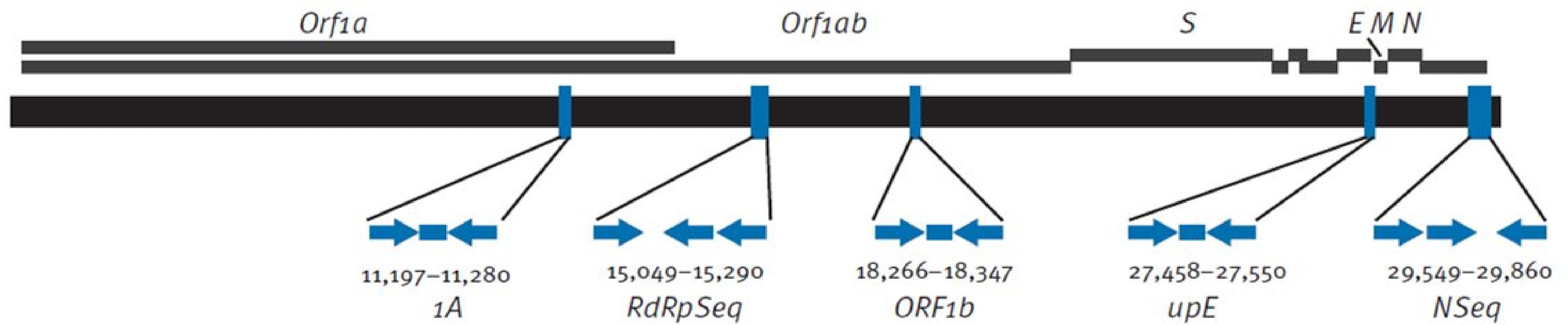
Citation style for this article:
Corman VM, Müller MA, Costabel U, Timm J, Binger T, Meyer B, Kreher P, Lattwein E, Eschbach-Bludau M, Nitsche A, Bleicker T, Landt O, Schweiger B, Drexler JF, Osterhaus AD, Haagmans BL, Dittmer U, Bonin F, Wolff T, Drosten C. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. Euro Surveill. 2012;17(49):pii=20334. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20334

Article submitted on 05 December 2012 / published on 6 December 2012

FIGURE 1

RT-PCR target regions for screening, confirmation and sequencing of novel human coronavirus (hCoV-EMC)



N: nucleocapsid; *Orf*: open reading frame; *RdRp*: RNA-dependent RNA polymerase; RT-PCR: reverse transcription-polymerase chain reaction.

The figure shows the relative positions of amplicon targets presented in this study, as well as in [2]. Primers are represented by arrows, probes as blue bars. Numbers below amplicon symbols are genome positions according to the hCoV-EMC/2012 prototype genome presented in [1].

The *1A* assay is the confirmatory real-time RT-PCR test presented in this study (target in the *ORF1a* gene). The *RdRpSeq* assay is a hemi-nested sequencing amplicon presented in this study (target in the *RdRp* gene). The *ORF1b* assay is a confirmatory real-time RT-PCR presented in [2]. The *upE* assay is a real-time RT-PCR assay recommended for first-line screening as presented in [2] (target upstrem of *E* gene). The *NSeq* assay is a hemi-nested sequencing amplicon presented in this study (target in *N* gene).

Nested protocol

RT-PCR for sequencing in the *N* gene (*NSeq* assay)

The assay employed the same conditions as the *RdRpSeq* assay, except that the primer sequences were *NSeq*-Fwd (CCT TCG GTA CAG TGG AGC CA) and *NSeq*-Rev (GAT GGG GTT GCC AAA CAC AAA C) for the first round and *NSeq*-Fnest (TGA CCC AAA GAA TCC CAA CTA C) and *NSeq*-Rev (the same as in the first round) for the second round. The second round was only done if no product was visible by agarose gel electrophoresis after the first round.

Virus quantification by real-time RT-PCR using in-vitro transcribed RNA

In-vitro transcribed RNA was prepared as described previously [2]. Serial 10-fold dilutions of this RNA were amplified in parallel with samples in a Roche LightCycler 480II after entering the known RNA concentrations of standards in the quantification module of the operation software. Virus concentrations in terms of genome copies per ml of original sample were extrapolated using a conversion factor of 85.7, as explained previously [2].

Virus growth, infection and titration

Virus stocks of the clinical isolate hCoV-EMC/2012

25 µl per well. As a positive control, a macaque-anti-hCoV-EMC (day 14 post infection), provided by author B. H. was used in a 1:20 dilution. Slides were incubated at 37 °C for 1 hour (rapid slides) or at room temperature for 30 minutes (conventional coverslips) and washed three times with phosphate-buffered saline (PBS)-Tween (0.1%) for 5 minutes. The secondary antibody was a goat-anti human Cy2-labelled immunoglobulin G conjugate. After incubation at 37 °C (spotted slides) or room temperature (conventional coverslips) for 30 minutes, they were washed three times with PBS-Tween for 5 minutes, rinsed with water and mounted with DAPI ProLong mounting medium (Life Technologies).

Recombinant assays for confirmatory IFA and western blot analysis

The hCoV-EMC/2012 spike (*S*) and *N* genes were amplified from cDNA. For PCR amplification of FLAG-tagged *N* and *S* and subsequent cloning into a pCG1 vector (kindly provided by Georg Herrler, TIHO, Hannover), the following primers were used: 2c-nhCoV-SflagN-BamHI-F (TACGGATCCGCCACCATGGATTACAAGGATGACGATGACAA GGGAGGCATACACTCAGTGTTTCTACTGATGT), 2c-nhCoV-S-SalI-R (AGCGTCGACTTAGTGAACATGAAC CTTATGCGG), 2c-nhCoV-NflagN-BamHI-F (TACGGATCCGCCACCATGGATTACAAGGATGACGATG

MERS

RAPID COMMUNICATIONS

Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction

V M Corman[1,2], I Eckerle[1], T Bleicker[1], A Zaki[3], O Landt[4], M Eschbach-Bludau[1], S van Boheemen[5], R Gopal[6], M Ballhause[4], T M Bestebroer[5], D Muth[1], M A Müller[1], J F Drexler[1], M Zambon[6], A D Osterhaus[5], R M Fouchier[5], C Drosten (drosten@virology-bonn.de)[1]

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Corman VM, Eckerle I, Bleicker T, Zaki A, Landt O, Eschbach-Bludau M, van Boheemen S, Gopal R, Ballhause M, Bestebroer TM, Muth D, Müller MA, Drexler JF, Zambon M, Osterhaus AD, Fouchier RM, Drosten C. Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. Euro Surveill. 2012;17(39):pii=20285. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20285

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non-nested

Real-time reverse-transcription polymerase chain reaction screening assay upstream of E gene (upE assay)

A 25-µl reaction was set up containing 5 µl of RNA, 12.5 µl of 2 X reaction buffer provided with the Superscript III one step RT-PCR system with Platinum Taq Polymerase (Invitrogen; containing 0.4 mM of each dNTP and 3.2 mM Magnesium sulfate), 1 µl of reverse transcriptase/Taq mixture from the kit, 0.4 µl of a 50 mM magnesium sulfate solution (Invitrogen – not provided with the kit), 1 µg of non-acetylated bovine serum albumin (Sigma), 400 nM concentrations of primer upE-Fwd (GCAACGCGCGATTCAGTT) and primer upE-Rev (GCCTCTACACGGGACCCATA), as well as 200 nM of probe upE-Prb (6-carboxyfluorescein [FAM])-CTCTTCACATAATCGCCCCGAGCTCG-6-carboxy-N,N,N,N´-tetramethylrhodamine [TAMRA]). All oligonucleotides were synthesized and provided by Tib-Molbiol, Berlin. Thermal cycling involved 55°C for 20 min, followed by 95°C for 3 min and then 45 cycles of 95°C for 15 s, 58°C for 30 s.

It should be mentioned that common one-step real-time RT-PCR kits formulated for application with probes should all provide satisfactory results with default

instrument with plastic tubes. The component only serves the purpose of enabling glass capillary-based PCR cycling.

Real-time reverse-transcription polymerase chain reaction confirmatory assay (open reading frame (ORF)1b gene)

The assay had the same conditions as for the upE RT-PCR, except primer and probe sequences were ORF1b-Fwd (TTCGATGTTGAGGGTGCTCAT), primer ORF1b-Rev (TCACACCAGTTGAAAATCCTAATTG), and probe ORF1b-Prb (6-carboxyfluorescein [FAM])- CCCGTAATGCATGTGGCACCAATGT-6-carboxy-N,N,N,N´-tetramethylrhodamine [TAMRA]). This target gene did not overlap with those of known pan-CoV assays [3-5].

In-vitro transcribed RNA controls

PCR fragments covering the target regions of both assays, and some additional flanking nucleotides ('peri-amplicon fragments'), were generated using primers CTTCTCATGGTATGGTCCCTGT and AAGCCATACACACCAAGAGTGT for the upE assay, and CGAGTGATGAGCTTTGCGTGA and

https://www.fda.gov/media/85951/download

Novel Coronavirus 2012 Real-Time RT-PCR Assay

Centers for Disease Control and Prevention

For Use Under an Emergency Use Authorization Only

Instructions for Use

Intended Use

The CDC Novel Coronavirus 2012 Real-time RT-PCR Assay (NCV-2012 rRT-PCR) is intended for the *in vitro* qualitative detection of MERS-CoV RNA in respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, sputa, lower respiratory aspirates/washes), sera and stool from individuals meeting MERS-CoV clinical and/or epidemiological criteria (for example, clinical signs and symptoms associated with MERS-CoV infection, contact with a probable or confirmed MERS-CoV case, history of travel to geographic locations where MERS-CoV cases were detected, or other epidemiologic links for which MERS-CoV testing may be indicated as part of a public health investigation).

Testing with the NCV-2012 rRT-PCR Assay should not be performed unless the patient meets clinical and/or epidemiologic criteria for testing suspect specimens.

Results are for the presumptive identification of MERS-CoV. Laboratories are required to report results to the Centers for Disease Control and Prevention (CDC). The definitive identification of MERS-CoV requires additional testing and confirmation to be performed by CDC. The diagnosis of MERS-CoV infection must be made based on history, signs, symptoms, exposure likelihood, and other laboratory evidence, in addition to the detection of MERS-CoV RNA.

Negative NCV-2012 rRT-PCR Assay results do not preclude MERS-CoV infection and should not be used as the sole basis for patient management decisions. In asymptomatic individuals, a negative result does not exclude the possibility of future illness and does not demonstrate that an individual is not infectious.

The NCV-2012 rRT-PCR Assay is intended for use by trained laboratory personnel who are proficient in performing real-time RT-PCR assays. The level of MERS-CoV that would be

controles

- Keep reagent tubes and reactions capped as much as possible.
- Clean surfaces using an acceptable surface decontaminant (see above).
- Do not bring extracted nucleic acid or PCR amplicons into the assay setup area.
- Use aerosol barrier (filter) pipette tips only.
- Use optical strip 8-cap strips only. Do not use PCR plate sealing film.

Assay Controls

Assay Controls should be run concurrently with all test samples.

- VTC – NCV-2012 rRT-PCR Assay Positive Control
- NTC_1 – A known negative template control (sterile, nuclease-free water) added during rRT-PCR reaction set-up.
- NTC_2 – A known negative template control (sterile, nuclease-free water) that is **extracted concurrently** with the test samples and included as a sample during rRT-PCR set-up.
- RP – All clinical samples should be tested for human RNAse P gene (using the LRN RNase P primer and probe set) to control for specimen quality and extraction.

Table 4: NCV-2012 rRT-PCR Assay Test Interpretation and Reporting Instructions

<table>
<tr><th colspan="8">Interpretation of NCV-2012 rRT-PCR Assay Results</th></tr>
<tr><th colspan="3">Testing Algorithm Part 1</th><th colspan="2">Algorithm Part 2</th><th></th><th></th><th></th></tr>
<tr><th>NCV.upE</th><th>NCV.N2</th><th>RP</th><th>NCV.N3</th><th>RP</th><th>Interpretation</th><th>Reporting</th><th>Actions</th></tr>
<tr><td>-</td><td>-</td><td>+</td><td colspan="2">Not Done</td><td>MERS-CoV Negative</td><td>MERS-CoV RNA not detected by rRT-PCR</td><td>Report results through LRN Results Messenger</td></tr>
<tr><td>-</td><td>-</td><td>-</td><td colspan="2">Not Done</td><td>Inconclusive</td><td>Inconclusive for MERS-CoV RNA by rRT-PCR. An inconclusive result may occur in the case of an inadequate specimen.</td><td>If there are no additional specimens available for the patient, request collection of additional specimens.

Report results through LRN Results Messenger</td></tr>
<tr><td>-</td><td>+</td><td rowspan="3">+/-</td><td rowspan="3">+</td><td rowspan="3">+/-</td><td rowspan="3">MERS-CoV Presumptive Positive</td><td rowspan="3">MERS-CoV RNA detected by rRT-PCR. Confirmatory testing required.
Specimen will be referred to CDC for further analysis.</td><td rowspan="3">Send specimen to CDC for confirmatory testing.

Report results thorugh LRN Results Messenger</td></tr>
<tr><td>+</td><td>-</td></tr>
<tr><td>+</td><td>+</td></tr>
<tr><td>-</td><td>+</td><td rowspan="3">+/-</td><td rowspan="3">-</td><td rowspan="3">+/-</td><td rowspan="3">Equivocal</td><td rowspan="3">NCV-2012 rRT-PCR testing was equivocal. Additional analysis may be conducted by CDC.</td><td rowspan="3">Contact CDC for consultation.

Report results thorugh LRN Results Messenger</td></tr>
<tr><td>+</td><td>-</td></tr>
<tr><td>+</td><td>+</td></tr>
</table>

NOTE: All test results generated using the NCV-2012 rRT-PCR Assay must be sent to CDC using LRN Results Messenger. Please refer to the LRN Data Messaging Policy (found under Documents/LRN Specific Information/LRN Policy Statements on the LRN

WHO protocollen

Summary table of available protocols in this document

Institute	Gene targets
China CDC, China	ORF1ab and N
Institut Pasteur, Paris, France	Two targets in RdRP
US CDC, USA	Three targets in N gene
National Institute of Infectious Diseases, Japan	Pancorona and multiple targets, Spike protein
Charité, Germany	RdRP, E, N
HKU, Hong Kong SAR	ORF1b-nsp14, N
National Institute of Health, Thailand	N

***Disclaimer**: The order on the list is by country of the hosting institution and does not imply any preference of WHO. Neither the names of vendors or manufacturers included in the protocols are preferred/endorsed by WHO. The protocols have not yet been validated through a WHO process.*

No.		Name	direction	sequence (5′ to 3′)	Expected size (bp)
ORF1a set					
1	1st	NIID_WH-1_F501	Sense	TTCGGATGCTCGAACTGCACC	413
2	1st	NIID_WH-1_R913	Antisense	CTTTACCAGCACGTGCTAGAAGG	
3	2nd	NIID_WH-1_F509	Sense	CTCGAACTGCACCTCATGG	346
4	2nd	NIID_WH-1_R854	Antisense	CAGAAGTTGTTATCGACATAGC	
5	Seq	NIID_WH-1_Seq_F519	Sense	ACCTCATGGTCATGTTATGG	
6	Seq	NIID_WH-1_Seq_R840	Antisense	GACATAGCGAGTGTATGCC	
S set					
7	1st	WuhanCoV-spk1-f	Sense	TTGGCAAAATTCAAGACTCACTTT	547
8	1st	WuhanCoV-spk2-r	Antisense	TGTGGTTCATAAAAATTCCTTTGTG	
9	2nd	NIID_WH-1_F24381	Sense	TCAAGACTCACTTTCTTCCAC	493
10	2nd	NIID_WH-1_R24873	Antisense	ATTTGAAACAAAGACACCTTCAC	
11	Seq	NIID_WH-1_Seq_F24383	Sense	AAGACTCACTTTCTTCCACAG	
12	Seq	NIID_WH-1_Seq_R24865	Antisense	CAAAGACACCTTCACGAGG	

De PCR-testen voor SARS-CoV-2 in Nederland zijn oorspronkelijk gericht op twee targets: het E-gen en het RdRP-gen. Met de ervaring die daarmee is opgedaan, hebben de meeste laboratoria ervoor gekozen om alleen nog voor het meest gevoelige E-gen te testen om tijd te besparen en het gebruik van reagentia bij schaarste in te perken. Inmiddels zijn er protocollen waarin de originele 'Corman' RdRP-gen-PCR geheel SARS-CoV-2-specifiek en vergelijkbaar gevoelig als de E-gen-PCR gemaakt is (Corman 2020).

De amplificatiecurves dienen goed te worden beoordeeld (zoals bij iedere PCR-analyse en zeker bij hogere Ct-waarden boven Ct 35). Is de curve afwijkend, onbetrouwbaar of moeilijk te interpreteren, dan is zeker bij een epidemiologisch onverwachte positieve uitslag confirmatie nodig. Er is nu een diversiteit aan in-house en commerciële RT-PCR-testen die verschillende genen, één of meerdere, als target gebruikt. In validaties met kwaliteitspanels van het RIVM en kwaliteitsrondzendingen georganiseerd door het RIVM zijn deze vergelijkbaar gevoelig (zie voor meer detail de bijlage Aanvullende informatie diagnostiek en de rapportages van november 2020 en februari 2021).

Een positieve PCR-uitslag met een hoge Ct-waarde is voor patiëntendiagnostiek en -behandeling onverminderd relevant. Voor screening in teststraten en in BCO kunnen positieve PCR-uitslagen met hoge Ct-waarden mogelijk duiden op een mindere besmettelijkheid van bijbehorende geteste personen, zoals o.a. wat langer na infectie. Om GGD'en handvatten te geven wordt geadviseerd aan het laboratorium om per workflow regelmatig de Ct-verdeling in kaart te brengen om de Ct-grens op het 90ste percentiel vast te stellen waarboven een PCR-resultaat als zwak-positief geduid kan worden. Bij rapportage van het PCR-resultaat in CoronIT kan dit in een commentaarveld bij de uitslag meegegeven worden. De zwak positieve testuitslag kan door de GGD-arts samen met de anamnese beoordeeld worden, om het verdere beleid te bepalen.

Mede om tekorten op specifieke reagentia beter te kunnen opvangen, gebruiken verschillende labs verschillende testsystemen van diverse aanbieders. Sommige labs hebben meerdere testsystemen tot hun beschikking. De diverse testsystemen kunnen verschillende targets voor amplificatie gebruiken, soms andere genen, soms andere delen van genen. Niet van alle testsystemen is de precieze locatie van primers en probes in de targetgenen door de fabrikant bekend gemaakt. De ervaring met validatie van de diverse testsystemen door het RIVM, in-house of met commerciële PCR of andere NAAT, leert dat de grootste meerderheid van deze testen een gevoeligheid hebben met een 'limit of detection' (LOD) die ligt tussen de 82.6 en 8.26 digitale kopieën RdRP-RNA per milliliter VTM (gemeten met hitte geïnactiveerd virus). Targetgenen die gebruikt worden zijn E, N1, N2, niet nader gespecificeerd N, RdRP, niet naar gespecificeerd liggend op ORF1a/b of S. Alhoewel E-gen RT-PCR door Corman et al. is ontworpen om generiek SARS-like betacoronavirussen te detecteren, zijn er ook testsystemen/kits die de locatie van primers en probes op het E-gen hebben verplaatst naar SARS-CoV-2-specifiek gebied. De testsystemen met targetgenen E, N1, N2, N en S kunnen afhankelijk van tijdstip na infectie gevoeliger zijn dan testen gebaseerd op het RdRP of ORF1a/b. Dit heeft ermee te maken dat van de E, N1, N2, N en S genen 'subgenome messengers' (subgenomisch mRNA) worden gemaakt en van RdRP en ORF1a/b niet. Met RdRP en ORF1a/b wordt alleen het genomische virale RNA gedetecteerd (Parker 2020). Vier tot vijf dagen na infectie lijken 'subgenome messengers' tot niet detecteerbaar niveau afgenomen te zijn (Wolfel 2020). Het vinden van een enkel gentarget positiefmonster bij (heel) lage virale load als voor meerdere targets wordt getest, is daarmee hoogstwaarschijnlijk de resultante van het tijdstip van afname van een monster na infectie, (subtiele) verschillen in gevoeligheid van de PCR-testen op verschillende genen en de statistische kans dat de aanwezigheid van RNA in een monster wordt gedetecteerd rond of beneden de LOD van een PCR-test.