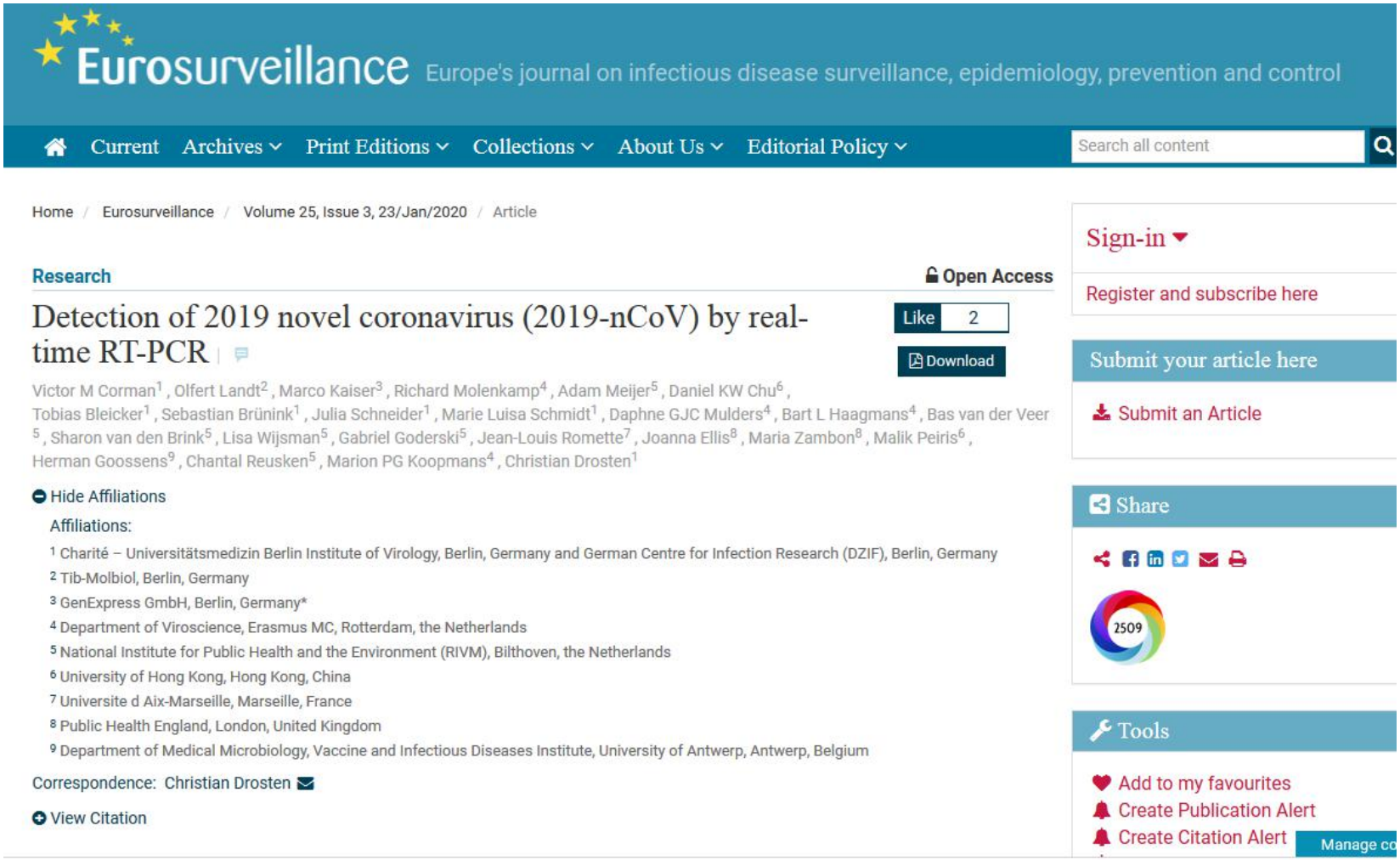
Wat is er mis met de RT-PCR Test ?



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Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR

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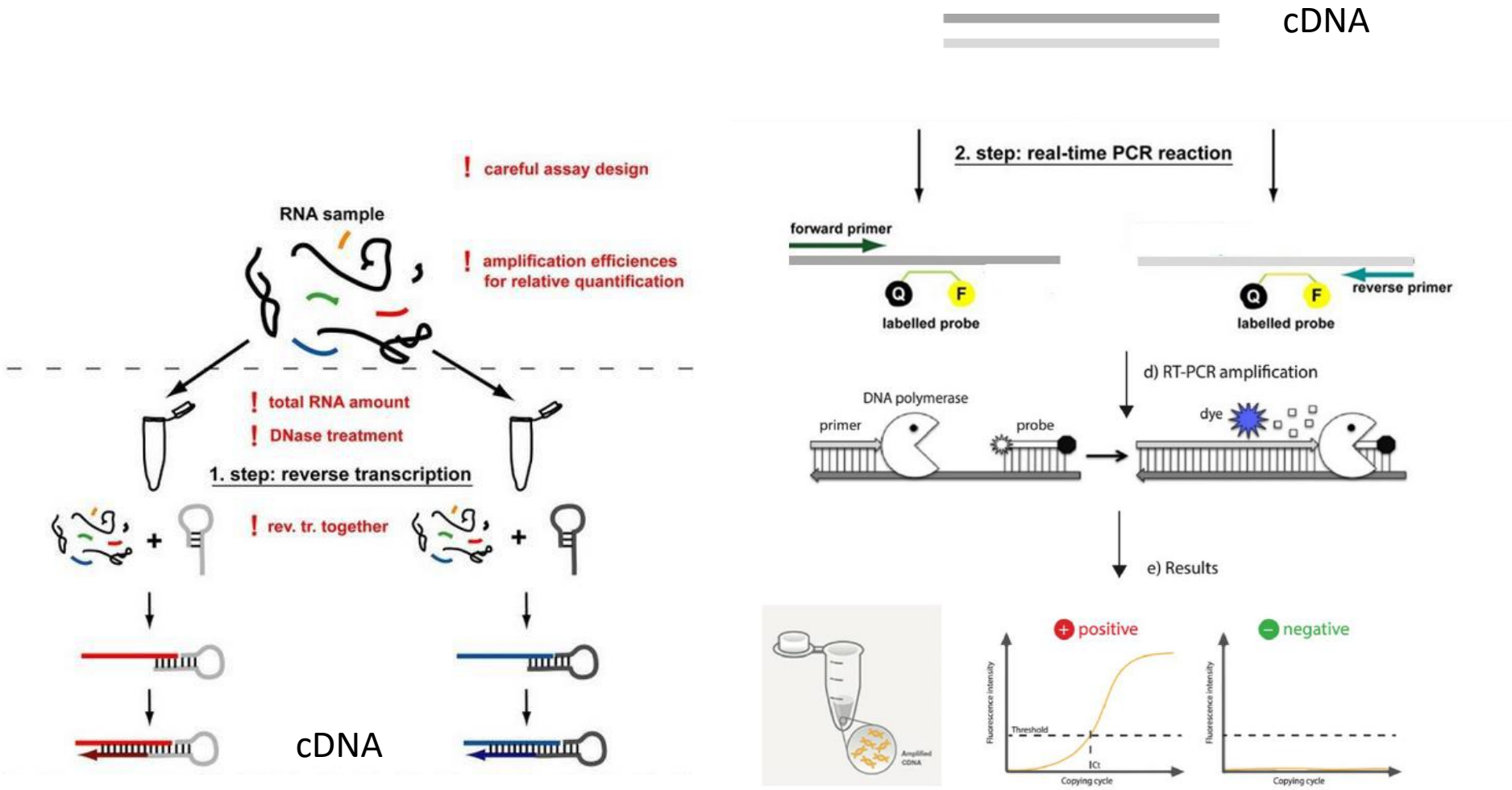
Wat is een RT-PCR Test ?



https://www.globalbiotechinsights.com/articles/20247/the-worldwide-test-for-covid-19

https://www.researchgate.net/publication/265174763_3%27_IsomiR_Species_and_DNA_Contamination_Influence_Reliable_Quantification_of_MicroRNAs_by_Stem-Loop_Quantitative_PCR/figures?lo=1

Peter Borger & Bobby Rajesh Malhotra

Wat is belangrijk als je een RT-PCR Test ontwerpt?

1) De primers
   → moeten specifiek zijn voor je target (=het gen dat je wilt amplificeren);
   → CG gehalte van je primers (minimaal 40%, maximaal 60%)
   → de concentratie van de primers (100-200 nM)
   →minimaal 3 genen testen (liefst zo ver mogelijk uit elkaar gelegen).

2) De temperatuur waarbij alle reactie plaatsvinden
   → DNA smelt temperatuur (>92%)
   → Tm; De annealing temperatuur (de T waarbij de primers het target binden/loslaten, (per primerpaar mag die niet meer dan 1˚ C (maximaal 2˚C) verschillen)

3) Het aantal amplificatiecycli (minder dan 35; liefst 25-30 cycli)

4) Positieve en negatieve controles en moleculairbiologische validaties

5) Zeer belangrijk: Er moet een «Standard operational procedure» (SOP) voorhanden zijn, die bovenstaande parameters vastlegd, zodat alle Laboratoria dezelfde testcondities gebruiken.

Peter Borger & Bobby Rajesh Malhotra

Ontwerpfout 1: Variabele Primer concentraties

Table 1. Primers and probes, real-time RT-PCR for 2019 novel coronavirus

Toggle display: ▾ Open fullscreen ⤢

Assay/use	Oligonucleotide	Sequence[a]	Concentration[b]
RdRP gene	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG	Use 600 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
	RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA	Use 800 nM per reaction
E gene	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	Use 400 nm per reaction
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	Use 200 nm per reaction
	E_Sarbeco_R	ATATTGCAGCAGTACGCACACA	Use 400 nm per reaction
N gene	N_Sarbeco_F	CACATTGGCACCCGCAATC	Use 600 nm per reaction
	N_Sarbeco_P	FAM-ACTTCCTCAAGGAACAACATTGCCA-BBQ	Use 200 nm per reaction
	N_Sarbeco_R	GAGGAACGAGAAGAGGCTTG	Use 800 nm per reaction

[a] W is A/T; R is G/A; M is A/C; S is G/C. FAM: 6-carboxyfluorescein; BBQ: blackberry quencher.

[b] Optimised concentrations are given in nanomol per litre (nM) based on the final reaction mix, e.g. 1.5 µL of a 10 µM primer stock solution per 25 µL total reaction volume yields a final concentration of 600 nM as indicated in the table.

Ontwerpfout 2: Aspecifieke («Wobbly») Primers

Table 1. Primers and probes, real-time RT-PCR for 2019 novel coronavirus

Toggle display: ▦ ▾ Open fullscreen ⤢

Assay/use	Oligonucleotide	Sequence[a]	Concentration[b]
RdRP gene 2	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG	Use 600 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
	RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA	Use 800 nM per reaction
E gene 1	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	Use 400 nm per reaction
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	Use 200 nm per reaction
	E_Sarbeco_R	ATATTGCAGCAGTACGCACACA	Use 400 nm per reaction
N gene 3	N_Sarbeco_F	CACATTGGCACCCGCAATC	Use 600 nm per reaction
	N_Sarbeco_P	FAM-ACTTCCTCAAGGAACAACATTGCCA-BBQ	Use 200 nm per reaction
	N_Sarbeco_R	GAGGAACGAGAAGAGGCTTG	Use 800 nm per reaction

[a] W is A/T, R is G/A, M is A/C, S is G/C. FAM: 6-carboxyfluorescein; BBQ: blackberry quencher.

[b] Optimised concentrations are given in nanomol per litre (nM) based on the final reaction mix, e.g. 1.5 µL of a 10 µM primer stock solution per 25 µL total reaction volume yields a final concentration of 600 nM as indicated in the table.

Ontwerpfout 3-5: GC-gehalte van de Primers

Normal ranges for GC%: 40 - 60 %
Normal ranges for TM: 55 - 65°
Best-practise for qPCR in our case:
***60°* for both primers (reverse & forward)**

Table 1. Primers and probes, real-time RT-PCR for 2019 novel coronavirus

Toggle display: ▾ Open fullscreen ⤢

Assay/use	Oligonucleotide	Sequence[a]	Concentration[b]
RdRP gene	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG	Use 600 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
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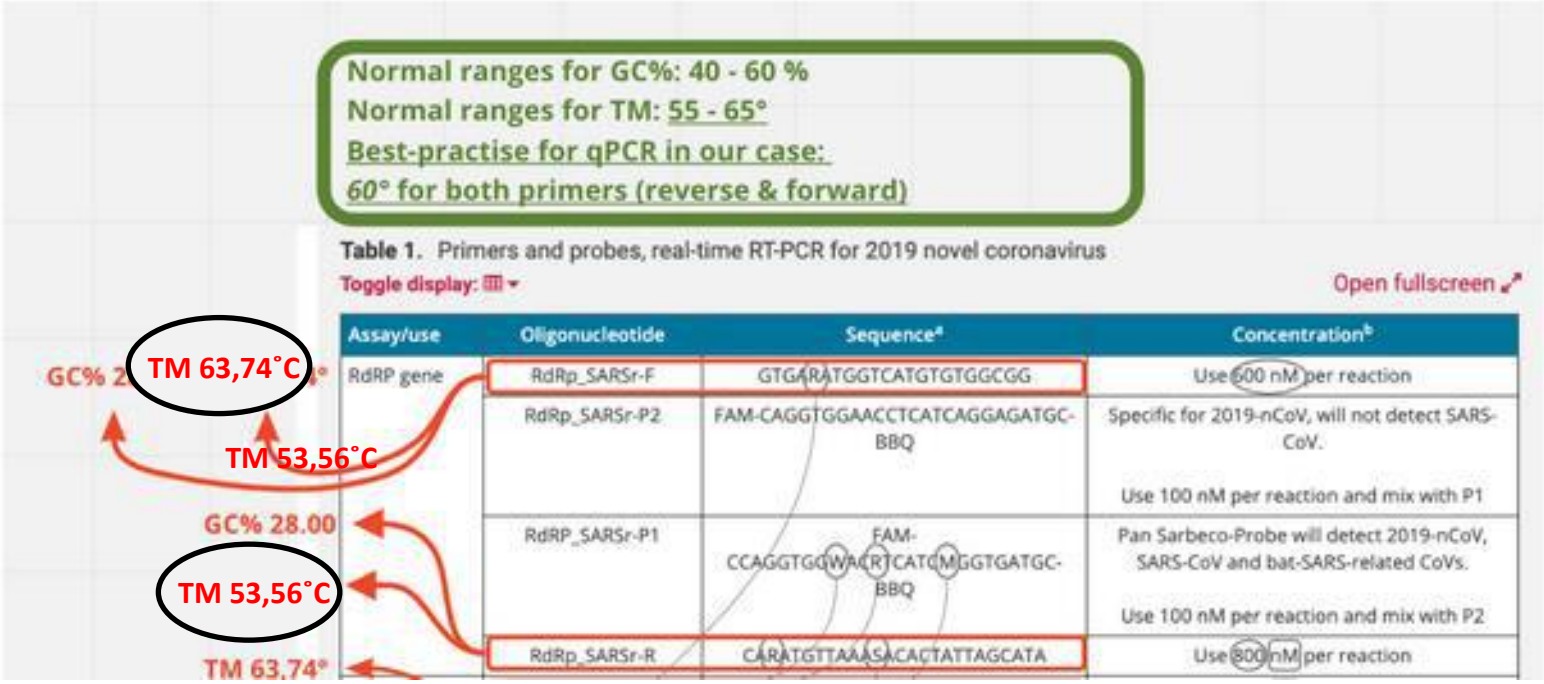
	RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA	Use 800 nM per reaction
E gene	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	Use 400 nm per reaction
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	Use 200 nm per reaction
	E_Sarbeco_R	ATATTGCAGCAGTACGCACACA	Use 400 nm per reaction
N gene	N_Sarbeco_F	CACATTGGCACCCGCAATC	Use 600 nm per reaction
	N_Sarbeco_P	FAM-ACTTCCTCAAGGAACAACATTGCCA-BBQ	Use 200 nm per reaction
	N_Sarbeco_R	GAGGAACGAGAAGAGGCTTG	Use 800 nm per reaction

GC% 28.00 TM 63,74°
GC% 28.00
TM 63,74°
TM 63,74°
GC% 34,6
GC% 45,5

[a] W is A/T; R is G/A; M is A/C; S is G/C. FAM: 6-carboxyfluorescein; BBQ: blackberry quencher.

[b] Optimised concentrations are given in nanomol per litre (nM) based on the final reaction mix, e.g. 1.5 µL of a 10 µM primer stock solution per 25 µL total reaction volume yields a final concentration of 600 nM as indicated in the table.

Ontwerpfout 6: Annealing (Tm) Primerpaar RdRp Gen



Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity
Forward primer	GTGAGATGGTCATGTGTGGCGG	22	63.74	59.09	4.00
Reverse primer	CAGATGTTAAACACATATTAGCATA	25	53.56	28.00	7.00

Ontwerpfout 7: Grootste deel van virus niet gedekt

Not covered by Corman-Drosten RT-PCR Test

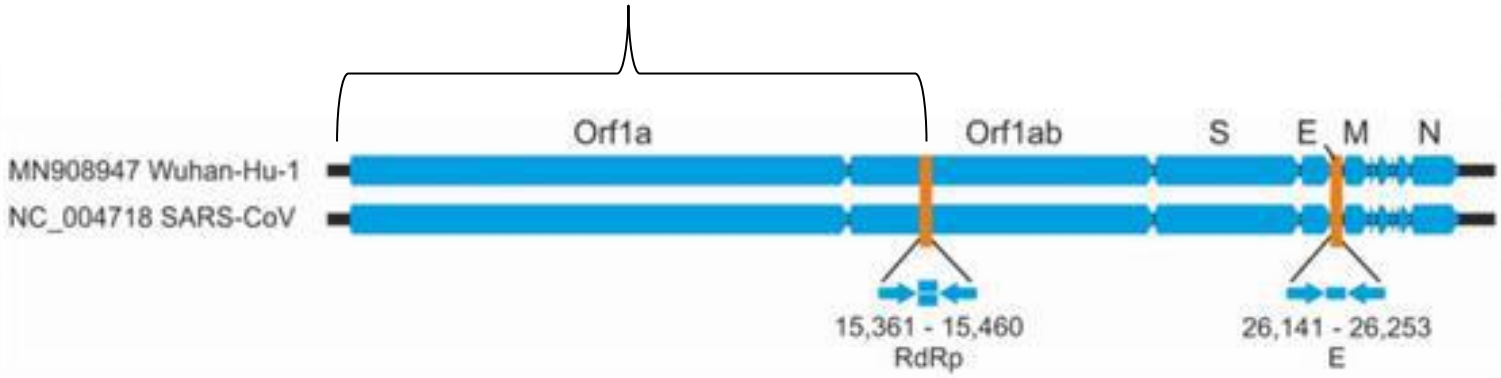


Figure 1 relative positions of amplicon targets on SARS-CoV an 2019-nCoV genome. ORF: open reading frame; RdRp: RNA-dependent RNA polymerase. Numbers below amplicon are genome positions according to SARS-CoV, NC_004718.

Ontwerpfout 8: Geen negatieve & positieve controles

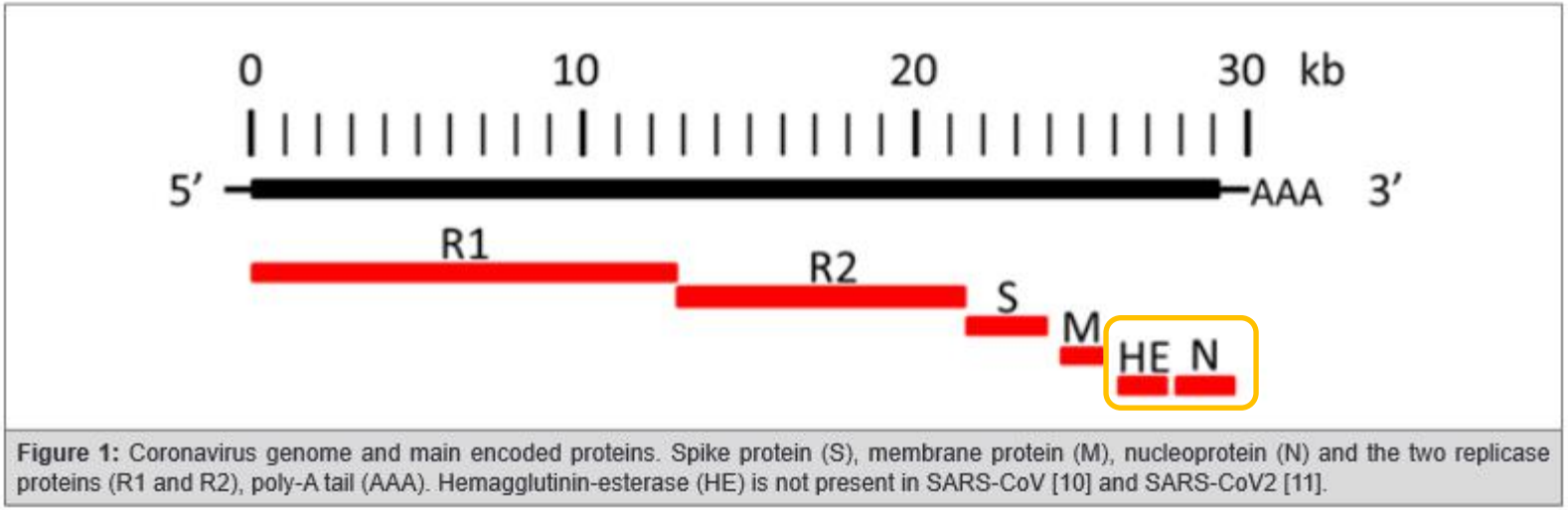


Figure 1: Coronavirus genome and main encoded proteins. Spike protein (S), membrane protein (M), nucleoprotein (N) and the two replicase proteins (R1 and R2), poly-A tail (AAA). Hemagglutinin-esterase (HE) is not present in SARS-CoV [10] and SARS-CoV2 [11].

Borger P, Am J Biomed Sci Res 2020

Negatieve Controle = Corona HE gen, want het komt niet in SARS-CoV1/2 voor

Positieve Control = N gen met de onderstaande SARS-CoV1/2 specifieke sequentie

SARS2003: **KTFPPTEPKKDKKKK**

COVID19: **KTFPPTEPKKDKKKK**

Ontwerpfout 9: aanbevolen 45 amplificatie cycli

“The present report describes the establishment of a diagnostic workflow for detection of an emerging virus in the absence of physical sources of viral genomic nucleic acid.”

Corman et al, Eurosurveillance 2020

* Thermo Fischer/Invitrogen: SuperScriptIII OneStep RT-PCR System with Platinum® Taq DNA Polymerase
** MgSO4 (50 mM) [Sigma], This component is not provided with the OneStep RT-PCR kit
*** non-acetylated [Roche].

Cycler:

55°C	10’	
94°C	3’	
94°C	15”	45x
58°C	30”	

Wat bewerken deze ontwerpfouten? Aspecieke amplificatie!

→ Is daar bewijs voor?

Exclusivity of 2019 novel coronavirus based on clinical samples pre-tested positive for other respiratory viruses

Using the E and RdRp gene assays, we tested a total of 297 clinical samples from patients with respiratory disease from the biobanks of five laboratories that provide diagnostic services (one in Germany, two in the Netherlands, one in Hong Kong, one in the UK). We selected 198 samples from three university medical centres where patients from general and intensive care wards as well as mainly paediatric outpatient departments are seen (Germany, the Netherlands, Hong Kong). The remaining samples were contributed by national public health services performing surveillance studies (RIVM, PHE), with samples mainly submitted by practitioners. The samples contained the broadest range of respiratory agents possible and reflected the general spectrum of virus concentrations encountered in diagnostic laboratories in these countries (Table 2). In total, this testing yielded no false positive outcomes. In four individual test reactions, weak initial reactivity was seen but they were negative upon retesting with the same assay. These signals were not associated with any particular virus, and for each virus with which initial positive reactivity occurred, there were other samples that contained the same virus at a higher concentration but did not test positive. Given the results from the extensive technical qualification described above, it was concluded that this initial reactivity was not due to chemical instability of real-time PCR probes but most probably to handling issues caused by the rapid introduction of new diagnostic tests and controls during this evaluation study.

Compleet aspecifiek opgezet?

"To show that the assays can detect other bat-associated SARS-related viruses, we used the E gene assay to test six bat-derived faecal samples available from Drexler et al. [13] und Muth et al. [14]. These virus-positive samples stemmed from European rhinolophid bats. Detection of these phylogenetic outliers within the SARS-related CoV clade suggests that all Asian viruses are likely to be detected. This would, theoretically, ensure broad sensitivity even in case of multiple independent acquisitions of variant viruses from an animal reservoir."

Corman et al, Eurosurveillance 2020

De Corman-paper werd niet gepeer-reviewed?!

RESEARCH

Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR

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Citation style for this article:
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Article submitted on 21 Jan 2020 / accepted on 22 Jan 2020 / published on 23 Jan 2020

Voor een diagnostische test is de peerreview extreem belangrijk!

Corman-paper werd niet moleculairbiologisch gevalideerd!

Nergens in de Corman paper wordt beschreven hoe de verkegen amplificatie produkten werden gevalideerd.

Validatie van amplifatieprodukten kan op twee manieren:

1) het amplificatie-produkt op een gel runnen met een DNA ladder, zodat de grootte van het produkt kan worden geschat. Deze moet overeenkomen met de berekende grootte van de amplificatie produkt. Maar beter is nog...

2) Het amplificatieprodukt sequencen. Dit geeft 100% zeker uitsluitsel m.b.t. Identiteit van het amplificatieprodukt.

Voor een diagnostische test is deze validatie extreem belangrijk!

Drosten (RKI) en Reusken (RIVM) zijn zowel auteurs als editors.

RESEARCH

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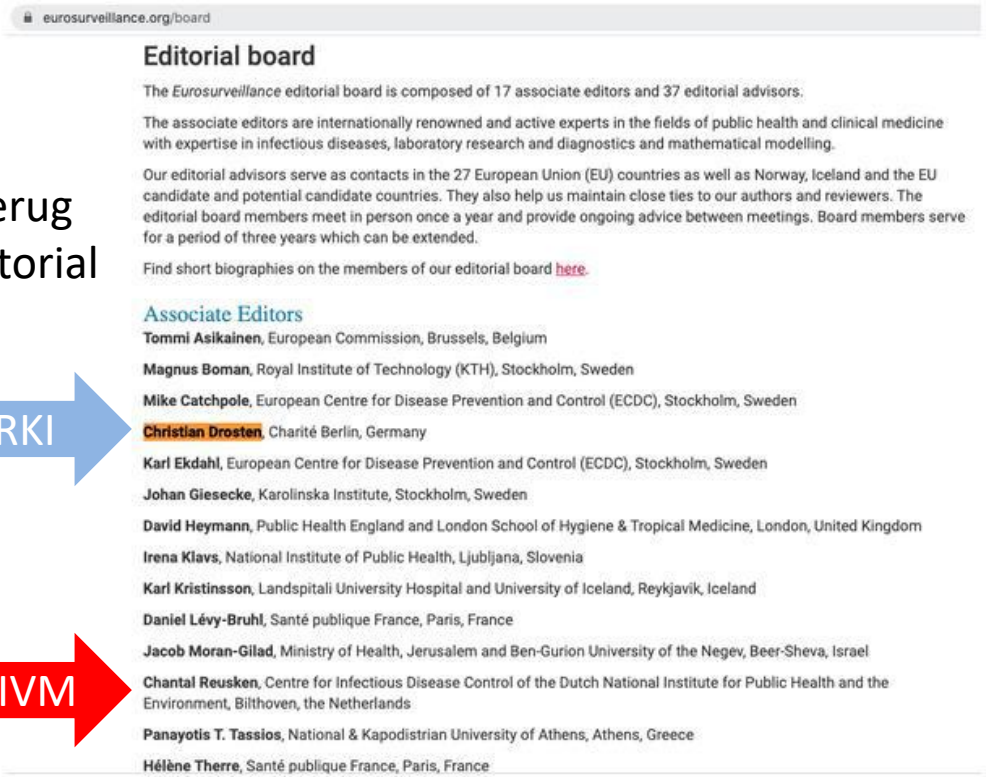
Het RKI en RIVM vinden we terug bij zowel auteurs als in de editorial board.



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20-4957: peer-review rapport request

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With reference to the above dates, please note that ECDC is closed for holidays on 02/11/2020.

Yours faithfully,

ECDC Access to Documents team

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