25 november 2020

Onjuist testbeleid ondersteunt de volksgezondheid niet, de weg naar proportionaliteit

Onder de motorkap van de (RT) PCR Tests

Waarom moet diagnosestelling terug naar de eerstelijnszorg? Juiste diagnosestelling is de basis van medische wetenschap. Zijn we niet zeker, dan zijn we vrij voor een second opinion te opteren, alvorens we een beslissing nemen voor een ingreep of medicatie. We willen immers niet dat er een vergissing wordt gemaakt en we chronisch van de zorg afhankelijk worden. Dit heeft alles te maken met 'veilig voelen' en 'vertrouwen in wetenschap en artsenij'. De patiënt staat centraal en wij als individu nemen uiteindelijk de eindbeslissing. Dat heet zelfbeschikking.
Dit hele diagnose proces hebben we in deze crisis even aan de kant gezet. Sterker nog, we hebben de diagnose overgeleverd aan een test, waarvoor deze niet bedoeld is; De (RT) PCR test. Deze test is ontwikkeld om van iets kleins iets groots te maken. Binnen marges van vergroting geeft het ons informatie, daarbuiten niet. De test heeft echter ineens het label diagnose gekregen en wordt vrij vertaald naar woorden als 'besmet' of 'besmettelijk. Dat kan helemaal niet. De test kan best van toegevoegde waarde zijn, maar dan moet hij wel goed gepositioneerd worden.

Wat komt aan bod: We gaan in ons schrijven dieper in op de (RT) PCR test en waar deze aan moet voldoen, onder de motorkap dus, maar het worstcasescenario komt ook aan bod. In dat scenario zitten we bijna gevangen, indien we de ingezette koers niet wijzigen. Termen als ontwerp van de test en standard operating procedure (**SOP**) zullen nader worden toegelicht en we zullen Het Volkswagen schandaal als passend metafoor gebruiken. Tot slot doen we een aanbeveling die aansluit bij het alternatief van het artsen collectief covid19 bestaande uit hoogleraren, medisch specialisten en huisartsen.

Hoe kunnen we ons herpakken? De diagnostisering moet traditioneel weer naar de eerstelijnszorg worden teruggebracht, te beginnen bij de huisarts. Na de eerste fysieke controle kan de huisarts samen met de patiënt besluiten tot het (preventief) voorschrijven van voedingssupplementen en/of medicatie en/of doen van aanvullende checks, tests en controles om de diagnose te bevestigen en/of door te verwijzen etc. In het griepseizoen

wordt er op meerdere virussen getest. De eerstelijnszorg zou dus nu ook de diagnose moeten stellen, waarna de (RT) PCR test ter bevestiging zou kunnen plaatsvinden. Dit soort tests moeten echter wel **alleen bij echt zieken**, bij ouderen en bij kwetsbaren met COVID-19 symptomen, op uniforme wijze worden uitgevoerd. Hierbij moeten we zowel kwalitatieve als kwantitatieve criteria van de tests onder controle hebben, anders baseren we ons op nutteloze informatie. Dat is de basis van waaruit de test ons kan helpen om overzicht te krijgen en rust terug te brengen in de samenleving. Hiermee nemen we afscheid van massa - "test, trace, isolate and treat", - van 'snotneuzen in quarantaine' - van gezonde mensen uitsluiten en – van ouderen opsluiten.

Ontwerp en standard operating procedure: Vanuit de observatie, hoe de testen nu verlopen en in welke context de resultaten worden geplaatst, is het niet meer dan normaal, het ontwerp van de (RT) PCR test, de peer-review, het peer-review proces en de standard operating procedure (SOP) voor testlocaties en laboratoria aan stringente controle te onderwerpen. Op dit moment blijken echter meer dan 8 fouten in het (RT) PCR test ontwerp te zitten en is er geen goede SOP, met referte naar het onderzoek van moleculair geneticus Dr. Peter Borger. We gaan hier verder op in.

Waar moet een (RT) PCR test aan voldoen?

1. De primers:
   - **A**: De primers moeten specifiek zijn voor het target (=het gen dat je wil amplificeren/vergroten)
   - **B**: GC-gehalte van de primers (min 40% max 60%) zegt iets over de verschillende onderdelen welke van het gen (genoom) van het virus gedetecteerd worden en wanneer deze loslaat;
   - **C**: De concentratie van de primers (100-200nM) zegt iets over de verbinding. Indien te hoog geconcen treerd is er gerede kans op verkeerde verbinding of verbinding met andere deeltje (virussen);
   - **D**: minimaal 3 gen testen (waarbij de gedeelte het liefst zo ver mogelijk uit elkaar liggen) Het liefst 6 primers voor verschillende gedeelte van het virus, zodat we vanuit meerdere punten bevestigen dat het virus heel is en we zeker weten dat we het over het juiste RNA virus hebben.
2. De temperatuur waarbij alle reacties plaatsvinden
   - **A**: DNA smelt bij temp > 92° C
   - **B**: Tm De annealing temperature: de T waarbij de primers aan het target binden/loslaten. Per primer paar mag het niet meer dan 2 graden verschillen. (De forward en de reverse primer).
3. **Het aantal amplificatie cycli** (vergrotingscycli): *Tussen de 25 – 28 geeft de test betrouwbare informatie. Daarboven is het lezen van de curve belangrijk. Tussen de 30 en 35 is de kans op een fout positief resultaat al substantieel en boven de 35 zeggen de resultaten niets meer en spreek je per definitie over fout positieve testuitslagen;*
4. **Controles** moeten uitgevoerd worden op positieve en negatieve resultaten

Zeer belangrijk: Er moet een "standard operating procedure"(SOP) zijn, die al deze aspecten vast heeft gelegd en is getest voor laboratoria. Dr. Peter Borger stelt vast dat dat niet het geval is.

De toelichting op het ontwerp van de (RT) PCR test vindt u **tot min 16:50** *in een online college van Dr. Peter Borger. Hij gaat in op de voorwaarden van een (RT) PCR test. Aan bod komen respectievelijk het Eurosurveillance, het EU-orgaan waar de (RT) PCR test paper voor peer-review is ingediend, de ontwerp criteria, en standard operating procedure.*
https://youtu.be/ajVS9kKoFQY of https://youtu.be/6DW76e10r2I

Het is cruciaal voor de betrouwbaarheid van de uitkomst van de (RT) PCR test, dat deze voldoet aan boven-staande criteria en dat deze test gepositioneerd wordt in de traditionele diagnosestelling van de eerstelijnszorg voordat we de resultaten vertalen naar beleid. Deze criteria bieden leveranciers kaders om aan de kwali-teitseisen en bandbreedten te voldoen. Een goede SOP stelt testomgevingen en laboratoria instaat binnen de kaders te testen en bij te dragen aan het aanleveren van bruikbare informatie.

Op alle fronten zijn er ontwerpfouten van de (RT) PCR test en SOP (meer dan 8). En er zijn valide vragen te stellen over het peer-review proces.

Hieronder een opsomming van de ontwerpfouten:

1. Variabele Primer Concentraties moet tussen 100 en max 200 liggen. De primer concentraties liggen veel te hoog op 400, 600 en 800 en deze zijn variabel. Hierdoor tast men in het duister over welke verbindingen in de test ontstaan en wat er wordt vergroot.

2. Aspecifieke ook wel Wobbly- Primers in het ontwerp heeft tot gevolg dat er verkeerde primers aange leverd worden. Dit veroorzaakt grote variabiliteit in test methode. De test detecteert niet alleen het Covid19 Gen maar ook ander Gen. Er is geen uniformiteit en specificiteit

3 t/m 5. GC-Gehalte van de primers is te laag. Met respectievelijk 28% en 34,6% liggen deze ruim onder de bandbreedte van de 40 en 60%. Door een te laag GC-gehalte laat de verbinding eerder los.

6. Annealing™ Primerpaar RdRP Gen forward- en reverse temperatuurverschillen van maar liefst 10 graden in plaats van het criterium binnen 2 graden verschil (94-96° C). De tijd dat de primers blijven zitten wijken af. Men kan Aspecifieke producten verwachten.

7. Primer target dekt het virus niet af. Meer dan de helft van het Gen van het virus wordt niet gedetec teerd. Een ½ virus is geen virus. De vraag blijft bestaan of de volledige sequentie aanwezig is; er sprake is van een heel virus. Het is volstrekt onvoldoende dat in Nederland de meeste laboratoria op 1 target wordt getest.

8. 45 amplificatie Cycli geeft alleen onbruikbare informatie dus geeft alleen fout positieven (zie **voorwaarde 3**).

Waar deze fouten toe leiden wordt in de analyse van Dr P. Borger ook belicht, namelijk:

- De (RT) PCR test is aspecifieke opgezet! De test detecteert niet alleen het Covid GEN;
- Aspecifieke amplification. Het aantal aanbevolen amplificatie cycli van 45 leidt tot onbruikbare informatie = fout-positieven resultaten; Het vergroot virusdeeltjes waar je niet naar op zoek bent;
- Is daar bewijs voor? De (RT) PCR test blijkt compleet Aspecifiek opgezet. Dit wordt notabene in het Paper bevestigd, dat bij Eurosurveillance ingediend is voor peer review: “This would theoretically, ensure broad sensitivity even in case of multiple independent acquisitions of variant viruses from animal reservoir”.
- *Ten aanzien van de peer review van Het bij Eurosurveillance ingediende (RT) PCR-paper.* Deze werd op 21 januari 2020 ingediend en was binnen 24 uur al peer-reviewed, wat praktisch onmogelijk is. Conclusie?! De Corman – Paper werd niet peer-reviewed!? De auteur (2) blijkt ook editor (het eigen stuk te peer reviewen?!)

<u>De toelichting</u> ten aanzien van de ontwerpfouten vindt u ***<u>vanaf minuut 16:50 tot minuut 31:15</u>*** *in het online college van Dr. Peter Borger en in de daarbij behorende PowerPoint vindt u in de bijlage. Dr. Borger is molecuair geneticus met specifieke ervaring op het gebied van longaandoeningen en (RT) PCR test heeft frequent onderzoeken peer-reviewed. Zijn bevindingen spreken voor zich waarbij niets uitgesloten wordt.*

Is de (RT) PCR test vergelijkbaar met het Volkswagen schandaal!? Dat is een vraag waar we u meer gevoel bij gaan geven. Dat ons zicht wordt vertroebeld door de technische discussie over een discutabele test is een understatement. Maar we lijken wel gek dat we met zijn alle de PCR test willen begrijpen en kolossaal willen promoveren. De ellelange discussie houdt ons aan het lijntje en ondertussen dendert de trein met overheids-maatregelen door. We hoeven het eigenlijk helemaal niet meer te hebben over de complexiteit van de (RT) PCR test, respectievelijk de fouten in het ontwerp en de standard operating procedure. In dit geval begint en eindigt het voor ons, als voormalig leken, voordat zo’n PCR test überhaupt op de markt komt. We zouden moeten kunnen vertrouwen op de kwaliteitscontrole van het ingediende Paper bij Euroserveillance en de peer-review. Het is namelijk logisch dat een niet kloppende test onbetrouwbare informatie oplevert en indien deze informatie in de verkeerde context wordt geplaatst en serieus wordt genomen om beleid op te baseren, het beleid aan alle kanten rammelt en dus de samenleving en bedrijfsleven wordt gemangeld.

Dat is nu exact de reden waarom we het Volkswagen schandaal ter illustratie gebruiken. U herinnert zich het

Volkswagen emissie schandaal, dat in 2015 in de VS werd ontdekt. Het nieuwe model kwam door de keuring, door aan de knoppen te draaien van het ontwerp, beter bekend als sjoemelsoftware. Zo bleef de uitstoot van stikstofoxiden (NOx) in de test onder een bepaalde grenswaarde, waardoor Volkswagen toestemming kreeg om het model als 'clean diesel' te verkopen. NOx is zeer schadelijk voor milieu en gezondheid. Toen dit aan het licht kwam moest het Volkswagen concern uiteindelijk in Europa alleen al 8,4 miljoen wagens terughalen en in de VS zo'n elf miljoen en betaalde het concern bijna 20 miljard aan boetes en schikkingen in de VS en Europa. Iedereen kan zich hierbij voorstellen dat je met je VW, Audi, Skoda, Porsche of Seat, teruggaat naar de dealer. Een onderzoekscommissie van het Europees Parlement concludeerde uiteindelijk dat de Europese Commissie nalatig is geweest door jarenlang geen actie te ondernemen en dit terwijl er sinds 2010 meerdere aanwijzingen voor waren. Vier jaar na 'dieselgate is het er echter niet beter op geworden en de politiek laat de fraudeurs er toch mee wegkomen.

Als we dan onder de motorkap van de (RT) PCR test kijken zijn fouten in het ontwerp voor een microbioloog gemakkelijk te detecteren. Er blijkt aan de knoppen van het ontwerp (de software) van de (RT) PCR test gedraaid te zijn, waardoor deze niet betrouwbaar is. De test is met alle ontwerpfouten op de markt geslingerd. Zelfs een willekeurige ondernemer kan op dit moment een teststraat starten. Lang leve de volksgezondheid en aan mijn lijf geen polonaise is dan een logische reactie!

U mag zelf oordelen of wetenschappers onderdeel van de bouw en het proces van de (RT) PCR test mogen zijn; of ze auteur mogen zijn van het bij Eurosurveillance ingediende Paper en tegelijkertijd editor van de peer-review; en ze tegelijkertijd ook nog verbonden mogen zijn aan instituten als het RIVM, of daar eerder onderdeel van hebben uitgemaakt; het adviesorgaan voor overheid en het Ministerie van VWS etc. etc. Dit alleen verplicht ons al tot het stellen van vragen over de peer-review, die binnen 24 uur na acceptatie van de Paper bevestigd was, waar normaal weken aan ten grondslag liggen. Naast de constateringen door Dr. Peter Borger zou een onderzoekscommissie van het Europees Parlement tot dezelfde conclusie moeten komen ten aanzien van nalatigheid van de Europese Commissie door 10-tallen jaren geen actie te ondernemen tegen wetenschappelijke 'sjoemelpeer-reviews' ten behoeve van de wetenschap en farmacie. Dit dient hard aangepakt te worden en te worden herijkt. Wat heel normaal is in de automobielindustrie om auto's terug te nemen naar fabriek, zou om dezelfde reden weer heel normaal moeten worden met ontwerpen in de farmacie. De tests moeten dus uit de markt worden genomen. *Een bron van kopzorgen voor ons allen en de volksgezondheid.* We kunnen niet met onze gezondheid en veiligheid spelen, zoals men dat ook al met de Mexicaanse griep in 2009 op grote schaal trachtte te doen. 11 jaar na de 'griep gate' is er net als na de 'dieselgate' niets veranderd en "tuinen we er wel allemaal in". *Met deze (RT) PCR test weet u helemaal niet waar u aan toe bent; de test zegt niets over uw gezondheid; u gaat niet naar start! Maar u gaat direct en onterecht 10 dagen in quarantaine. Het testbeleid zet de zorg op het verkeerde been en daarmee het beleid en de samenleving.*

'Never ending circle': Op het moment dat we zo'n testontwerp toestaan, welke totaal niet aan de criteria voldoet, en we vervolgens onbruikbare resultaten vertalen naar de cockpit van Ministerie van VWS, kunnen we in een 'never-ending-circle' van maatregelen terecht komen". Zelfs als we kern gezond zijn geeft deze test namelijk fout positieve resultaten. Ten aanzien van beleid geldt dus "*garbage in = garbage out*", zoals dat ook voor een managementinformatiesysteem geldt. Op deze manier worden we continu op het verkeerde been gezet en ontstaat een maatschappij waar burger en economie continu aan het infuus liggen. Een samenleving die in continue waakzaamheid verkeert en door interventie gekarakteriseerd wordt; waar lichamelijke, psychische en sociale 'ziekten' chronisch zijn. Een samenleving waarbij de continue inbreuk op zelfbeschikking en persoonlijke integriteit kenmerkend is.

De economische schade neemt inmiddels catastrofale vormen aan. Een domino-effect met toename van verdeeldheid onder de bevolking. Ook de druk op de zorg nemen we op deze manier niet weg, waar de laatste 10 jaar continu op is bezuinigd. De afhankelijkheid naar de farmacie groeit en de democratie vervaagd. We

realiseren ons kennelijk niet, dat we met de acceptatie van de voorwaarden, onszelf de afgelopen 9 maanden beetje bij beetje weggeven hebben en daardoor in een test samenleving terecht komen. De farmacie en wetenschap moeten onmiddellijk aan een vernieuwde & stringente controle onderworpen worden en we moeten nieuwe normen stellen, waardoor de farmacie de samenleving definitief onbetwist ondersteunt. Na deze crisis zal de rekening richting partijen en personen opgemaakt moeten worden.

Aanbevelingen:
De (RT) PCR test en het peer-review proces zal met spoed herijkt moeten worden wil deze überhaupt relevantie hebben. De (RT) PCR test bevat grove ontwerpfouten en zet ons land mede op het slot. Gezien het feit dat de gevolgen voor de samenleving desastreus zijn als we testresultaten verkeerd blijven interpreteren als basis voor beleid, illustreert deze, verkorte weergegeven analyse, hoe belangrijk het is de diagnose terug te brengen naar de eerstelijnszorg, waarbij we onze zelfbeschikking en onze persoonlijke integriteit niet uit het oog verliezen. De eerstelijns diagnostisering kan door een test ondersteund worden, indien deze goed gepositioneerd wordt. De eerstelijnszorg moet deze verantwoordelijkheid ook wel weer actief terugpakken en gaan staan voor de eed die zij hebben afgelegd.

Gezonde mensen moeten in de regel niet getest worden, omdat we zo naar een testmaatschappij neigen, waarbij de overheid stuurt op basis van sociale in- en uitsluiting. Een maatschappij waarbij mondkapjes, (snel)-tests en vaccins exemplarisch zijn. Een maatschappij die op deze manier in een stuip schiet bij een virus met een IFR van 0,05 % tot 70 jaar en 0,23% totaal. We zouden voor Sars-Cov-2 alleen de oudere en kwetsbare proportioneel moeten beschermen. De cockpit van het Ministerie van VWS en het daaruit voortvloeiend beleid moet derhalve met urgentie proportioneel worden aangepast in crisistijd. We moeten er goed over na blijven denken welke golf en crisis we uiteindelijk aan het bestrijden zijn.

Voor nu moet de economie en de zorg intelligent ondersteund worden, zodat we weer echt samen kunnen leven, in plaats van dat we het bureaucratisch systeem voeden en kapitaal vernietigen. De samenleving moet versneld open conform het voorgesteld alternatief uit het artsen collectief. Een samenleving die uitgaat van levenskwaliteit, zelfbeschikking, persoonlijke integriteit, keuzevrijheid en proportionaliteit. Zo was Nederland ingericht en daar moeten we snel weer naar terug. Een samenleving, waarbij we met een gerust hart onder ‘de motorkap’ moeten kunnen kijken, ongeacht welke!

Met Hoogachting,
Een delegatie van het regenboogteam

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Though the whole world relies on RT-PCR to "diagnose" Sars-Cov-2 infection, the science is clear: they are not fit for purpose. https://mdvrdrmnblog.wpcomstaging.com/pcr/

- How to Mislead All Humanity. Using a "Test" To Lock Down Society By Dr. Pascal Sacré Global Research, November 05, 2020 https://www.globalresearch.ca/covid-19-rt-pcr-how-to-mislead-all-humanity-using-a-test-to-lock-down-society/5728483

- RT-PCR, the science is clear: they are not fit for purpose. https://off-guardian.org/2020/06/27/covid19-pcr-tests-are-scientifically-meaningless/

- The Truth about PCR Test Kit from the Inventor and Other Experts https://mdvr-drmnblog.wpcomstaging.com/wp-content/uploads/2020/09/Coronavirus-The-Truth-about-PCR-Test-Kit-from-the-Inventor-and-Other-Experts-Weblyf.pdf https://bestarticle.net/the-truth-about-pcr-test-kit-from-the-inventor-and-other-experts-6581.html

- BBC news geen directe link - interview Professor Allyson Pollock in het overzicht– tests have not been properly evaluated https://mdvrdrmnblog.wpcomstaging.com/pcr/

- Your Coronavirus Test Is Positive. Maybe It Shouldn't Be. https://www.nytimes.com/2020/08/29/health/coronavirus-testing.html

- Up to 90% Who've Tested COVID-Positive Wrongly Diagnosed NY Times https://redstate.com/michael_thau/2020/09/03/ny-times-up-to-90-of-people-who-tested-positive-for-c19-not-infected-truth-a-whole-lot-worse-pt-3-n253332 https://mdvrdrmnblog.wpcomstaging.com/wp-content/uploads/2020/10/Up-to-90-Whove-Tested-COVID-Positive-Wrongly-Diagnosed.pdf

- False positives in reverse transcription PCR testing for SARS-CoV-2 https://www.medrxiv.org/content/10.1101/2020.04.26.20080911v1.full.pdf https://mdvrdrmnblog.wpcomstaging.com/wp-content/uploads/2020/10/False-positive-COVID19-results-hidden-problems-and-costs.pdf

- Mass PCR testing of the healthy and asymptomatic is counter-productive https://rationalground.com/why-mass-pcr-testing-of-the-healthy-and-asymptomatic-is-currently-counter-productive/ The Lancet: False-positive COVID-19 results: hidden problems and costs https://www.thelancet.com/action/showPdf?pii=S2213-2600%2820%2930453-7

- Are you infectious if you have a positive PCR test result for COVID-19? https://www.cebm.net/covid-19/infectious-positive-pcr-test-result-covid-19/

- PCR – how the positive predictive value is influenced by disease prevalence https://youtu.be/QqgJHryKOSU

- Dr Andrew Kaufman April 17, 2020 (Why PCR test is useless) https://youtu.be/ue-2BxapzM8
- Londoreal.tv Dr. Andrew Kaufman Unmasking the Covid19 lies https://mdvrdrmnblog.wpcomstaging.com/wp-content/uploads/2020/07/David-Crow-Coronavirus-Panic.pdf
- David Crowe Flaws in Coronavirus Pandemic Theory https://mdvrdrmnblog.wpcomstaging.com/wp-content/uploads/2020/07/David-Crow-Coronavirus-Panic.pdf
- Dr. med Claus Köhnlein PCA-based test, where false positives are programmed in. https://player.vimeo.com/video/404203138
- Coronavirus: The Truth about PCR Test Kit https://bestarticle.net/the-truth-about-pcr-test-kit-from-the-inventor-and-other-experts-6581.html
- The Problem to determine SARS-CoV-2 infections using PCR Tests https://vimeo.com/443416775
- Was the COVID-19 Test Meant to Detect a Virus? https://mdvrdrmnblog.wpcomstaging.com/wp-content/uploads/2020/08/Was-the-COVID-19-Test-Meant-to-Detect-a-Virus-UncoverDC.pdf
- Recovered patients: detection specimens for up to 12 weeks. https://mdvrdrmnblog.wpcomstaging.com/wp-content/uploads/2020/07/recovered-patients-can-continue-to-have-SARS-CoV-2-RNA-detected.pdf
- Test do not rule out bacterial infection or co-infection https://mdvrdrmnblog.wpcomstaging.com/wp-content/uploads/2020/07/CDC-2019-Novel-Coronavirus-Real-Time-RT-PCR.pdf
- COVID diagnostic test: worst test ever devised? https://mdvrdrmnblog.wpcomstaging.com/wp-content/uploads/2020/09/COVID-diagnostic-test-worst-test-ever-devised-Jon-Rappoports-Blog.pdf https://blog.nomorefakenews.com/2020/09/10/covid-diagnostic-test-worst-test-ever-devised/
- In search of the SARS-COV-2 identification records FOI response from Canada's National Research Council: No record of "COVID-19 virus" (SARS-COV-2) isolation by anyone, anywhere on the planet. (I FOI'd them b/c Health Canada said they "might" have such a record lol.) https://twitter.com/Christi45657364/status/1283126240254341121?ref_src=twsrc%5Etfw%7Ctwcamp%5Etweetembed%7Ctwterm%5E1283126240254341121%7Ctwgr%5E&ref_url=https%3A%2F%2Fmdvrdrmnblog.wpcomstaging.com%2Fpcr%2F
- Exosome versus Virus https://bit.ly/2A25TbA https://youtu.be/V-dNs3lljrY
- Exosomes explained https://www.britannica.com/science/exosome
- COVID19 -Centers for disease control and Prevention https://www.fda.gov/media/134922/download https://www.cdc.gov/coronavirus/2019-ncov/testing/diagnostic-testing.html
- CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel https://mdvrdrmnblog.wpcomstaging.com/wp-content/uploads/2020/08/CDC-2019-Novel-Coronavirus-2019-nCoV-Real-Time-RT-PCR-Diagnostic-Panel.pdf
- Test for Past Infection (Antibody Test) https://mdvrdrmnblog.wpcomstaging.com/wp-content/uploads/2020/07/Test-for-Past-Infection-Antibody-Test-CDC.pdf
- Viral Test https://www.cdc.gov/coronavirus/2019-ncov/testing/diagnostic-testing.html
- Guidance on interpreting COVID-19 test results Protocol https://www.who.int/docs/default-source/coronaviruse/real-time-rt-pcr-assays-for-the-detection-of-sars-cov-2-institut-pasteur-paris.pdf?sfvrsn=3662fcb6_2
- BOMBSHELL: WHO Coronavirus PCR Test Primer Sequence is Found in All Human DNA https://pieceofmindful.com/2020/04/06/bombshell-who-coronavirus-pcr-test-primer-sequence-is-found-in-all-human-dna/
- https://pieceofmindful.com/2020/04/06/bombshell-who-coronavirus-pcr-test-primer-sequence-is-found-in-all-human-dna/

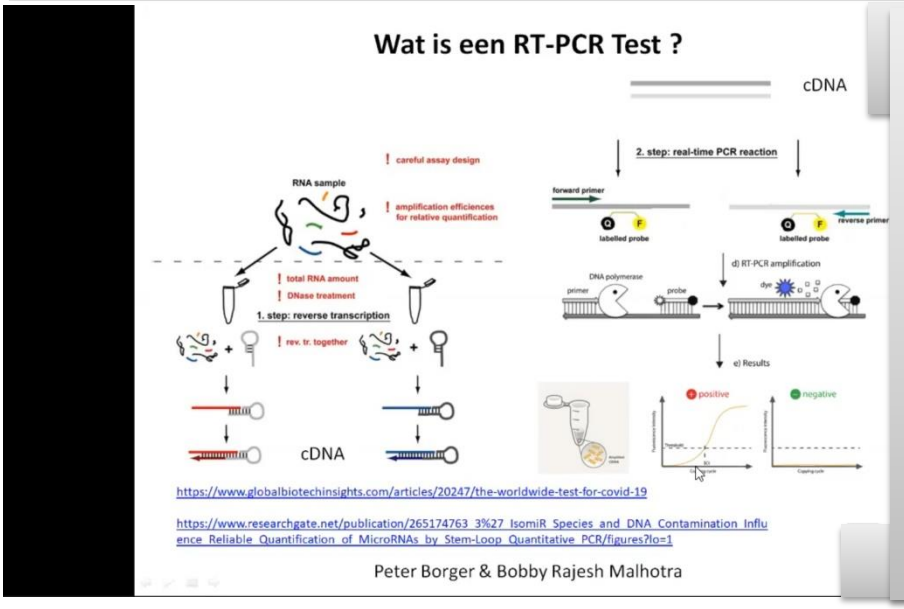
Bijlage 1

Onder de motorkap van de RT PCR Test

8+ fouten in het Ontwerp van RT PCR Test en het belang van een SOP

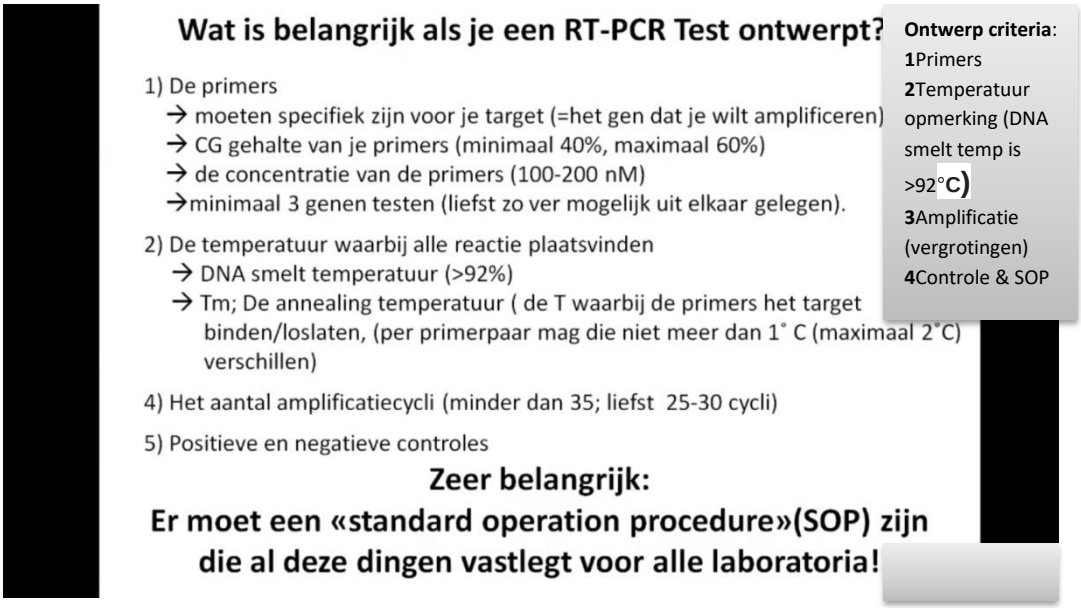
(RT PCR Test Analyse (Dr Peter Borger)

https://youtu.be/6DW76e10r2ICR



Wat doet de PCR Test.
1 en 2 links, 3 en 4 rechts
1- RNA Sample (slijm in buisje)
2- RNA naar cDNA,
3- Primers toevoegen,
4-Amplificatie (vergroten)

Kwaliteit ontwerp bepaald betrouwbaarheid test



Ontwerp criteria:
1Primers
2Temperatuur opmerking (DNA smelt temp is >92°C)
3Amplificatie (vergrotingen)
4Controle & SOP

Ontwerpfout 1: Variabele Primer concentraties

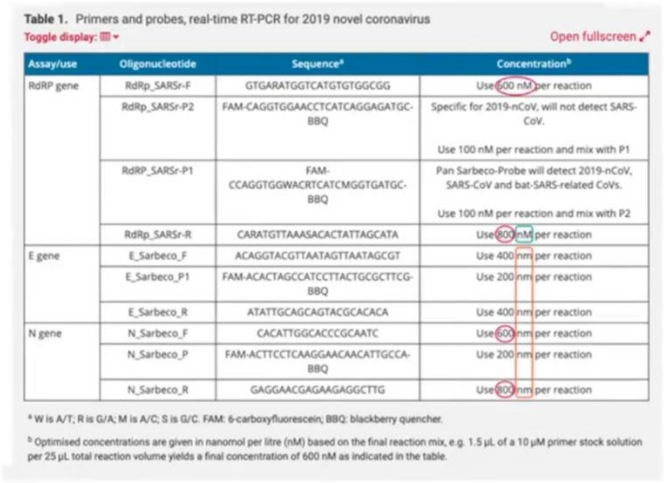


Table 1. Primers and probes, real-time RT-PCR for 2019 novel coronavirus

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 600 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 600 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.

Primer concentraties variabel en veel te hoog.

Hierdoor tast men in het duister over welke verbindingen zijn ontstaan en wat is vergroot.

= grove ontwerp fout!

Ontwerpfout 2: Aspecifieke («Wobbly») Primers

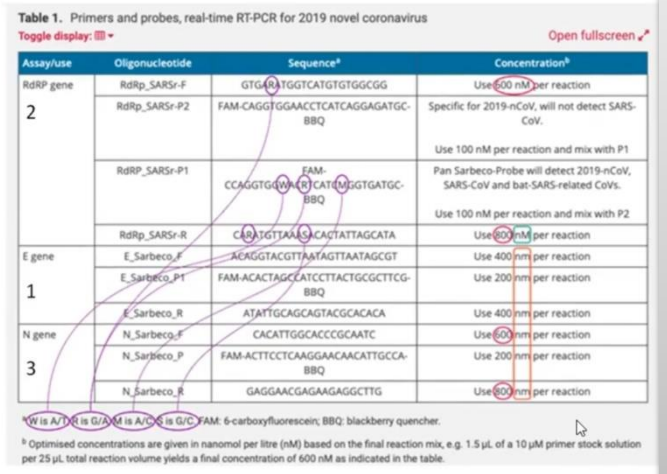


Table 1. Primers and probes, real-time RT-PCR for 2019 novel coronavirus

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 600 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 600 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.

Primers Aspecifiek. Ontwerp geeft geen specifieke primer aan.

Gevolgen: er worden verkeerde primers aangeleverd/ gemaakt. Veroorzaakt grote variabiliteit in test methode. De test detecteert niet alleen het Covid GEN In de test.

<u>Geen uniformiteit en specificiteit</u>

Ontwerpfout 3-5: GC-gehalte van de Primers

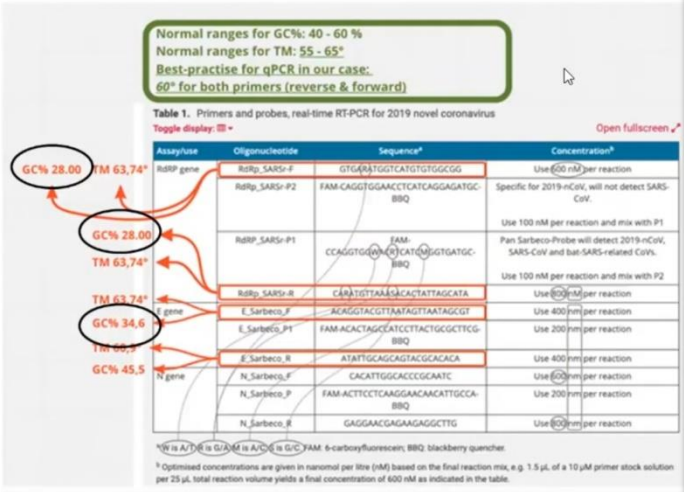


Table 1. Primers and probes, real-time RT-PCR for 2019 novel coronavirus

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	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 600 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 600 nm per reaction

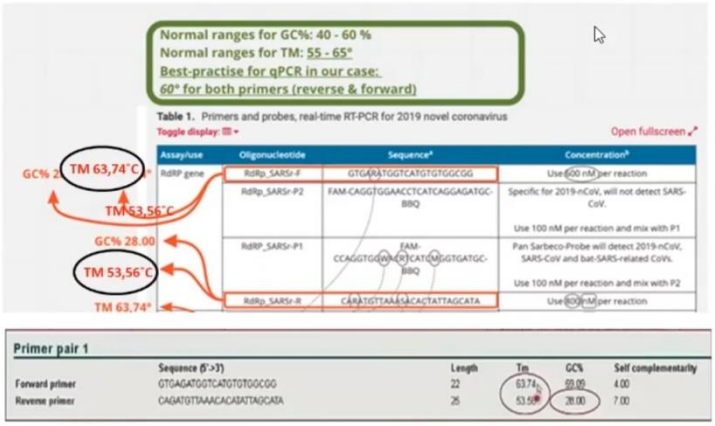
[a] W is A/T; R is G/A; M is A/C; S is G/C. FAM: 6-carboxyfluorescein; BBQ: blackberry quencher.

[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.

GC gehalte wijkt af.

Te laag waardoor verbinding eerder loslaat.

Ontwerpfout 6: Annealing (Tm) Primerpaar RdRp Gen



Groot temperatuur Verschil.

Gevolgen:

Tijd dat de primers blijven zitten wijken af.

Men kan Aspecifieke producten verwachten.

Ontwerpfout 7: Grootste deel van virus niet gedekt

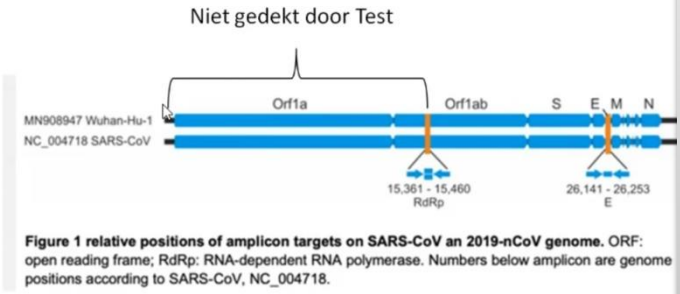


Figure 1 relative positions of amplicon targets on SARS-CoV an 2019-nCoV genome. ORF: open reading frame; RdRp: RNA-dependent RNA polymerase. Numbers below amplicon are genome positions according to SARS-CoV, NC_004718.

Een ½ virus is geen virus.

Meer dan de helft van het Gen van het virus wordt niet gedetecteerd -Primer target dekt virus niet af. Vraag blijft of de volledige sequentie aanwezig is. Er zijn meer primers nodig om fout positief te voorkomen.
Conclusie: Info uit test waardeloos

Ontwerpfout 8: 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"
58°C 30" | 45x

Aanbevolen amplificatie Cycli te hoog.

Afkap waarde te hoog. Deeltjes worden vergroot die geen waarde hebben. Er kan geen waarde aan toegekend worden.

Wat bewerken deze ontwerpfouten? Aspecieke amplificat

→ 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.

Veel Aspecifieke uitslagen als gevolg.

Dit wordt door ontwerpers in het Paper zelf bevestigd.

Compleet aspecifiek opgezet?

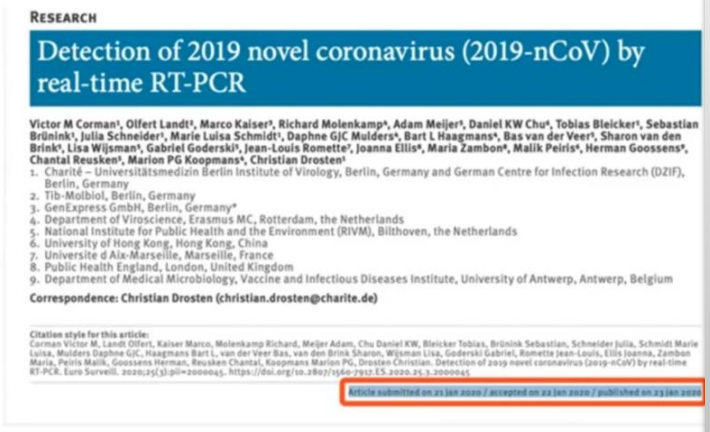
"To show that the assays can detect other bat-associated SARS-related viruses, we used the E gene assay to test six bat-derived faecal samples available from Drexler et al. [13] und Muth et al. [14]. These virus-positive samples stemmed from European rhinolophid bats. Detection of these phylogenetic 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

Test Compleet Aspecifiek opgezet. Je detecteert ook andere Corona virus. Corona is een vleermuisvirus (komt 96% overeen met Sars Cov1)

"All Asian virusus are likely to be detected" uit paper ontwerpers

De Corman-paper werd niet gepeer-reviewed?!



RESEARCH

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

Victor M Corman[1], Olfert Landt[2], Marco Kaiser[3], Richard Molenkamp[4], Adam Meijer[5], Daniel KW Chu[6], Tobias Bleicker[1], Sebastian Brünink[1], Julia Schneider[1], Marie Luisa Schmidt[1], Daphne GJC Mulders[4], Bart L Haagmans[4], Bas van der Veer[5], Sharon van den Brink[5], Lisa Wijsman[5], Gabriel Goderski[3], Jean-Louis Romette[7], Joanna Ellis[8], Maria Zambon[8], Malik Peiris[6], Herman Goossens[9], Chantal Reusken[5], Marion PG Koopmans[4], Christian Drosten[1]

1. Charité – Universitätsmedizin Berlin Institute of Virology, Berlin, Germany and German Centre for Infection Research (DZIF), Berlin, Germany
2. Tib-Molbiol, Berlin, Germany
3. GenExpress GmbH, Berlin, Germany*
4. Department of Viroscience, Erasmus MC, Rotterdam, the Netherlands
5. National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
6. University of Hong Kong, Hong Kong, China
7. Universite d Aix-Marseille, Marseille, France
8. Public Health England, London, United Kingdom
9. Department of Medical Microbiology, Vaccine and Infectious Diseases Institute, University of Antwerp, Antwerp, Belgium

Correspondence: Christian Drosten (christian.drosten@charite.de)

Citation style for this article:
Corman Victor M, Landt Olfert, Kaiser Marco, Molenkamp Richard, Meijer Adam, Chu Daniel KW, Bleicker Tobias, Brünink Sebastian, Schneider Julia, Schmidt Marie Luisa, Mulders Daphne GJC, Haagmans Bart L, van der Veer Bas, van den Brink Sharon, Wijsman Lisa, Goderski Gabriel, Romette Jean-Louis, Ellis Joanna, Zambon Maria, Peiris Malik, Goossens Herman, Reusken Chantal, Koopmans Marion PG, Drosten Christian. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020;25(3):pii=2000045. https://doi.org/10.2807/1560-7917.ES.2020.25.3.2000045

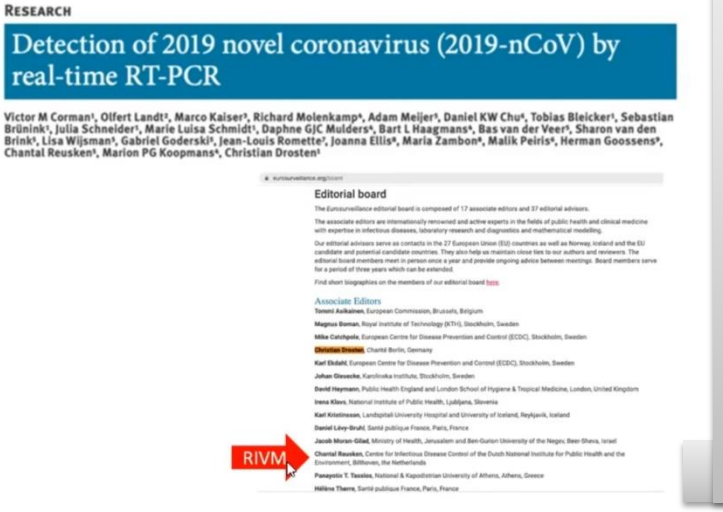
Article submitted on 21 Jan 2020 / accepted on 22 Jan 2020 / published on 23 Jan 2020

21 januari als test ingestuurd

22 januari geaccepteerd.

Binnen 24 uur + onmogelijk Voor Peer Review staat minimaal 2 weken!

Laatste auteur (Drosten, RKI) = Editor van Eurosurveillan



RESEARCH

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RIVM

Is de test verder gekomen dan het Editorial Office? Peer review kan gezien het aantal fouten nooit plaats gevonden hebben. Voor een Moleculair Geneticus te eenvoudig! Dat kan bijna niet. **Auteur zit in Editorial board??**

Peer review kan niet hebben plaatsgevonden

'Hoe testen we ons de Lock Down in'

8+ fouten in het Ontwerp van RT PCR Test en het belang van een SOP

(RT PCR Test Analyse (Dr Peter Borger)

https://youtu.be/6DW76e10r2ICR

Research

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

Victor M Corman[1], Olfert Landt[2], Marco Kaiser[3], Richard Molenkamp[4], Adam Meijer[5], Daniel KW Chu[6], Tobias Bleicker[1], Sebastian Brünink[1], Julia Schneider[1], Marie Luisa Schmidt[1], Daphne GJC Mulders[4], Bart L Haagmans[4], Bas van der Veer[5], Sharon van den Brink[5], Lisa Wijsman[5], Gabriel Goderski[5], Jean-Louis Romette[7], Joanna Ellis[8], Maria Zambon[8], Malik Peiris[6], Herman Goossens[9], Chantal Reusken[5], Marion PG Koopmans[4], Christian Drosten[1]

1. Charité – Universitätsmedizin Berlin Institute of Virology, Berlin, Germany and German Centre for Infection Research (DZIF), Berlin, Germany
2. Tib-Molbiol, Berlin, Germany
3. GenExpress GmbH, Berlin, Germany*
4. Department of Viroscience, Erasmus MC, Rotterdam, the Netherlands
5. National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
6. University of Hong Kong, Hong Kong, China
7. Universite d Aix-Marseille, Marseille, France
8. Public Health England, London, United Kingdom
9. Department of Medical Microbiology, Vaccine and Infectious Diseases Institute, University of Antwerp, Antwerp, Belgium

Correspondence: Christian Drosten (christian.drosten@charite.de)

Citation style for this article:
Corman Victor M, Landt Olfert, Kaiser Marco, Molenkamp Richard, Meijer Adam, Chu Daniel KW, Bleicker Tobias, Brünink Sebastian, Schneider Julia, Schmidt Marie Luisa, Mulders Daphne GJC, Haagmans Bart L, van der Veer Bas, van den Brink Sharon, Wijsman Lisa, Goderski Gabriel, Romette Jean-Louis, Ellis Joanna, Zambon Maria, Peiris Malik, Goossens Herman, Reusken Chantal, Koopmans Marion PG, Drosten Christian. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020;25(3):pii=2000045. https://doi.org/10.2807/1560-7917.ES.2020.25.3.2000045

Article submitted on 21 Jan 2020 / accepted on 22 Jan 2020 / published on 23 Jan 2020

Background: The ongoing outbreak of the recently emerged novel coronavirus (2019-nCoV) poses a challenge for public health laboratories as virus isolates are unavailable while there is growing evidence that the outbreak is more widespread than initially thought, and international spread through travellers does already occur. **Aim:** We aimed to develop and deploy robust diagnostic methodology for use in public health laboratory settings without having virus material available. **Methods:** Here we present a validated diagnostic workflow for 2019-nCoV, its design relying on close genetic relatedness of 2019-nCoV with SARS coronavirus, making use of synthetic nucleic acid technology. **Results:** The workflow reliably detects 2019-nCoV, and further discriminates 2019-nCoV from SARS-CoV. Through coordination between academic and public laboratories, we confirmed assay exclusivity based on 297 original clinical specimens containing a full spectrum of human respiratory viruses. Control material is made available through European Virus Archive – Global (EVAg), a European Union infrastructure project. **Conclusion:** The present study demonstrates the enormous response capacity achieved through coordination of academic and public laboratories in national and European research networks.

Introduction

According to the World Health Organization (WHO), the WHO China Country Office was informed of cases of pneumonia of unknown aetiology in Wuhan City, Hubei Province, on 31 December 2019 [1]. A novel coronavirus currently termed 2019-nCoV was officially announced as the causative agent by Chinese authorities on 7 January. A viral genome sequence was released for immediate public health support via the community online resource virological.org on 10 January (Wuhan-Hu-1, GenBank accession number MN908947 [2]), followed by four other genomes deposited on 12 January in the viral sequence database curated by the Global Initiative on Sharing All Influenza Data (GISAID). The genome sequences suggest presence of a virus closely related to the members of a viral species termed severe acute respiratory syndrome (SARS)-related CoV, a species defined by the agent of the 2002/03 outbreak of SARS in humans [3,4]. The species also comprises a large number of viruses mostly detected in rhinolophid bats in Asia and Europe.

As at 20 January 2020*, 282 laboratory-confirmed human cases have been notified to WHO [5]. Confirmed cases in travellers from Wuhan were announced on 13 and 17 January in Thailand as well as on 15 January in Japan and 19 January in Korea. The extent of human-to-human transmission of 2019-nCoV is unclear at the time of writing of this report but there is evidence of some human-to-human transmission.

Among the foremost priorities to facilitate public health interventions is reliable laboratory diagnosis. In acute respiratory infection, RT-PCR is routinely used to detect causative viruses from respiratory secretions. We have previously demonstrated the feasibility of introducing robust detection technology based on real-time RT-PCR in public health laboratories during international

Table 1

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

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.

health emergencies by coordination between public and academic laboratories [6-12]. In all of these situations, virus isolates were available as the primary substrate for establishing and controlling assays and assay performance.

In the present case of 2019-nCoV, virus isolates or samples from infected patients have so far not become available to the international public health community. We report here on the establishment and validation of a diagnostic workflow for 2019-nCoV screening and specific confirmation, designed in absence of available virus isolates or original patient specimens. Design and validation were enabled by the close genetic relatedness to the 2003 SARS-CoV, and aided by the use of synthetic nucleic acid technology.

Methods

Clinical samples and coronavirus cell culture supernatants for initial assay evaluation

Cell culture supernatants containing typed coronaviruses and other respiratory viruses were provided by Charité and University of Hong Kong research laboratories. Respiratory samples were obtained during 2019 from patients hospitalised at Charité medical centre and tested by the NxTAG respiratory pathogen panel (Luminex, S´Hertogenbosch, The Netherlands) or in cases of MERS-CoV by the MERS-CoV upE assay as published before [10]. Additional samples were selected from biobanks at the Rijksinstituut voor Volksgezondheid en Milieu (RIVM), Bilthoven, at Erasmus University Medical Center, Rotterdam, at Public Health England (PHE), London, and at the University of Hong Kong. Samples from all collections comprised sputum as well as nose and throat swabs with or without viral transport medium.

Faecal samples containing bat-derived SARS-related CoV samples (identified by GenBank accession numbers) were tested: KC633203, Betacoronavirus BtCoV/Rhi_eur/BB98–98/BGR/2008; KC633204, Betacoronavirus BtCoV/Rhi_eur/BB98–92/BGR/2008; KC633201, Betacoronavirus BtCoV/Rhi_bla/BB98–22/BGR/2008; GU190221 Betacoronavirus Bat coronavirus BR98–19/BGR/2008; GU190222 Betacoronavirus Bat coronavirus BM98–01/BGR/2008; GU190223, Betacoronavirus Bat coronavirus BM98–13/BGR/2008. All synthetic RNA used in this study was photometrically quantified.

RNA extraction

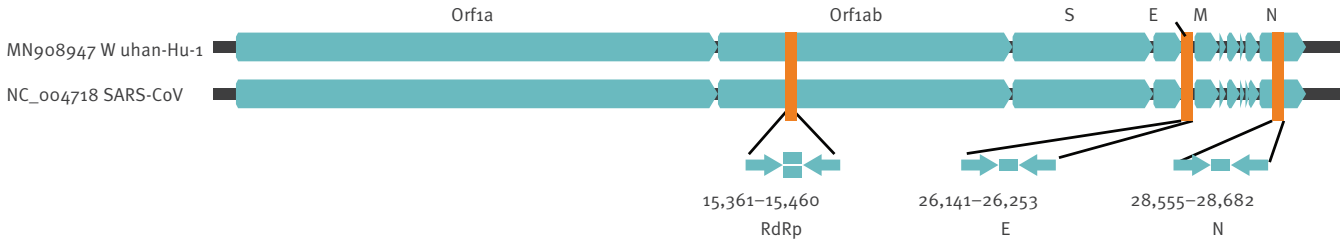
RNA was extracted from clinical samples with the MagNA Pure 96 system (Roche, Penzberg, Germany) and from cell culture supernatants with the viral RNA mini kit (QIAGEN, Hilden, Germany).

Real-time reverse-transcription PCR

A 25 µL reaction contained 5 µL of RNA, 12.5 µL of 2 × reaction buffer provided with the Superscript III one step RT-PCR system with Platinum Taq Polymerase (Invitrogen, Darmstadt, Germany; containing 0.4 mM of each deoxyribont triphosphates (dNTP) and 3.2 mM magnesium sulphate), 1 µL of reverse transcriptase/Taq mixture from the kit, 0.4 µL of a 50 mM magnesium sulphate solution (Invitrogen), and 1 µg of nonacetylated bovine serum albumin (Roche). Primer and probe sequences, as well as optimised concentrations are shown in Table 1. All oligonucleotides were synthesised and provided by Tib-Molbiol (Berlin,

Figure 1

Relative positions of amplicon targets on the SARS coronavirus and the 2019 novel coronavirus genome



E: envelope protein gene; M: membrane protein gene; N: nucleocapsid protein gene; ORF: open reading frame; RdRp: RNA-dependent RNA polymerase gene; S: spike protein gene.

Numbers below amplicons are genome positions according to SARS-CoV, GenBank NC_004718.

Germany). Thermal cycling was performed at 55 °C for 10 min for reverse transcription, followed by 95 °C for 3 min and then 45 cycles of 95 °C for 15 s, 58 °C for 30 s. Participating laboratories used either Roche Light Cycler 480II or Applied Biosystems ViiA7 instruments (Applied Biosystems, Hong Kong, China).

Protocol options and application notes

Laboratories participating in the evaluation used the TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher) with the same oligonucleotide concentrations and cycling conditions. The QIAGEN One-Step RT-PCR Kit was also tested and found to be compatible.

The intended cross-reactivity of all assays with viral RNA of SARS-CoV allows us to use the assays without having to rely on external sources of specific 2019-nCoV RNA.

For a routine workflow, we recommend the E gene assay as the first-line screening tool, followed by confirmatory testing with the RdRp gene assay. Application of the RdRp gene assay with dual colour technology can discriminate 2019-nCoV (both probes positive) from SARS-CoV RNA if the latter is used as positive control. Alternatively, laboratories may choose to run the RdRp assay with only the 2019-nCoV-specific probe.

Ethical statement

The internal use of samples for diagnostic workflow optimisation was agreed under the medical ethical rules of each of the participating partners.

Results

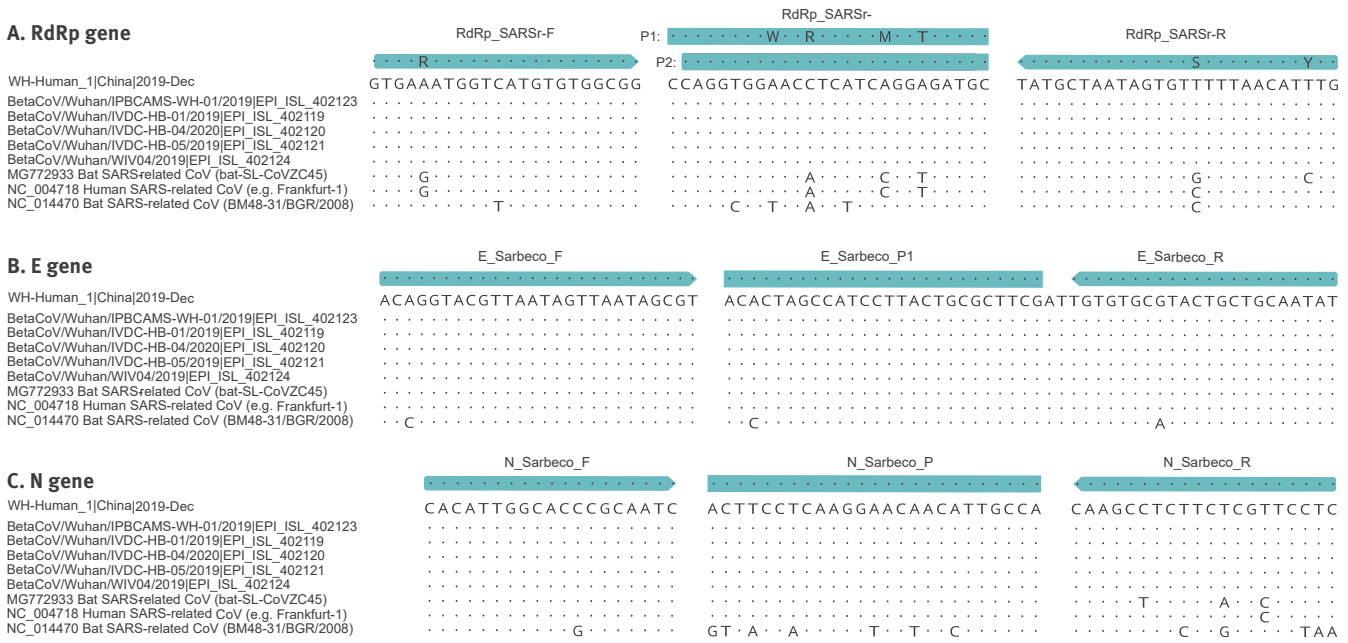
Before public release of virus sequences from cases of 2019-nCoV, we relied on social media reports announcing detection of a SARS-like virus. We thus assumed that a SARS-related CoV is involved in the outbreak. We downloaded all complete and partial (if>400 nt) SARS-related virus sequences available in GenBank by 1 January 2020. The list (n=729 entries) was manually checked and artificial sequences (laboratory-derived, synthetic, etc), as well as sequence duplicates were removed, resulting in a final list of 375 sequences. These sequences were aligned and the alignment was used for assay design (Supplementary Figure S1). Upon release of the first 2019-nCoV sequence at virological.org, three assays were selected based on how well they matched to the 2019-nCoV genome (Figure 1). The alignment was complemented by additional sequences released independently on GISAID (https://www.gisaid.org), confirming the good matching of selected primers to all sequences. Alignments of primer binding domains with 2019-nCoV, SARS-CoV as well as selected bat-associated SARS-related CoV are shown in Figure 2.

Assay sensitivity based on SARS coronavirus virions

To obtain a preliminary assessment of analytical sensitivity, we used purified cell culture supernatant containing SARS-CoV strain Frankfurt-1 virions grown on Vero cells. The supernatant was ultrafiltered and thereby concentrated from a ca 20-fold volume of cell culture supernatant. The concentration step simultaneously reduces the relative concentration of background nucleic acids such as not virion-packaged viral RNA. The virion preparation was quantified by real-time RT-PCR using a specific in vitro-transcribed RNA quantification standard as described in Drosten et al. [8]. All assays were subjected to replicate testing in order to determine stochastic detection frequencies at each assay's sensitivity end point (Figure 3A and B). All assays were highly sensitive, with best results obtained for the E gene and RdRp gene assays (5.2 and 3.8 copies per reaction at 95% detection probability, respectively). These two assays were chosen for further evaluation. One of the laboratories participating in the external evaluation used other basic RT-PCR reagents (TaqMan Fast Virus 1-Step Master Mix) and repeated the sensitivity study, with equivalent results (E gene: 3.2 RNA copies/reaction (95% CI: 2.2–6.8); RdRP: 3.7 RNA copies/reaction (95% CI: 2.8–8.0). Of note, the N gene assay also performed well but was not subjected

Figure 2

Partial alignments of oligonucleotide binding regions, SARS-related coronaviruses (n = 9)



The panels show six available sequences of 2019-nCoV, aligned to the corresponding partial sequences of SARS-CoV strain Frankfurt 1, which can be used as a positive control for all three RT-PCR assays. The alignment also contains a closely related bat virus (Bat SARS-related CoV isolate bat-SL-CoVZC45, GenBank accession number MG772933) as well as the most distant member within the SARS-related bat CoV clade, detected in Bulgaria (GenBank accession number NC_014470). Dots represent identical nucleotides compared with the WH_Human_1 sequence. Nucleotide substitutions are specified. Blue arrows: oligonucleotides as specified in Table 1. More comprehensive alignments can be found in the Supplement.

to intensive further validation because it was slightly less sensitive (Supplementary Figure S2)

Sensitivity based on in vitro-transcribed RNA identical to 2019 novel coronavirus target sequences

Although both assays detected 2019-nCoV without polymorphisms at oligonucleotide binding sites (Figure 2), we additionally generated in vitro-transcribed RNA standards that exactly matched the sequence of 2019-nCoV for absolute quantification and studying the limit of detection (LOD). Replicate reactions were done at concentrations around the detection end point determined in preliminary dilution experiments. The resulting LOD from replicate tests was 3.9 copies per reaction for the E gene assay and 3.6 copies per reaction for the RdRp assay (Figure 3C and D). These figures were close to the 95% hit rate of 2.9 copies per reaction, according to the Poisson distribution, expected when one RNA molecule is detected.

Discrimination of 2019 novel coronavirus from SARS coronavirus by RdRp assay

Following the rationale that SARS-CoV RNA can be used as a positive control for the entire laboratory procedure, thus obviating the need to handle 2019-nCoV RNA, we formulated the RdRp assay so that it contains two probes: a broad-range probe reacting with SARS-CoV and 2019-nCoV and an additional probe that reacts only with 2019-nCoV. By limiting dilution experiments, we confirmed that both probes, whether used individually or in combination, provided the same LOD for each target virus. The specific probe RdRP_SARSr-P2 detected only the 2019-nCoV RNA transcript but not the SARS-CoV RNA.

Detection range for SARS-related coronaviruses from bats

At present, the potential exposure to a common environmental source in early reported cases implicates the possibility of independent zoonotic infections with increased sequence variability [5]. 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.

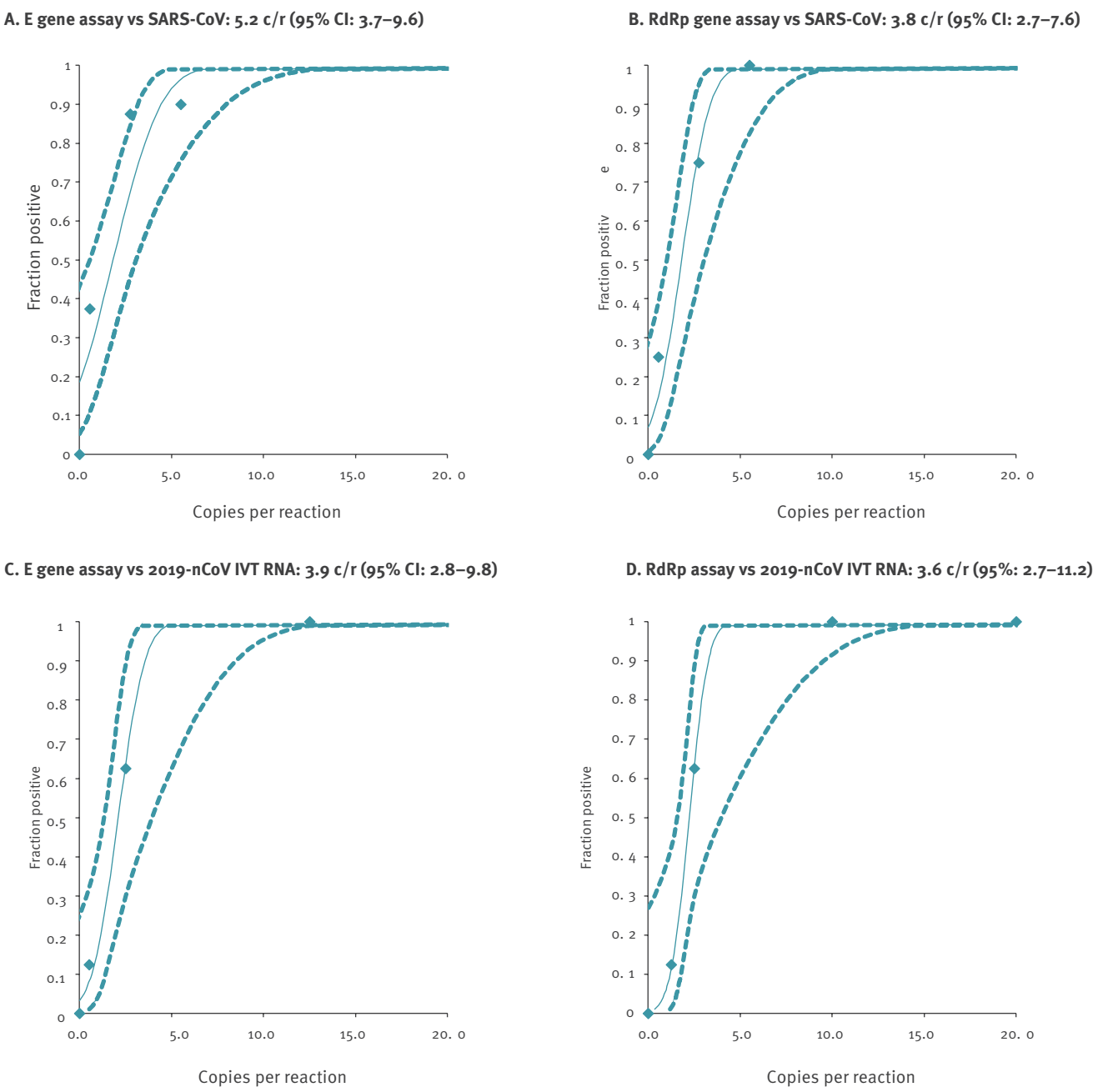
Specificity testing

Chemical stability

To exclude non-specific reactivity of oligonucleotides among each other, causing artificial fluorescent

Figure 3

Determination of limits of detection based on SARS coronavirus genomic RNA and 2019 novel coronavirus-specific in vitro transcribed RNA



CI: confidence intervals; c/r: copies per reaction; IVT: in vitro-transcribed RNA.

A: E gene assay, evaluated with SARS-CoV genomic RNA. B: RdRp gene assay evaluated with SARS-CoV genomic RNA. C: E-gene assay, evaluated with 2019-nCoV-specific in vitro-transcribed RNA standard. D: RdRp gene assay evaluated with 2019-nCoV-specific in vitro-transcribed RNA standard.

The x-axis shows input RNA copies per reaction. The y-axis shows positive results in all parallel reactions performed, squares are experimental data points resulting from replicate testing of given concentrations (x-axis) in parallels assays (eight replicate reactions per point).

Technical limits of detection are given in the panels headings. The inner line is a probit curve (dose-response rule). The outer dotted lines are 95% CI.

Table 2

Tests of known respiratory viruses and bacteria in clinical samples and cell culture preparations for cross-reactivity in 2019 novel coronavirus E and RdRp gene assays (n = 310)

Clinical samples with known viruses	Clinical samples[a]	Virus isolates[b]
HCoV-HKU1	14	1[c]
HCoV-OC43	16	2[d]
HCoV-NL63	14	1[e]
HCoV-229E	18	2[f]
MERS-CoV	5	1[g]
Influenza A(H1N1)pdm09	17	1
Influenza A(H3N2)	16	1
Influenza A (untyped)	11	NA
Influenza A(H5N1)	1	1
Influenza A(H7N9)	0	1
Influenza B (Victoria or Yamagata)	31	1
Rhinovirus/enterovirus	31	NA
Respiratory syncytial virus (A/B)	33	NA
Parainfluenza 1 virus	12	NA
Parainfluenza 2 virus	11	NA
Parainfluenza 3 virus	14	NA
Parainfluenza 4 virus	11	NA
Human metapneumovirus	16	NA
Adenovirus	13	1
Human bocavirus	6	NA
Legionella spp.	3	NA
Mycoplasma spp.	4	NA
Total clinical samples	**297**	**NA**

[a] For samples with multiple viruses detected, the virus with highest concentration is listed, as indicated by real-time PCR Ct value.

[b] Directly quantified or spiked in human negative-testing sputum.

[c] 1×10^5 RNA copies/mL, determined by specific real-time RT-PCR. Isolated from human airway epithelial culture.

[d] 1×10^{10} RNA copies/mL, determined by specific real-time RT-PCR of one isolate. The other isolate was not quantified but spiked in human negative-testing sputum.

[e] 4×10^9 RNA copies/mL, determined by specific real-time RT-PCR.

[f] 3×10^9 RNA copies/mL, determined by specific real-time RT-PCR of one isolate. The other isolate was not quantified spiked in human negative-testing sputum.

[g] 1×10^8 RNA copies/mL, determined by specific real-time RT-PCR.

signals, all assays were tested 120 times in parallel with water and no other nucleic acid except the provided oligonucleotides. In none of these reactions was any positive signal detected.

Cross-reactivity with other coronaviruses

Cell culture supernatants containing all endemic human coronaviruses (HCoV)229E, NL63, OC43 and HKU1 as well as MERS-CoV were tested in duplicate in all three assays (Table 2). For the non-cultivable HCoV-HKU1, supernatant from human airway culture was used. Viral RNA concentration in all samples was determined by specific real-time RT-PCRs and in vitro-transcribed RNA standards designed for absolute quantification of viral load. Additional undiluted (but not quantified) cell culture supernatants were tested as summarised in Table 2. These were additionally mixed into negative human sputum samples. None of the tested viruses or virus preparations showed reactivity with any assay.

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.

Discussion

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. Effective assay design was enabled by the willingness of scientists from China to share genome information before formal publication, as well as the availability of broad sequence knowledge from ca 15 years of investigation of SARS-related viruses in animal reservoirs. The relative ease with which assays could be designed for this virus, in contrast to SARS-CoV in 2003, proves the huge collective value of descriptive studies of disease ecology and viral genome diversity [8,15-17].

Real-time RT-PCR is widely deployed in diagnostic virology. In the case of a public health emergency, proficient diagnostic laboratories can rely on this robust technology to establish new diagnostic tests within their routine services before pre-formulated assays become available. In addition to information on

reagents, oligonucleotides and positive controls, laboratories working under quality control programmes need to rely on documentation of technical qualification of the assay formulation as well as data from external clinical evaluation tests. The provision of control RNA templates has been effectively implemented by the EVAg project that provides virus-related reagents from academic research collections [18]. SARS-CoV RNA was retrievable from EVAg before the present outbreak; specific products such as RNA transcripts for the here-described assays were first retrievable from the EVAg online catalogue on 14 January 2020 (https://www.european-virus-archive.com). Technical qualification data based on cell culture materials and synthetic constructs, as well as results from exclusivity testing on 75 clinical samples, were included in the first version of the diagnostic protocol provided to the WHO on 13 January 2020. Based on efficient collaboration in an informal network of laboratories, these data were augmented within 1 week comprise testing results based on a wide range of respiratory pathogens in clinical samples from natural infections. Comparable evaluation studies during regulatory qualification of in vitro diagnostic assays can take months for organisation, legal implementation and logistics and typically come after the peak of an outbreak has waned. The speed and effectiveness of the present deployment and evaluation effort were enabled by national and European research networks established in response to international health crises in recent years, demonstrating the enormous response capacity that can be released through coordinated action of academic and public laboratories [18-22]. This laboratory capacity not only supports immediate public health interventions but enables sites to enrol patients during rapid clinical research responses.

*Author's correction

The sentence As at 20 January 2020, 282 laboratory-confirmed human cases have been notified to WHO was originally published with a wrong date (As at 20 January 2019...). This mistake was corrected on 8 April 2020.

On 29 July 2020 the correct affiliation of Marco Kaiser was added and the remaining affiliations were renumbered.

**Addendum

The Conflict of interest section was updated on 29 July 2020.

Acknowledgements

This work was funded by European Union DG Research through projects Prepare (GA602525), Compare (GA643476), and EVAg (GA653316); by European Union DG SANCO through EVD-LabNet, as well as by the German Ministry of Research through projects RAPID (01KI1723A) and DZIF (301-4-7-01.703).

We gratefully acknowledge the authors, the originating and submitting laboratories for their sequence and metadata shared through GISAID, on which this research is based.

All authors of data may be contacted directly via www.gisaid.org: National Institute for Viral Disease Control and Prevention, China CDC (Wenjie Tan, Xiang Zhao, Wenling Wang, Xuejun Ma, Yongzhong Jiang, Roujian Lu, Ji Wang, Weimin Zhou, Peihua Niu, Peipei Liu,Faxian Zhan, Weifeng Shi, Baoying Huang, Jun Liu, Li Zhao, Yao Meng, Xiaozhou He, Fei Ye, Na Zhu, Yang Li, Jing Chen, Wenbo Xu, George F. Gao, Guizhen Wu); Wuhan Institute of Virology, Chinese Academy of Sciences (Peng Zhou, Xing-Lou Yang, Ding-Yu Zhang, Lei Zhang, Yan Zhu, Hao-Rui Si, Zhengli Shi); Institute of Pathogen Biology, Chinese Academy of Medical Sciences and Peking Union Medical College (Lili Ren, Jianwei Wang, Qi Jin, Zichun Xiang, Yongjun Li, Zhiqiang Wu, Chao Wu, Yiwei Liu); and National Institute for Communicable Disease Control and Prevention (ICDC), China CDC (Zhang Y-Z, Wu, F, Chen Y-M, Pei Y-Y, Xu L, Wang W, Zhao S, Yu B, Hu Y, Tao Z-W, Song Z-G, Tian J-H, Zhang Y-L, Liu Y, Zheng J-J, Dai F-H, Wang Q-M, She J-L and Zhu T-Y)

We thank Marta Zuchowski, Sigrid Kersten, and Joerg Hofmann for help with sample logistics. In vitro-transcribed control RNA for the E gene assay can be acquired from author C. D. through the European Virus Archive platform (www.european-virus-archive.com),

Conflict of interest **

Olfert Landt is CEO of Tib-Molbiol; Marco Kaiser is senior researcher at GenExpress and serves as scientific advisor for Tib-Molbiol.

Authors' contributions

VMC: Planned and conducted experiments, conceptualised the laboratory work

OL: Planned and conducted experiments, conceptualised the laboratory work

MK: Planned and conducted experiments

RM: Planned and conducted experiments, conceptualised the laboratory work

AM: Planned and conducted experiments, conceptualised the laboratory work

DKWC: Planned and conducted experiments

TB: Planned and conducted experiments

SB: Planned and conducted experiments

JS: Planned and conducted experiments

MLS: Planned and conducted experiments

DGJCM: Planned and conducted experiments

BLH: Planned and conducted experiments

BvdV: Planned and conducted experiments

SvdB: Planned and conducted experiments

LW: Planned and conducted experiments

GG: Planned and conducted experiments

JLR: Contributed to overall study conceptualization

JE: Planned and conducted experiments, conceptualised the laboratory work

MZ: Planned laboratory work, contributed to overall study conceptualization

MP: Planned laboratory work, contributed to overall study conceptualization

HG: Contributed to overall study conceptualization

CR: Planned experiments, conceptualised the laboratory work

MPGK: Planned experiments, conceptualised the laboratory work

CD: Planned experiments, conceptualised the laboratory work, conceptualised the overall study, wrote the manuscript draft.

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3′ IsomiR Species and DNA Contamination Influence Reliable Quantification of MicroRNAs by Stem-Loop Quantitative PCR

Article *in* PLoS ONE · August 2014

DOI: 10.1371/journal.pone.0106315 · Source: PubMed

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3′ IsomiR Species and DNA Contamination Influence Reliable Quantification of MicroRNAs by Stem-Loop Quantitative PCR

Anita Schamberger[1], **Tamás I. Orbán**[1,2]*

1 Institute of Enzymology, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Budapest, Hungary, **2** Chemical Technology Transfer Ltd., Budapest, Hungary

Abstract

MicroRNAs (miRNAs) are ~20–24 nucleotide-long regulatory RNAs that have been proven to play important roles in many cellular processes. Since their discovery, a number of different techniques have been developed to detect and accurately quantify them. For individual mature miRNA measurements, quantitative stem-loop real-time PCR represents a widely used method. Although there are some data on optimization of this technique, there are still many factors that have not been investigated yet. In this study, we have thoroughly optimized this technique and pointed out several important factors that influence reliable quantification. First, we found that total RNA input can affect the measurements. Second, our data showed that carryover DNA contamination could also mislead the detection in a sequence-specific manner. Additionally, we provided evidence that different 3′ isomiR species of a particular miRNA can be reverse transcribed and cross-detected even by specifically targeted assays. Besides these, we have investigated the measurement of reaction efficiencies from total RNA samples and the accuracy of simultaneous reverse transcription reactions for increasing reliability and cost effectiveness without the loss of sensitivity and specificity. In summary, we provide a detailed, refined protocol for reliable detection of microRNA species by quantitative stem-loop PCR.

Citation: Schamberger A, Orbán TI (2014) 3′ IsomiR Species and DNA Contamination Influence Reliable Quantification of MicroRNAs by Stem-Loop Quantitative PCR. PLoS ONE 9(8): e106315. doi:10.1371/journal.pone.0106315

Editor: Pedro Gonzalez, Duke University, United States of America

Received November 26, 2013; **Accepted** August 5, 2014; **Published** August 29, 2014

Funding: Anita Schamberger is a recipient of the Jedlik Ányos predoc fellowship. This research was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4. A/1-11-1-2012-0001 'National Excellence Program' and also supported by the TransRat grant (KMR_12-2012-0112) from the Hungarian National Development Agency given to Tamás I. Orbán. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Author Tamás I. Orbán is employed by Chemical Technology Transfer Ltd. Chemical Technology Transfer Ltd provided support in the form of salary for author TIO, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific role of this author is articulated in the ''author contributions'' section.

Competing Interests: Author Tamás I. Orbán is employed by Chemical Technology Transfer Ltd. There are no patents, products in development or marketed Cover Letter products to declare. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

* Email: orban.tamas@ttk.mta.hu

Introduction

MicroRNAs (miRNAs) are short, non-coding regulatory RNA molecules that control mRNA stability and translation by targeting the 3′ untranslated region of given mRNA species [1,2]. They influence various cellular functions and now are believed to form a crucial and extensive regulatory network similar to that of transcription factors [3]. The biogenesis of miRNAs consists of different, subsequent processing steps during which mature miRNA is liberated from longer precursor RNA forms [4–6]. In order to understand proper regulation and function, the different RNA forms can be studied and measured by various techniques. In the general laboratory practice, however, it is often sufficient to measure individual mature miRNA steady state levels. Nevertheless, measurements are challenging due to their short size, and sequence specific detection methods are more limited than in the case of mRNA molecules. Traditional hybridization techniques using radioactively or fluorescently labeled nucleic acids are generally applied, including in situ hybridization [7,8] or Northern blotting [9–11]. Their sensitivity can be strongly increased by using specifically modified artificial nucleotides, such as locked nucleic acids (LNAs) [12–15], but miRNAs with low abundance can still be beyond the sensitivity of these methods [16,17].

Similarly to mRNA detection and quantification, measuring the expression level of miRNA species by real-time PCR represents one of the most sensitive and accurate methods developed so far for such purposes. However, due to the short nature of miRNAs, a specific stem-loop real-time PCR technique has been developed among other methodologies [18–20]. The detection of mature miRNAs by this technique is composed of two main steps (Figure 1). The first step is a specifically targeted cDNA synthesis when a sequence specific stem-loop primer is hybridized to the mature miRNA and used to initiate the reverse transcription reaction. The second step is the real-time PCR during which the extended and transcribed miRNA is quantified using oligos specific for the miRNA and the primer loop sequences. This technique is fast and could be standardized for high-throughput purposes. However, this method has the *a priori* assumption that the miRNA in question has a well-defined 3′ end. Conversely, based on deep sequencing results, recent reports described significant sequence length heterogeneity of miRNAs originating from a given locus, often having significant variability of their 5′

and/or 3′ ends [21,22]. Moreover, the distribution of such isomiRs seems to vary among cell types or physiological statuses of the cells [23,24]. Therefore, such 3′ end variability could seriously influence miRNA detection by stem-loop PCR by interfering with the very first step, the sequence specific reverse transcription. There are several data on optimization of miRNA detection from discussing RNA isolation techniques to comparing various platforms [25–30]. Nevertheless, there are many other factors during individual mature miRNA detection by the widely used stem-loop quantitative PCR that are not discussed yet, although they play important roles in the accuracy and reproducibility of the measurements.

In this study, we intended to systematically investigate the stem-loop real-time PCR detection method of small RNA molecules. Careful optimization of this technique pointed to a previously underestimated aspect, that total RNA input and DNA contamination could severely influence the accurate detection. Moreover, we provide evidence that 3′ isomiR species are not exclusively measured by the stem-loop qRT-PCR methodology, and thereby can be cross-detected. This latter problem could not be overcome even by using the poly(A)-tailing-based qRT-PCR methodology. On the other hand, simultaneous reverse transcription of the target miRNA and the endogenous control does not necessarily influence the outcome of the results and may be a more accurate and cost effective approach for miRNA level quantitation. Based on our experiments, we suggest a refined protocol of miRNA detection by stem-loop real-time PCR technology.

Results

Relative quantification, reaction efficiency and the amount of reverse transcribed RNA

In quantitative RT-PCR applications, determination of the target is based on absolute or relative quantification. For individual miRNA measurements, relative quantification is the commonly used method, when the amount of the target is determined relative to an endogenous control. Since the target is compared to the control, they must be amplified with similar efficiencies. The accurate amplification efficiency in practice is calculated from the slope of a standard curve made by at least 5 points, encompassing the relevant concentration range of the application. Making an accurate and reproducible standard curve for miRNAs from total RNA samples (which is physiologically more relevant than using synthetic oligos) is challenging, since many miRNAs are present in low abundance. A sensitive balance has to be found between the sufficient dilution of the reverse transcription reaction (e.g.: for mRNA detection, it is a minimum of 1:10) and an optimal C_t value (delayed by the dilution of the reverse transcription reaction; Figure S1). Therefore, we recommend the employment of small dilution steps (e.g. 1.5×) with only 3 or 4 points in the strict range of the measurement. If there is appropriate correlation between the control and target, the relative quantification method can be used at the particular dilution range for the analysis of the measurements.

The next question is about the optimal amount of total RNA used for the reverse transcription reaction. As mentioned above, mature miRNA levels are often low in certain samples. Therefore, one could speculate to increase the amount of total RNA to increase the input of mature miRNAs in the reverse transcription reaction. To investigate this question, we measured more and less abundant miRNAs (abundance was estimated based on previous data: http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_089374.pdf and [17,30]) from different total RNA input, relative to the widely used U6 small nuclear RNA (snRNA) or to the miR-21-5p endogenous miRNA. Target and endogenous control samples were prepared simultaneously and measured in the same plate during the real-time PCR reaction. The increase of the total RNA amount resulted in a decrease of mature miRNA detection when applying the U6 endogenous snRNA control (Figure 2A). It has dropped significantly above 20 ng in general and the effect did not seem to depend on the abundance of the miRNA target. Considering the miR-21-5p endogenous control, the effect of the total RNA input on the measurements was less pronounced (Figure 2B). Finally, the lower range of RNA input was measured less accurately probably because the low template concentration leads to delayed C_t values. Summarizing the results, the optimal range of RNA input varies depending on endogenous controls and targets, therefore, should be optimized. Based on our data, however, 10 ng total RNA input can be appropriate when using U6 endogenous snRNA control and 20 ng with the application of miR-21-5p endogenous miRNA control.

Different targets can be reverse transcribed in the same reaction

For cDNA synthesis of miRNAs, the different small RNA targets have unique, sequence specific stem-loop primers to assist their reverse transcription. Although numerous miRNAs are reverse transcribed together in array experiments [31–33], it is indicated in the general protocol that for individual miRNA measurements, the endogenous control and the target have to be reverse transcribed in separate reactions (http://tools.lifetechnologies.com/content/sfs/manuals/cms_042167.pdf). To examine the feasibility of the simultaneous reactions, we compared real-time PCR measurements of simultaneously and separately reverse transcribed samples. We measured the level of several miRNAs including miR-1226-3p and miR-33b-5p in stably overexpressing HeLa cell lines, and the endogenous miR-21-5p in normal HeLa cell line. We found that there is no significant difference in the results when the reverse transcription was done separately or together with the endogenous control for the investigated assays (Figure 3). However, it is important to note that the long term storage of different hairpin primers mixed together in the same solution is not recommended as it may lead to a false positive detection of mature miRNAs (data not shown). In summary, the level of an individual miRNA can be determined by using cDNA samples in which the given target and the endogenous control are reverse transcribed together, thereby reducing potential pipetting errors and making the measurements more cost effective.

DNA contamination significantly influences the measurement of mature miRNAs

Next, we investigated the effect of genomic and plasmid DNA on miRNA measurements. Based on our previous data from transient transfections, we had indications that contaminating DNA might interfere with mature miRNA detection. Thus, we examined whether stem-loop qRT-PCR is specific to the present mature miRNA or it has false positive signal from samples which do not contain the particular target. We tested miR-1226-3p and miR-33b-5p assays on genomic DNA (gDNA), total RNA and plasmid DNA (encoding the corresponding miRNA) samples. The investigated plasmids differ only in their pre-miRNA coding sequence. Total RNA and gDNA samples were derived from mir-1226 and mir-33b overexpressing or parental HeLa cell lines. We compared DNase treated and non-treated parallels for each sample. After reverse transcription, we measured the mature

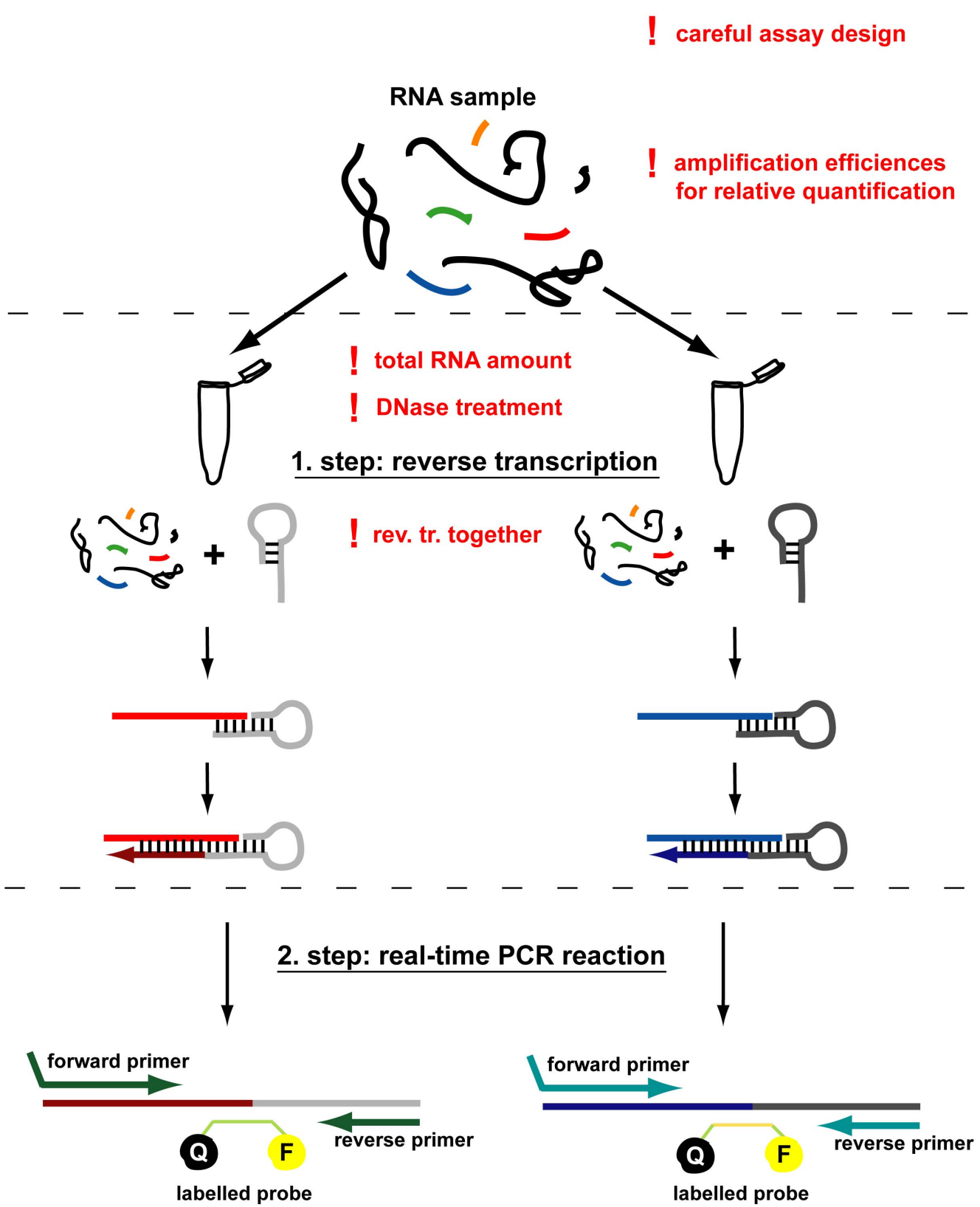
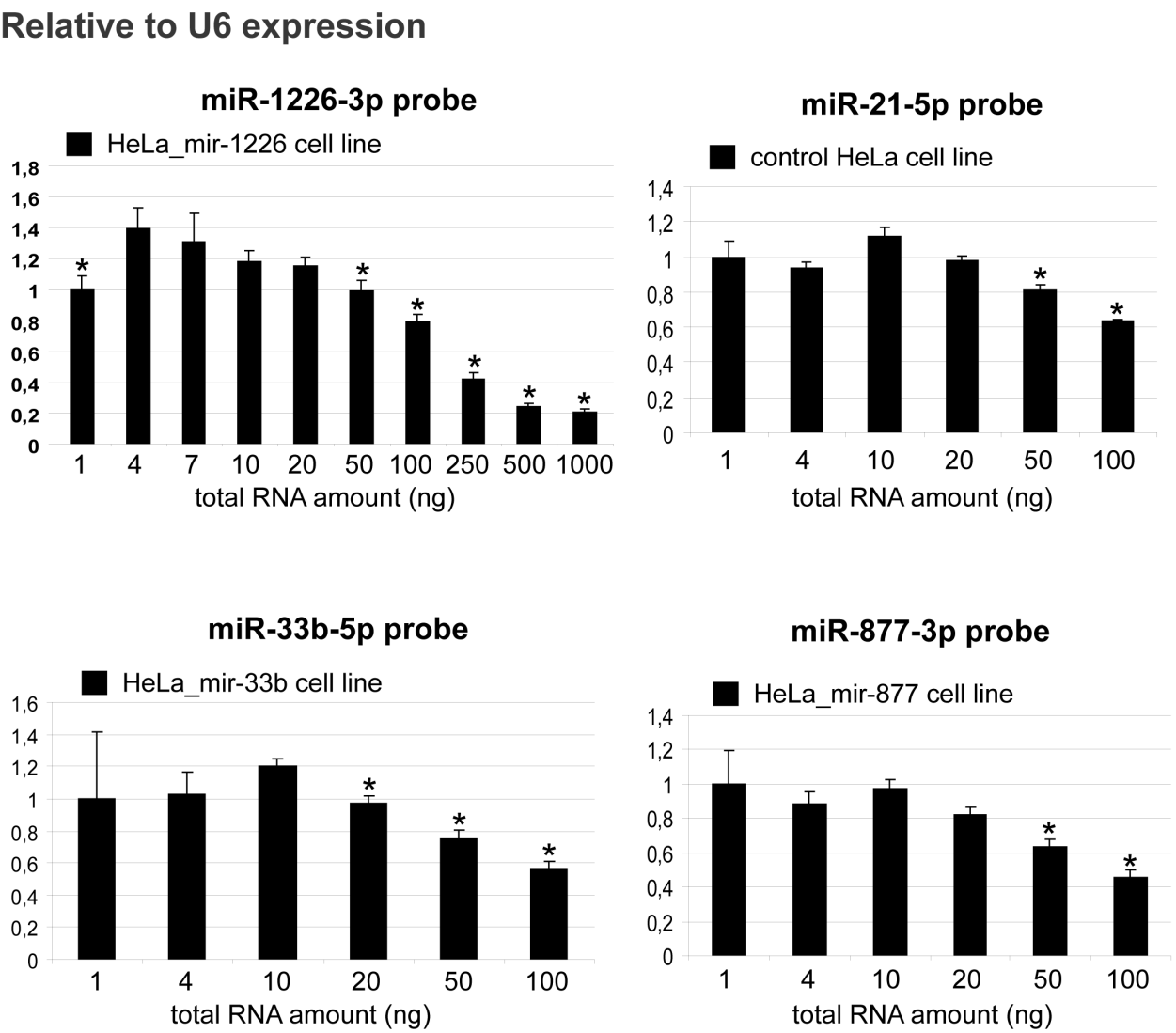


Figure 1. Schematic representation of stem-loop microRNA quantitative RT-PCR. The two main steps are reverse transcription and real-time PCR. In the first step, mature miRNA is extended and reverse transcribed by a sequence specific stem-loop primer. In the second step, the reverse transcribed miRNA is quantified by a fluorescently labeled hybridization probe using the strand replacement reaction. According to the previous protocol, all targets (e.g. endogenous control and target) should be reverse transcribed separately. In the dual-labeled probe based detection systems Q stands for quencher, F for fluorophore. Red exclamation marks indicate crucial points of the procedure that are discussed in this paper.
doi:10.1371/journal.pone.0106315.g001

A


Relative to U6 expression
miR-1226-3p probe
HeLa_mir-1226 cell line
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
1 4 7 10 20 50 100 250 500 1000
total RNA amount (ng)
miR-21-5p probe
control HeLa cell line
1,4
1,2
1
0,8
0,6
0,4
0,2
0
1 4 10 20 50 100
total RNA amount (ng)
miR-33b-5p probe
HeLa_mir-33b cell line
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
1 4 10 20 50 100
total RNA amount (ng)
miR-877-3p probe
HeLa_mir-877 cell line
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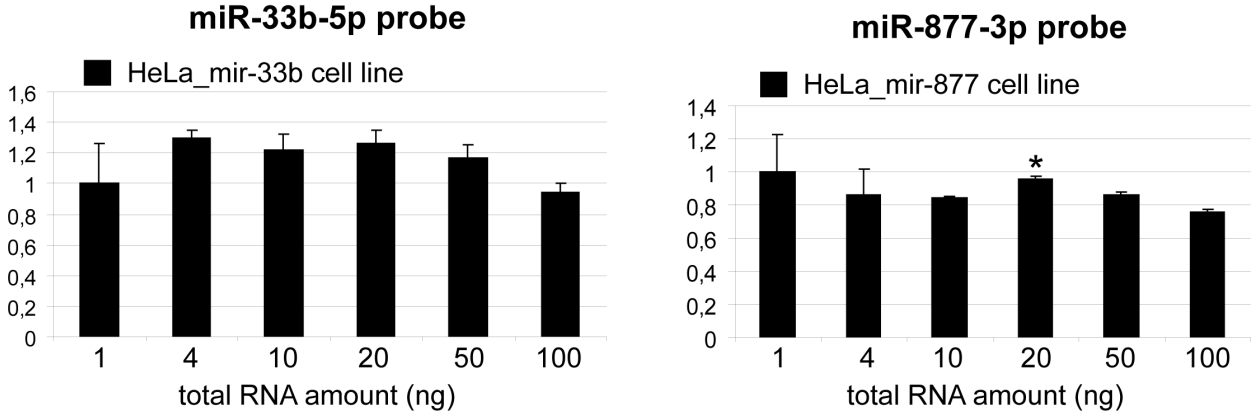
1 4 10 20 50 100
total RNA amount (ng)

B


Relative to miR-21-5p expression
miR-33b-5p probe
HeLa_mir-33b cell line
1,6
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total RNA amount (ng)
miR-877-3p probe
HeLa_mir-877 cell line
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1 4 10 20 50 100
total RNA amount (ng)

Figure 2. Mature miRNA detection from different amount of total RNA input. Different amount of total RNA samples were reverse transcribed for the detection of a particular mature miRNA by real-time PCR. MiRNAs with various abundance were measured, using relative quantification. The more abundant miR-1226-3p, miR-33b-5p and the less abundant miR-877-3p (mir-877*) were measured from cell lines stably overexpressing the corresponding miRNA, while the abundant endogenous miR-21-5p from parental HeLa cell line. Concerning the endogenous controls, the U6 snRNA (**A**) and the endogenous miR-21-5p (**B**) were applied. The optimal range of RNA input varies depending on endogenous controls and targets. The corresponding concentration series (controls and targets) were prepared and measured simultaneously. Mean values of three independent experiments (three biological parallels with three technical replicates) are shown. Error bars represent S.E.M.; samples are compared to a chosen optimal condition (10 ng of total RNA). *: $p<0.05$.
doi:10.1371/journal.pone.0106315.g002

miRNA levels in the above samples (Figure 4). The miR-1226-3p probe detected 4 fold higher amounts (2 C_t difference) of mature miRNA from the gDNA of mir-1226 overexpressing cell line compared to the two "non-relevant" gDNA samples (from HeLa and HeLa_mir-33b cell lines). In the case of the miR-33b-5p assay, the measured miRNA levels were similar among all gDNA samples. For RNA samples, as it was expected, both miR-1226-3p and miR-33b-5p probes resulted in significantly higher detected

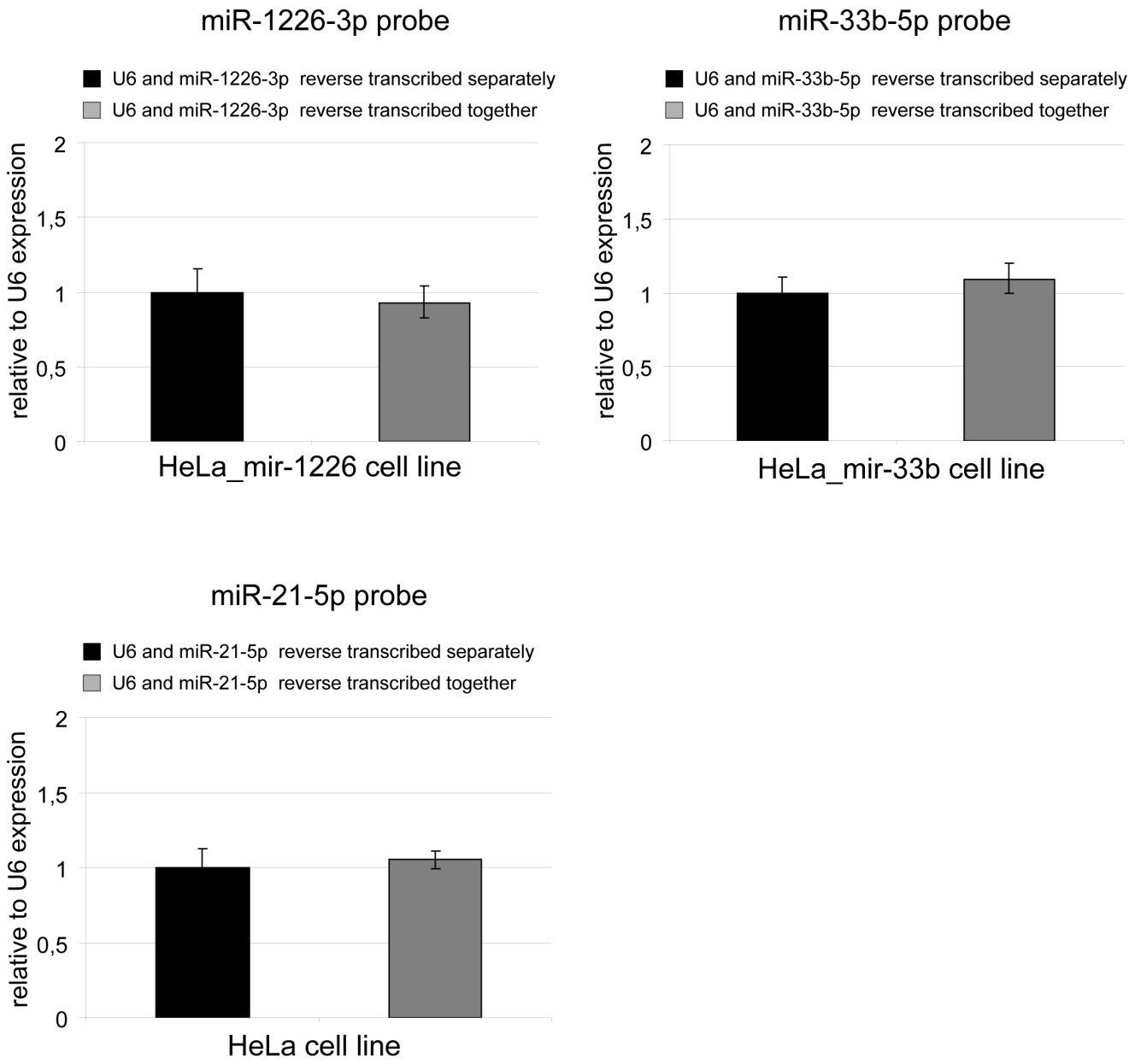


Figure 3. Reverse transcription of target and control can be done simultaneously. Mature miRNA levels of miR-1226-3p and miR-33b-5p were detected in stably overexpressing HeLa cell lines, whereas the endogenous miR-21-5p in parental HeLa cell line. cDNA samples were used from simultaneous or separate reverse transcription reactions of the endogenous control and the target. Experiments were carried out with three RT parallels and three technical replicates, error bars represent standard deviations.
doi:10.1371/journal.pone.0106315.g003

mature miRNA levels from the corresponding miRNA overexpressing cell line than in the controls. Concerning plasmid DNAs, apparently similar amount of miR-33b-5p was detected from mir-33b encoding plasmid as from mir-33b overexpressing cell line derived RNA. This striking false effect was even more pronounced in the case of miR-1226-3p when the mir-1226 expression plasmid served as a template. There was 9 C_t difference compared to the mir-1226 overexpressing cell line derived RNA sample, and about 14 C_t difference compared to the RNA backgrounds, representing an apparent 512× and 16384× higher miRNA amount, respectively. From plasmids encoding other "non-relevant" miRNA, very low signals were detected both for miR-1226-3p and miR-33b-5p. The above data indicate that the false positive signals from the relevant plasmid samples are miRNA sequence specific. Therefore, although mature miRNA molecules are not present, signals can be apparently detected from DNA containing the coding sequence of the corresponding pre-miRNA form. These results were also confirmed by experiments using miR-877-3p and miR-877-5p assays (data not shown).

Next, we intended to address the question that which part of the measurement (reverse transcription or real-time PCR) misleads the mature miRNA detection. To answer this question, first we made quantitative real-time PCR for miR-1226-3p from reverse transcribed and non-transcribed samples. We tested gDNA and RNA samples from mir-1226 overexpressing cell line and we also used mir-1226 encoding plasmid samples. Mir-33b overexpressing cell line and mir-33b encoding plasmid samples served as non-relevant controls. As shown in Figure 5A, there is a slight detection during the real-time PCR reaction from the relevant plasmid DNA without reverse transcription, but the majority of the false positive signal is detected only when the reverse transcription reaction is performed. We obtained similar results with the miR-33b-5p assay (Figure S2). In summary, these data reveal the unexpected fact that DNA may serve as a template during the reverse transcription reaction in a (stem-loop primer) sequence specific manner.

In further experiments, we tested whether the above phenomenon has a real relevance, for example when investigating miRNAs in transiently transfected cells. In such cases, RNA samples are prepared from cells containing the transfected plasmids. For these measurements we used RNA samples from HeLa cells transiently transfected with different amounts of a mir-1226 encoding plasmid (Figure 5B). The data showed that when samples were not treated with DNase, a significantly higher amount of miRNA was detected as compared to the DNase treated samples. This problem occurred not only by using the Trizol based total RNA isolation method, but also when applying a column-based isolation protocol such as the *mir*Vana Kit (Figure S3). These results indicate that there is plasmid DNA contamination in the total RNA samples which indeed misleads the accurate detection of mature miRNAs.

3′ isomiR forms of miRNAs are cross-detected

Emerging data strengthen the existence of isomiRs which are the results of the heterogeneous nature of miRNA processing, leading to variation in the length and/or sequence of mature miRNAs [23]. Since the exact 3′ end sequence seems to be crucial for stem-loop quantitative PCR, we investigated whether the different 3′ end variants of miRNAs can be exclusively detected by this technique. We applied different assays, designed for different 3′ isomiR species of a particular miRNA and tested the detection on various synthetic RNA oligonucleotides (~10^5 molecules/reaction).

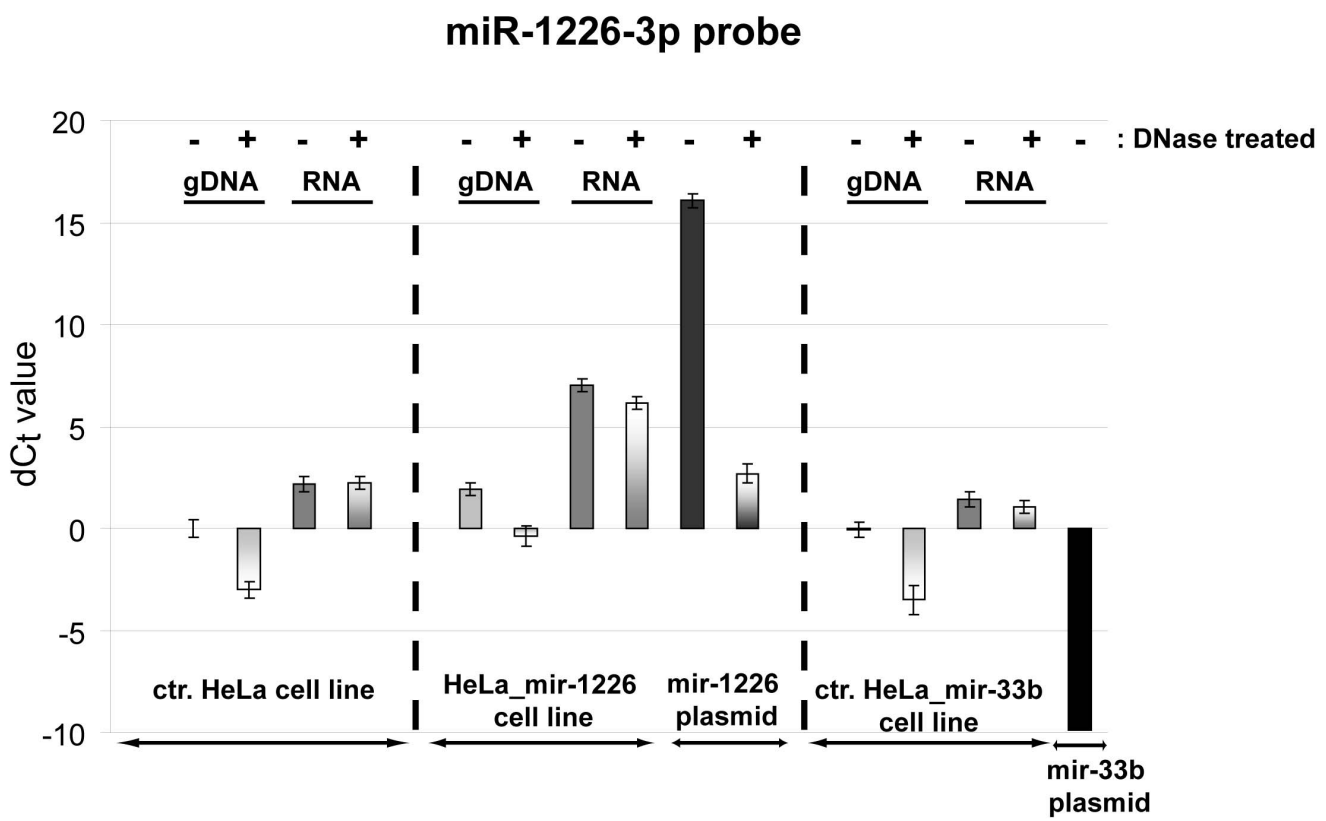
First, among numerous 3′ isomiRs of miR-877-5p, the three most abundant species were analyzed by specific assays (http://www.mirbase.org/cgi-bin/get_read.pl?acc = MI0005561, at date of November, 2013). Since there were no commercially available pre-designed assays for them, we used custom made TaqMan assays (Life Technologies, CA, USA). They were tested on synthetic RNA oligonucleotides, identical to the miR-877-5p isomiR sequences. Each assay was tested for each isomiR species, bearing nucleotide differences in their 3′ ends (Figure 6A). Assays specific for the "GACA" and "GAC" 3′ends detected both "GACA" and "GAC" ended RNAs similarly, while "GA" ending was detected with ~3 Ct delay. On the other hand, "GA" specific assay detected all three isoforms similarly. As concerning non-reverse transcribed (no RT) controls, signals were detected in the case of all three probes, indicating that these real-time PCR assays are somehow able to detect their synthetic RNA targets without reverse transcription. However, there were at least 10 Ct differences between the values of no RT controls and the reverse transcribed target containing samples.

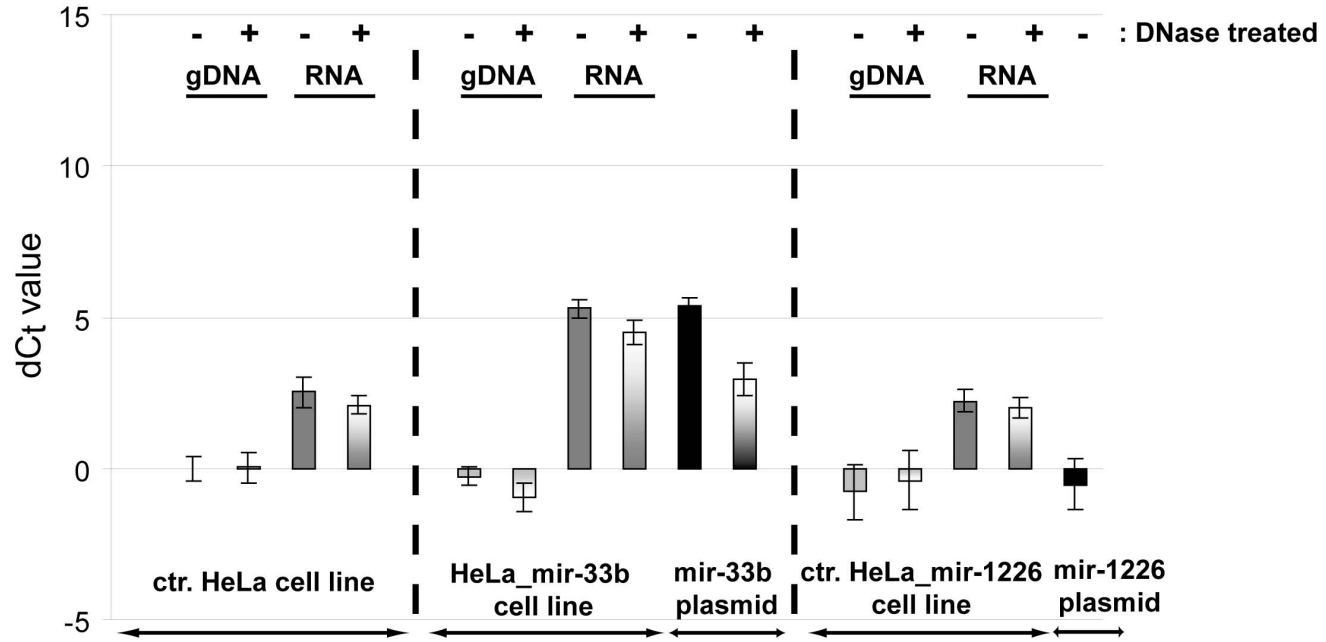
In other experiments, we tested miR-33b-5p isomiRs. There are two indicated 3′ isomiR forms of miR-33b-5p in the miRBase database, with 1 nt difference in their 3′ ends. The shorter mature miRNA is marked as the reference sequence but the longer form seems to be more abundant in investigated cell lines based on deep sequencing data (http://www.mirbase.org/cgi-bin/get_read.pl?acc = MI0003646, at date of November, 2013). We tested these variants by commercially available pre-designed TaqMan assays (Life Technologies, CA, USA). Both "GCA" and "GC" 3′ end specific assays detected the corresponding template better than the other isomiR, but the cross-detections were still considerable (2–4 C_t delayed; Figure 6B). To examine if a different miRNA detecting qRT-PCR approach might overcome this problem, we analyzed the above miR-33b-5p isomiRs by using the poly(A)-tailing-based method [19]. However, the isomiRs were also strongly cross-detected in those experiments (Figure S4), and even the melting curve analysis could not make reliable indication that more isomiRs are present when applying mixed isomiR population as a template for the different assays (data not shown).

The above data indicate that the examined quantitative real-time PCR methods for miRNA detection are not exclusively specific for a given isomiR, consequently 3′ isomiR species can be cross-detected in various extent. These results underline that careful selection of the assay is essential, since the accurate measurement of the given mature miRNA species strongly relies on the selected assay.

Discussion

In this study, we examined several factors in detail influencing accuracy and reliability of the miRNA quantitative stem-loop PCR. Considering the reverse transcription step of this methodology, our data indicate that the increase of the total RNA amount can result in a lower apparent miRNA expression level. This phenomenon could occur due to dissimilar reaction efficiencies of the target and the control in certain ranges of total RNA amount. Thus, it may lead to elevated detection of the endogenous control compared to the target (at higher concentration ranges, as suggested by the analysis of the raw data), therefore resulting in an apparent decrease in the level of the target. For a particular endogenous control/target pair the optimal amount of the total RNA for the reverse transcription reaction can vary, therefore pilot investigations are advisable prior to the real experiments. However, based on our experiments, 10–20 ng of total RNA might be adequate. In addition to these data, we provided evidence that the target of interest can be reverse transcribed


miR-1226-3p probe
20
15
10
5
0
-5
-10
dCt value
- + - + - + - + - + - + - + - + - : DNase treated
gDNA RNA gDNA RNA gDNA RNA
ctr. HeLa cell line
HeLa_mir-1226 cell line
mir-1226 plasmid
ctr. HeLa_mir-33b cell line
mir-33b plasmid


miR-33b-5p probe
15
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dCt value
- + - + - + - + - + - + - + - + - : DNase treated
gDNA RNA gDNA RNA gDNA RNA
ctr. HeLa cell line
HeLa_mir-33b cell line
mir-33b plasmid
ctr. HeLa_mir-1226 cell line
mir-1226 plasmid

Figure 4. The effect of DNA contamination on mature miRNA measurements. Mature miR-1226-3p and miR-33b-5p detection were tested on the indicated samples, with or without DNase treatment. On the y-axis, dC_t value is represented, calculated as the Ct difference between the examined samples and the gDNA of control HeLa cell line (Ct = 33,8 for miR-1226-3p and Ct = 34,9 for miR-33b-5p experiments). Signal was not detected up to 40 reaction cycles for the mir-33b plasmid control by the miR-1226-3p assays. One C_t difference represents about 2× higher detected mature miRNA level. The effect of DNA is probe specific and plasmid DNAs have more pronounced effect on the measurements than gDNA contaminations. Experiments were carried out with three replicates at least twice, one representative experiment is shown. Error bars represent standard deviations.
doi:10.1371/journal.pone.0106315.g004

together in one reaction with the appropriate endogenous control. Apart from lowering the costs of experiments, it has the advantage of reducing pipetting errors and thereby making the measurements more accurate.

Next, we found the unexpected result that contrary to the claims of the original protocol [18], DNA could serve as a template during mature miRNA measurements, mostly during the reverse transcription reaction. Our data suggest that the corresponding pre-miRNA coding sequence is detected by the stem-loop primer. There are data that reverse transcriptases can use (single stranded) DNA as a template, therefore this DNA dependent DNA polymerase activity might be an explanation for our observation. However, the extent of the DNA-derived false detection varied among different miRNA targeting assays, and plasmid DNAs had more pronounced effects on the detection than gDNA contaminations. The significance of DNA contamination is further underlined by the fact that miRNA expression studies are often carried out on transiently transfected cells, which contain a significant amount of plasmid DNA originating from the used expression vector. Additionally to this, DNA and RNA molecules are both detected at 260 nm by spectrophotometry, therefore DNA contamination also disturbs the accurate measurement of RNA concentration. All these factors imply that extensive DNase treatment is a critical part of this miRNA quantification protocol which cannot be omitted when using certain RNA isolation methods. Our data show, that in contrast to total RNA isolation using either Trizol reagent or *mir*Vana Kit, no significant DNA contamination present in the RNA samples when applying small RNA isolation by the *mir*Vana Kit (Figure S3).

In addition to the technical issues described above, the recently discovered isomiR species impose another challenge on miRNA detection by the stem-loop qPCR technique, as the sequence diversity of miRNA species could be quite extensive both at the 5′ and the 3′ ends. Although there are emerging data on the existence of this variability, neither all mechanisms responsible for the generation of isomiRs nor their potential functional differences are clear as yet. Even if 3′ variability appears to be redundant in function at present [23], it represents a problematic issue not only for stem-loop qRT-PCR, but also for miRNA detection by the poly(A)-tailing based methodology ([21]; Figure 6 and Figure S4). Therefore, the *a priori* knowledge of the exact 3′ sequence is a prerequisite for designing an accurate, specific assay for any particular small RNA species, and examining miRNA databases and available online deep sequencing data is strongly recommended. Additionally, we would like to point out that the indicated reference sequences in databases often represent only a small proportion of isomiRs, therefore it could mislead researchers in assay design. Thus, as it was shown in the case of miR-33b-5p and miR-877-5p, cautious selection or design of the assay is essential, since only one nucleotide difference in the 3′ end terminus can cause inaccurate detection, leading to false representation of a mature miRNA form.

In addition to our findings, we would like to note that besides the factors investigated here, there are other issues reported to influence reliable miRNA detection. For example, when applying the widely used Trizol reagent based RNA isolation method also for miRNAs, it is important to keep in mind that the extraction efficiency of miRNAs with low GC content or stable secondary structure is sensitive for the initial number of the cells [34].

Summarizing our results, we provide a detailed and improved protocol for proper application of quantitative stem-loop RT-PCR for the accurate detection of mature miRNA species (see Figure 1 and Materials and Methods).

Materials and Methods

Refined, detailed protocol for stem-loop quantitative RT-PCR of individual miRNAs

Assay design. Careful assay selection for the proper isomiR species is crucial to evade misleading data. If the desired isomiR species is not known for a given miRNA, several previously annotated abundant isoforms should be tested in parallel.

RNA isolation. If total RNA isolation is done by Trizol reagent, the usage of minimum $1–2\times10^6$ cell/ml Trizol is strongly recommended (see ref [34]). The assessment of the quality of the isolated RNA sample (e.g.: by BioAnalyzer, Agilent Technologies) is also advisable.

DNase treatment (strongly recommended for total RNA samples).

e.g. 5 μg of total RNA,
2 μl (4 unit) of DNase (New England Biolabs),
2 μl of 10× DNase buffer,
1 μl (40 unit) of RNasin (Life Technologies), in total volume of 20 μl.

Incubate at 37°C for 1 hour, inactivate at 75°C for 10 minutes, then put on ice. Quantification of RNA by spectrophotometry (e.g.: NanoDrop 2000 Spectrophotometer, Thermo Scientific).

cDNA preparation (TaqMan MicroRNA Reverse Transcription Kit, Life Technologies). For one reaction:

0.15 μl of 100 mM dNTP Mix,
1 μl of Reverse Transcriptase,
1.5 μl of 10× buffer,
0.19 μl of RNase inhibitor,
1.16 μl of H_2O.
Mix gently, then add 5 μl of total RNA (2 ng/μl).

Mix gently and add 3 μl of endogenous control specific RT primer and 3 μl of target specific RT primer.

Reverse transcribe the RNA according to the manufacturer's instructions (16°C for 30′, 42°C for 30′, 85°C 5′).

Important note: reverse transcription efficiency may vary among samples in different type of PCR tubes.

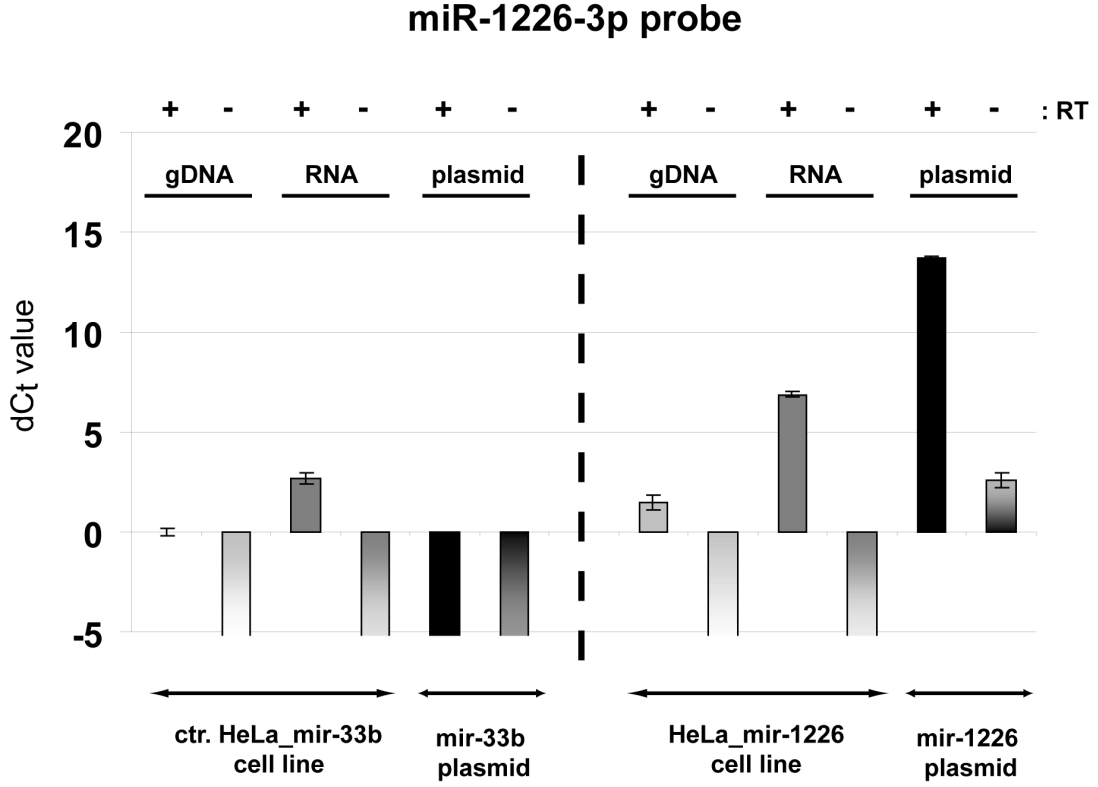
cDNA dilution for quantitative PCR. Dilute the total 15 μl of cDNA volume 5 times by adding 60 μl of H_2O.

Quantitative real-time PCR (using TaqMan MicroRNA Assays, Life Technologies). Perform the samples in triplicate in singleplex reactions, in a final volume of 20 μl.

for one reaction:

10 μl of 2× Mix (TaqMan Universal Master Mix II with UNG, Life Technologies),
1 μl of 20× probe,
9 μl of diluted cDNA.

A


miR-1226-3p probe
+ - + - + - + - + - + - : RT
20
15
10
5
0
-5
dCt value
gDNA
RNA
plasmid
gDNA
RNA
plasmid
ctr. HeLa_mir-33b cell line
mir-33b plasmid
HeLa_mir-1226 cell line
mir-1226 plasmid

B

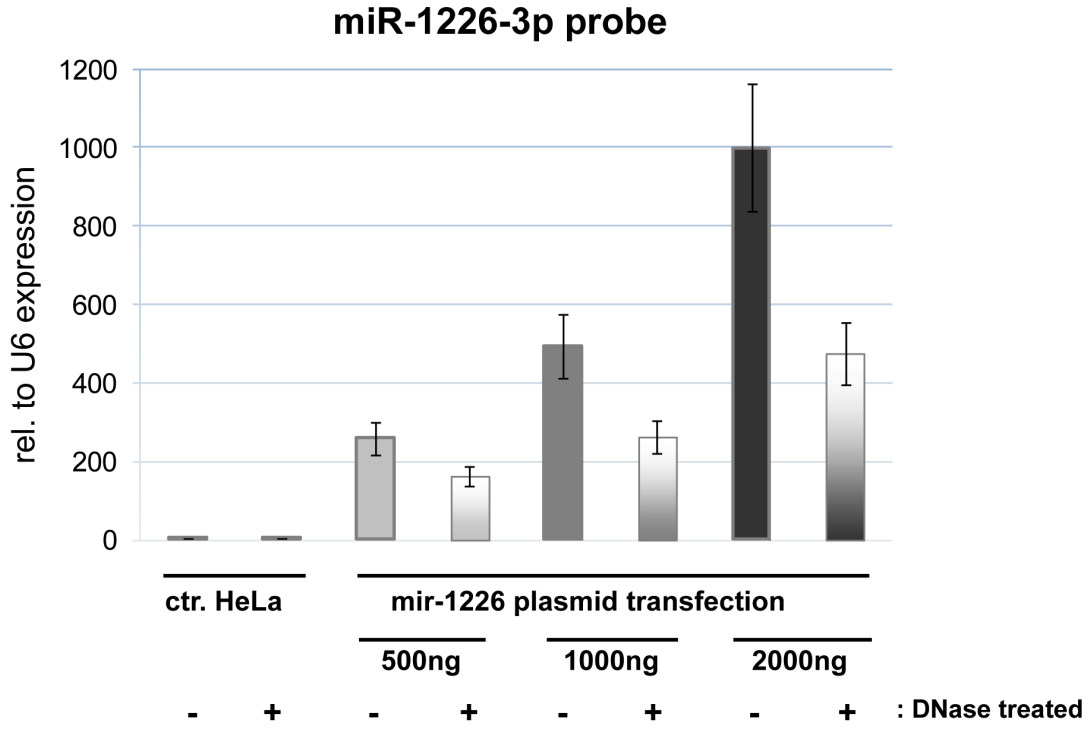

miR-1226-3p probe
1200
1000
800
600
400
200
0
rel. to U6 expression
ctr. HeLa
mir-1226 plasmid transfection
500ng
1000ng
2000ng
- + - + - + - + : DNase treated

Figure 5. DNA can serve as a template during miRNA detection. (**A**) False positive signal of DNA derives mainly from the reverse transcription reaction. Mature miR-1226-3p was tested in the indicated samples, with or without reverse transcription (RT). On the y-axis, dC_t value is represented, calculated as the Ct difference between the examined samples and the gDNA of control HeLa_mir-33b cell line (Ct = 35,9). One C_t difference represents about 2× higher detected mature miRNA level. (**B**) DNA contamination remains in total RNA samples during isolation by the widely used Trizol reagent. Total RNA samples were isolated from transiently transfected HeLa cells; the transfected plasmid DNA amounts are indicated. Samples were DNase treated and non-treated, then reverse transcribed and subjected to real-time PCR. Expression values relative to U6 snRNA are shown on the y-axis. Experiments were carried out with three replicates at least from three independent experiments; one representative experiment is shown, error bars represent standard deviations.
doi:10.1371/journal.pone.0106315.g005

The final dilution of the cDNA in the reaction is 11×. Always apply non-template controls for the different assays. Perform the PCR reaction according to the manufacturer's instructions (50°C for 2′, 95°C for 10′, in 40 cycles: 95°C for 15″, 60°C 1′).

Data analysis. If relative quantification is to be applied, make sure by standard curve analysis that it is indeed applicable for comparison of the particular assays. Always check the baseline and threshold values since big differences in Ct values of the samples or little contamination in the non-template control might cause false auto fit by the program.

Plasmid constructs and isolation

EGFP embedded mir-1226, mir-33b and mir-877 expression plasmids were cloned as described earlier [17]. Plasmid DNAs were isolated by QIAGEN Plasmid Midi Kit using EndoFree Plasmid Buffer Set.

Cell cultures and manipulation

Parental HeLa cell line [35] was kindly provided by Zsuzsanna Izsvák (Mobile DNA Group, Max-Delbrück Center, Berlin, Germany). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal calf serum, 1% of L-glutamine, and 1% of penicillin/streptomycin (Life Technologies) using standard cell culture methodology. Mir-1226, mir-33b and mir-877 stably expressing cell lines were established by the *Sleeping Beauty* transposon based gene delivery technology as described earlier [17]. For transient transfections, 3×10^5 HeLa cells per wells were seeded onto a 6-well plate for transfection on the next day by FuGENE HD reagent (Life Technologies) using plasmid DNAs as indicated (DNA:lipid reagent = 1 μg:3 μl). Transfection efficiencies were followed by EGFP fluorescence, detected by a IX51 fluorescence microscope (Olympus). Cells were collected for total RNA isolation 48 h after transfection.

Genomic DNA isolation

After trypsinization, cells were centrifuged and washed with 1× phosphate-buffered saline. Then, after careful removal of the liquid supernatant, cell pellets were stored at −80°C until further processing. Genomic DNAs were isolated from the cells by standard phenol-chloroform extraction after cell lysis and proteinase K digestion. To remove RNA contamination from genomic DNA, samples were RNaseA treated at 37°C for 1 hour before proteinase K treatment.

miRNA analysis

Total RNA was isolated from cultured cells using either the Trizol reagent or the *mir*Vana miRNA Isolation Kit (Life Technologies); small RNA samples were isolated using the *mir*Vana miRNA Isolation Kit (Life Technologies). $\sim2\times10^6$ of cells were harvested and prepared according to the manufacturer's instructions. To remove DNA contaminations, RNA samples were treated with DNaseI (New England Biolabs) at 37°C for 1 hour. When applying the stem-loop qRT-PCR for cDNA preparations, if not indicated otherwise, 10 ng of total RNA (or gDNA, or plasmid) was reverse transcribed with miRNA specific stem-loop primers using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies). For the poly(A)-tailing based qRT-PCR method, the miRCURY LNA Universal RT microRNA PCR Starter Kit from Exiqon was used following the manufacturer's protocol. "no RT" controls were prepared by inactivating the reverse transcriptase at 98°C for 20 minutes prior to adding it to the cDNA Mix (stem-loop qRT-PCR) or leaving out the enzyme mix from the reaction (Exiqon Kit). Related sample series were prepared simultaneously and measured in the same plate during the real-time PCR reaction. The general "cutoff" value was 40 cycles, as a standard used by the program of the used instruments. We always used three technical replicates for the real-time PCR measurements, and biological and RT parallels as indicated in the figure legends. When measuring miR-33b-5p and miR-877-3p with different total RNA input (shown in Figure2A and B), we used the same three independent RNA samples (isolated from the respective miRNA overexpressing HeLa cell line) for the experiments with the U6 and miR-21-5p endogenous controls. For isomiR detections, synthetic 5′-phosphate RNA oligos were purchased from Sigma. Assuming an average of 10^5 miRNA copies per 10 ng of total RNA [30], diluted RNA oligos were used either alone or supplemented with 10 ng of control HeLa total RNA samples for reverse transcription. Quantification was performed by quantitative real-time PCR using either TaqMan MicroRNA Assays and TaqMan Universal Master Mix II with UNG (Life Technologies) or LNA PCR primers sets and ExiLENT SYBR Green master mix (Exiqon). The real-time quantification reactions were performed on StepOne™ or StepOnePlus™ platforms (Life Technologies), according to the manufacturer's instructions; the data was analyzed by StepOne software (version 2.1; Life Technologies). Our data were represented as by the StepOne program (relative to an endogenous control, delta delta Ct values) or when it was not relevant, as delta Ct values (comparing to a control sample). For statistical analysis, two-sided Student's t-test was performed. The following TaqMan MicroRNA Assays were used in miRNA quantification, catalog numbers are in brackets: U6 small nuclear RNA [001973], hsa-miR-1226-3p [245467_mat], hsa-miR-33b-5p = hsa-miR-33b-5p "GC" [002085], hsa-miR-33b-5p "GCA" [001565], hsa-miR-21-5p [000397] and hsa-miR-877-3p = hsa-miR-877* [241029_mat]. For custom made miRNA assays, the following RNA sequences were used for assay design: 5′-GUAGAGGAGAUGGCG-CAGGGGACA for the hsa-miR-877-5p "GACA" isomiR (= hsa-miR-877-5p), 5′-GUAGAGGAGAUGGCGCAGGG-GAC for the hsa-miR-877-5p "GAC" isomiR and 5′-GUAGAG-GAGAUGGCGCAGGGGA for the hsa-miR-877-5p "GA" isomiR. For Exiqon LNA PCR primers sets, the following assays were used, catalog numbers are in brackets: has-miR-33b-5p "GC" [205860] and custom designed mir-33b-5p-GCA_1 "GCA" [206999].

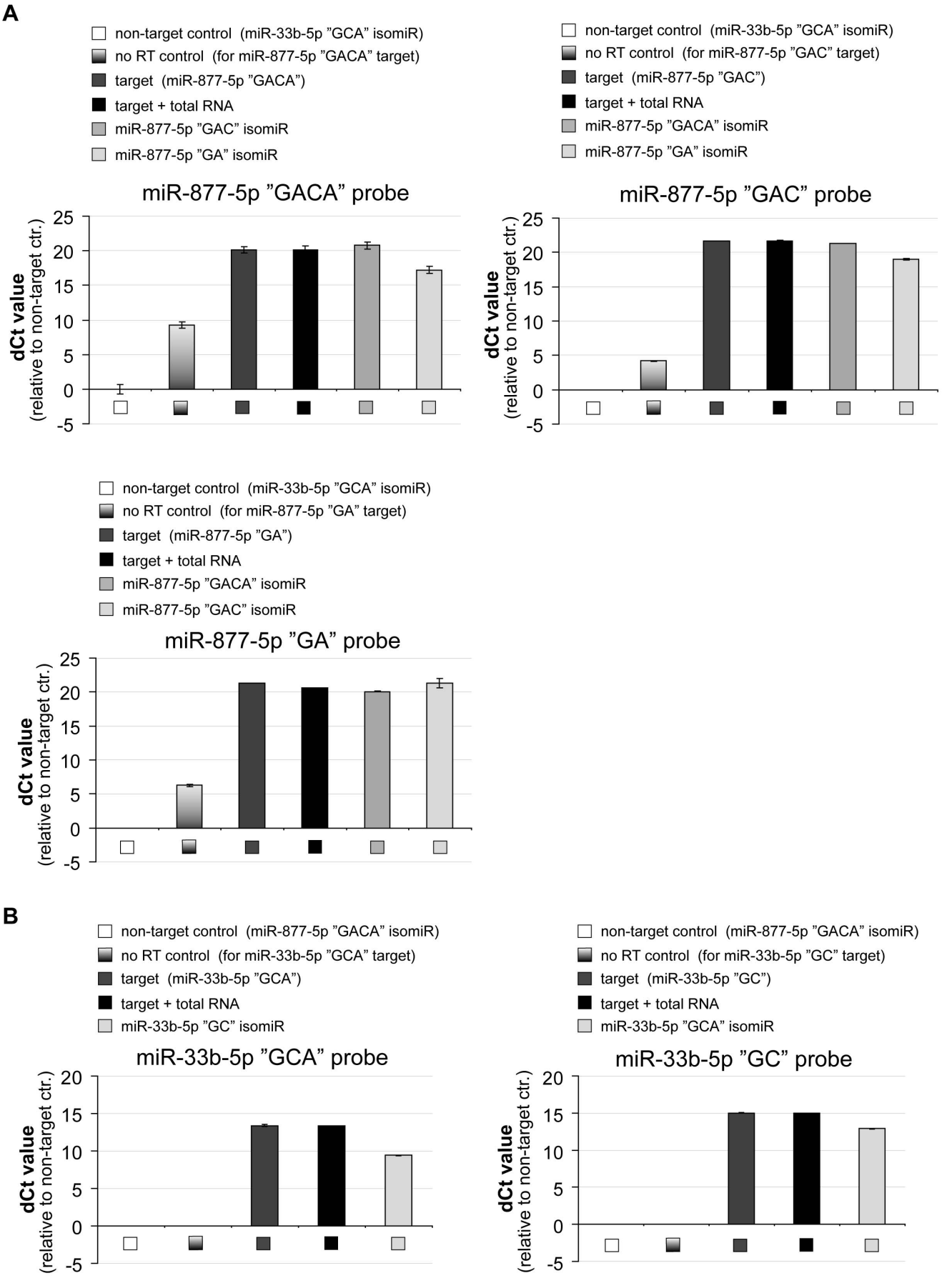

A
non-target control (miR-33b-5p "GCA" isomiR)
no RT control (for miR-877-5p "GACA" target)
target (miR-877-5p "GACA")
target + total RNA
miR-877-5p "GAC" isomiR
miR-877-5p "GA" isomiR
miR-877-5p "GACA" probe
dCt value
(relative to non-target ctr.)
25
20
15
10
5
0
-5
non-target control (miR-33b-5p "GCA" isomiR)
no RT control (for miR-877-5p "GAC" target)
target (miR-877-5p "GAC")
target + total RNA
miR-877-5p "GACA" isomiR
miR-877-5p "GA" isomiR
miR-877-5p "GAC" probe
dCt value
(relative to non-target ctr.)
non-target control (miR-33b-5p "GCA" isomiR)
no RT control (for miR-877-5p "GA" target)
target (miR-877-5p "GA")
target + total RNA
miR-877-5p "GACA" isomiR
miR-877-5p "GAC" isomiR
miR-877-5p "GA" probe
dCt value
(relative to non-target ctr.)
B
non-target control (miR-877-5p "GACA" isomiR)
no RT control (for miR-33b-5p "GCA" target)
target (miR-33b-5p "GCA")
target + total RNA
miR-33b-5p "GC" isomiR
miR-33b-5p "GCA" probe
dCt value
(relative to non-target ctr.)
20
15
10
5
0
-5
non-target control (miR-877-5p "GACA" isomiR)
no RT control (for miR-33b-5p "GC" target)
target (miR-33b-5p "GC")
target + total RNA
miR-33b-5p "GCA" isomiR
miR-33b-5p "GC" probe
dCt value
(relative to non-target ctr.)

Figure 6. 3′ isomiR species may not be distinguished by the stem-loop qRT-PCR. Detection of the 3′ isomiRs of miR-877-5p (**A**) and miR-33b-5p (**B**) by the corresponding stem-loop assays using synthetic RNA oligonucleotides as templates. Non-target controls were chosen as reference samples. (Ct values were 36/37/39,6 for the "GACA"/"GAC"/"GA" miR-877-5p isomiRs respectively, while signals were not detected up to 40 reaction cycles for the miR-33b isomiR assays.) The 3′ isomiRs of a particular miRNA locus are cross-detected using stem-loop qRT-PCR, although the extent varies among the different probes. Experiments were carried out at least in four independent measurements. One representative experiment is shown, error bars represent standard deviations.
doi:10.1371/journal.pone.0106315.g006

Supporting Information

Figure S1 Determination of reaction efficiencies of different targets. (**A**) Standard curves with 1.5× dilution series and 5 points. Template concentrations are presented in a logarithmic scale; R^2 values represent the correlation coefficients of the fitted lines. (**B**) Amplification efficiencies calculated from different ranges of the curves. 1–5 for five points; 2–5 for four points, omitting the obvious outlier of the measurement from the most concentrated template. (It is below the recommended minimum of 1:10 dilution of the cDNA sample in the qPCR reaction).
(TIF)

Figure S2 DNA can serve as a template for the reverse transcription reaction. False positive signal of DNA derives from the reverse transcription reaction. Mature miR-33b-5p assay was measured in the indicated samples, with or without reverse transcription (RT). On the y-axis, dC_t value is represented (calculated as the Ct difference between the examined samples and the gDNA of control HeLa_mir-1226 cell line). Control gDNA data are above C_t of 35; one C_t difference represents about 2× higher detected mature miRNA level. Experiments were carried out in three replicates; one representative experiment is shown, error bars represent standard deviations.
(TIF)

Figure S3 Residing DNA contamination in RNA samples prepared by different RNA isolation procedures by *mir*Vana miRNA Isolation Kit. RNA samples were isolated from parental (control) and transiently transfected HeLa cells. Samples were DNase treated and non-treated, then reverse transcribed and subjected to real-time PCR. Expression values relative to U6 snRNA are shown on the y-axis. Experiments were carried out with three technical replicates from three independent experiments (biol. repl.), error bars represent standard deviations. There is remaining DNA contamination in the total RNA samples (**A**), but not in the small RNA enriched samples (**B**) when prepared by the *mir*Vana Kit. The expression level of miR-1226-3p from total RNA (with DNase treatment) and from small RNA samples (with or without DNase treatment) is similar.
(TIF)

Figure S4 3′ isomiRs of miR-33b-5p are cross-detected using the poly(A)-tailing based quantitative RT-PCR method. 3′ isomiRs of miR-33b-5p were detected by isomiR-specific primer sets using synthetic RNA oligonucleotides as templates. Non-target controls served as references samples (Ct values >33). In the no RT control reactions, particularly no signals (Ct >39) were detected. The two different 3′ isomiRs are significantly cross-detected by the specific primer sets and even the post-PCR SYBR Green-based melting curve analysis could not make reliable distinction between the different isomiR-specific PCR products when applying mixed isomiR population as a template (data not shown). Experiments were carried out at least twice, one representative experiment is shown, error bars represent standard deviations.
(TIF)

Acknowledgments

We would like to thank Károly Fátyol for helpful discussions.

Author Contributions

Conceived and designed the experiments: AS TIO. Performed the experiments: AS TIO. Analyzed the data: AS TIO. Contributed reagents/materials/analysis tools: AS TIO. Wrote the paper: AS TIO.

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