the different therapeutic interventions that may be required for patients bearing one or the other condition (i.e., antithrombotic agents in the former or hemostatic agents in the latter). Although it is not possible to draw definite conclusions, it is reasonable to assume that using only 1 test to rule in or out LA when the clinical history of the patient being investigated is unknown may be risky, particularly if only 1 test is used. Two or more positive test results, particularly if they are from assays with different designs, probably are more informative and more likely to differentiate LA from anti-FVIII inhibitors.

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Use of Dual TaqMan Probes to Increase the Sensitivity of 1-Step Quantitative Reverse Transcription-PCR: Application to the Detection of SARS Coronavirus,

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Severe acute respiratory syndrome (SARS), caused by a novel coronavirus (SARS-CoV) *(1–5)*, has affected 8096 people and produced 774 deaths in 29 countries/regions *(6)*. The vital step in preventing and controlling future epidemics is to block transmission of infection through an effective quarantine policy, which in turn hinges on early diagnosis and confirmation of the disease, particularly by laboratory tests *(7)*. The need for rapid, ultrasensitive assays that can detect infection very early in the course of the disease is obvious.

The antibody response to SARS-CoV infection is detectable only after ~10 days of illness *(8)*; hence early laboratory diagnosis rests on early detection of the virus itself. Detection relies on reverse transcription followed by PCR (RT-PCR) *(7)*. We designed a 1-step real-time quantitative RT-PCR assay for SARS-CoV with the use of 2 TaqMan probes, instead of 1 probe, hybridizing to the same PCR product to further improve the sensitivity. This simple modification using dual TaqMan probes for quantification has wide applications in areas in which ultrasensitivity is critically required.

Our 1-step assay was designed to amplify the ORF1b regions of the SARS-CoV by TaqMan EZ RT-PCR Kit in a 7500 Real Time PCR System (Applied Biosystems). We compared assays using 1 and 2 TaqMan probes (Fig. 1, A and B). The 25-μL reaction mixture contained 1× TaqMan

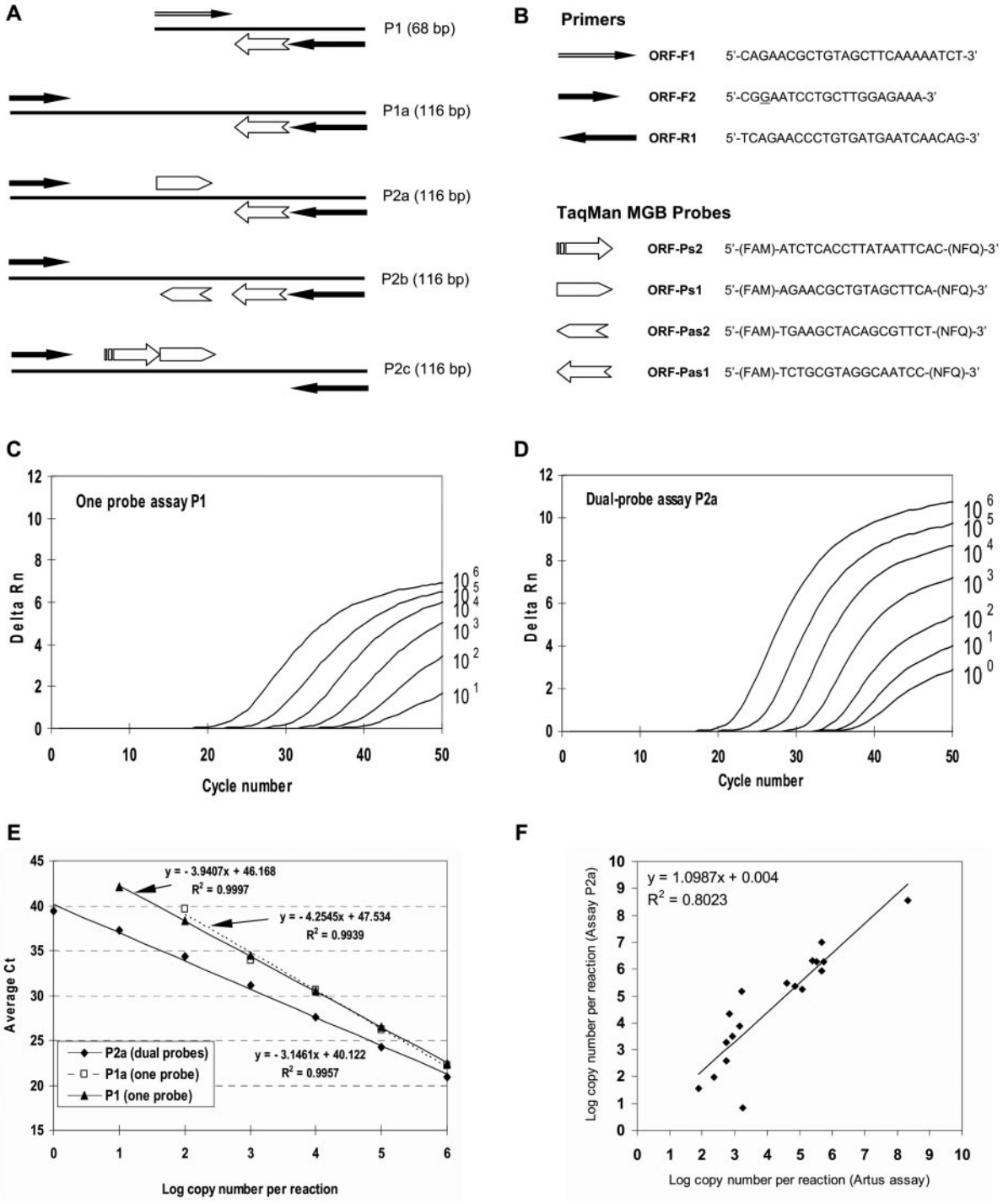

Fig.1. RT-PCR assay design and performance.

(*A*), 1-step RT-PCR assays with 1 (assay *P1*) or 2 TaqMan probes (assays *P2a* to *P2c*). The length of each amplicon is indicated in parentheses. The same forward primer was used for the 3 dual-probe assays and was upstream of that for the 1-probe assay. The *arrowheads* indicate the 3′ end of a primer or probe. (*B*), names and sequences of the primers and the TaqMan MGB probes shown together with the symbols used in *A*. Note that the TaqMan MGB probes (Applied Biosystems) are labeled with a 5′ reporter dye, 6-carboxyfluorescein (*FAM*), and a 3′ nonfluorescent quencher (*NFQ*) plus a minor grove binder (*MGB*) that stabilizes the probe–target duplex by binding the minor groove of double-stranded DNA *(31)*. (*C*), amplification plot of FAM fluorescence intensity against the PCR cycle for the P1 one-probe assay. *Delta Rn* (*y axis*) indicates the magnitude of the signal intensity generated by a given set of PCR conditions and is obtained from the equation: delta Rn = $(Rn^{+}) - (Rn^{-})$. The Rn^{+} value is obtained as a ratio of FAM fluorescence intensity to the fluorescence intensity of the passive reference dye (ROX) included in the reaction mixture for a PCR with template. The Rn^{-} value is similarly obtained as a ratio for a PCR without template (the no-template control). The RNA copy numbers per reaction are indicated on the *right* for each curve. (*D*), amplification plot of FAM fluorescence intensity against the PCR cycle for the P2a dual-probe assay. Assays P2b and P2c produced similar amplification plots (data not shown). (*E*), calibration curves for the P1 one-probe assay and the P2a dual-probe assay. Assays P2b and P2c produced calibration curves very similar to that for assay P2a (data not shown). (*F*), comparison of the RNA copy number per mL of input RNA sample determined by Artus assay (*x axis*) and the P2a dual-probe assay (*y axis*). The RNA samples were extracted from 18 confirmed SARS cases with 6 cases each providing stool, nasopharyngeal aspirate, and serum specimens.

EZ Buffer, 3 mM manganese acetate, 0.3 mM each deoxynucleotide triphosphate (except 1.2 mM for dUTP), 0.25 U of AmpErase UNG, 2.5 U of r*Tth* DNA polymerase, 0.8 μM each primer, 0.4 μM each probe, and 10 μL of extracted RNA (4 μL for the 1-probe assay). The 1-probe assay was based on a previous report *(9)*, but with slight modifications. Reactions were started by incubation at 50 °C for 2 min, followed by reverse transcription at 60 °C for 30 min, denaturation at 95 °C for 5 min, and amplification comprising 50 cycles of 95 °C for 15 s and 58 °C for 1 min. Calibrators were prepared from a concentrated RNA stock extracted from a SARS-CoV culture with the QIAamp Viral RNA Mini Kit (Qiagen) and quantified by RealArt HPA-Coronavirus LC RT-PCR Kit (Artus) in the Public Health Laboratory Centre. A no-template control was included in each run.

As expected, the final fluorescence intensity was up to twice as high and the threshold cycle number (Ct) smaller in the dual-probe assay (P2a; Fig. 1A) than in the 1-probe assay (P1; Fig. 1A) for a given input RNA copy number per reaction (Fig. 1, C and D). Comparison was made on the basis of copy number per reaction to account for the different input RNA volumes for the 2 assays. Moreover, the calibrator containing 1 RNA copy per reaction was detected 16 times out of 20 by the dual-probe assay but only 5 times out of 10 by the 1-probe assay (Fig. 1E). The improved sensitivity was not attributable to the change in the forward primer. On the contrary, assay P1a (Fig. 1A), which used the same forward primer as P2a but only the same 1 probe as in P1, hardly detected the calibrator containing 100 copies per reaction although it gave a calibration curve almost overlapping with that of P1 (Fig. 1E). This simple modification of using dual probes, instead of 1 probe, increased the sensitivity of the assay.

We further investigated the effect on the assay when the 2 probes hybridized to complementary target strands or to the same strand. In the P2a dual-probe assay, the 2 probes hybridized to complementary strands. In assay P2b, the 2 antisense probes hybridized to the same sense target strand, whereas the 2 sense probes in assay P2c hybridized to the same antisense target strand (Fig. 1A). Assays P2b and P2c gave the same results as assay P2a when the same series of calibrators was used. Thus, both cleavage efficiency and assay sensitivity were not affected whether the 2 probes were cleaved by the same polymerase molecule (as in assays P2b and P2c) or by 2 different polymerase molecules (as in assay P2a) for a given pair of complementary target strands in any single cycle. This in turn allows more flexible probe design even within a short stretch of sequences, provided that the 2 probes do not hybridize to each other.

We analyzed archived RNA samples from 18 SARS cases that were confirmed during the outbreak: 6 were extracted from stool (collected 3–21 days after onset of illness), 6 from nasopharyngeal aspirate (1–9 days after onset), and 6 from serum (1–10 days after onset). These RNA samples had previously been assayed by the Artus assay (concentrations, 7.7×10^1 to 2.1×10^8 per mL of RNA sample) in the Public Health Laboratory Centre, and all tested positive with our dual-probe assay P2a (concentrations, 6.9×10^1 to 3.7×10^8 per mL of RNA sample; Fig. 1F). This suggests that our dual-probe assay is at least as sensitive as the Artus assay because both could detect the SARS-CoV in clinical specimens collected in the early days after disease onset. To further evaluate the specificity of the P2a dual-probe assay, we analyzed RNA samples extracted from confirmed cases of influenza A (n = 10 each for H3N2 and H1N1), norovirus (n = 5), and from 2 other human coronaviruses (OC43 and 229E). All of these samples tested negative.

With improved RNA extraction methods *(10, 11)*, detection limits of 10 copies per RT-PCR reaction are common *(3, 9, 11–14)*, and a few studies have reported limits of detection of 5–8 copies per reaction *(15–17)*. An additional nested PCR step after RT-PCR *(18)* allowed detection of 1 copy per reaction but with a much reduced dynamic detection range *(19)*. Use of 2 TaqMan probes, instead of 1, increases the assay sensitivity without compromising the dynamic detection range. Even for an assay with a detection limit of 1 copy per reaction *(20)*, we speculate that the dual-probe strategy will increase the signal strengths and hence the assay reproducibility at the low detection range.

The use of 2 TaqMan probes labeled with 2 different reporter dyes in a single PCR for qualitative purposes such as allelic discrimination is well established, in which each allele-specific probe hybridizes only to an allele-specific amplicon but not the nonallelic amplicon *(21)*. As far as we are aware, this is the first report describing the use of 2 TaqMan probes labeled with the same reporter dye and hybridizing to the same amplicon for quantification purposes, which increases the sensitivity of the quantitative assay per se. Obviously, this strategy is applicable to both RNA and DNA as the input templates. This simple modification can also be applied to many areas in which ultrasensitivity or early detection of the target nucleic acids is of utmost importance, e.g., infections (SARS being just one example) and tumors. Increased assay sensitivity would counteract the dilution effect of pooling of donor plasmas for nucleic acid testing in the screening of transmissible infectious agents in blood donations *(22, 23)*. Quantification of residual tumor cells, e.g., leukemic cells, at early remission requires a sensitive assay *(24)*. Very limited amounts of target sequences are also encountered in paraffin-embedded tissue blocks *(25)*, ancient specimens *(26)*, forensic specimens taken from crime scenes *(26, 27)*, circulating nucleic acids *(28, 29)*, and engraftment of sex-mismatched organ transplantation *(30)*.

Our preliminary data indicate that the use of 3 probes did not further enhance the assay sensitivity, but rather increased the variability between duplicate readings. On the other hand, it is worth investigating whether the dual-probe strategy for quantification purposes can be extended to molecular beacons and hybridization probes.

In conclusion, we report the use of dual TaqMan probes for quantification purposes and apply it to the detection of

SARS-CoV with a detection limit of 1 copy RNA per reaction. This strategy is expected to be applicable to many areas requiring ultrasensitivity and/or early detection of target sequences.

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Clinical Relevance of Measurement of Antibodies to Individual snU_1-RNP Proteins, *Ariane Luyckx,[1] René Westhovens,[2] Els Oris,[1] Wolfgang Papisch,[3] and Xavier Bossuyt[1*]* ([1] Laboratory Medicine and [2] Internal Medicine, University Hospital Leuven, Leuven, Belgium; [3] Sweden Diagnostics, Freiburg, Germany; * address correspondence to this author at: Department of Laboratory Medicine, Immunology, University Hospital Leuven, Herestraat 49, B-3000 Leuven, Belgium; fax 32-13-347042, e-mail xavier.bossuyt@uz.kuleuven.ac.be)

Anti-ribonucleoprotein (RNP) antibodies are found in mixed connective tissue disease (MCTD), a syndrome characterized by features of systemic lupus erythematosus (SLE), inflammatory muscle disease, and scleroderma *(1)*. High titers of anti-RNP antibodies support the diagnosis of MCTD, and testing should be ordered when the diagnosis is suspected *(2)*. Anti-RNP antibodies are also found in rheumatic diseases such as SLE, Sjögren syndrome, rheumatoid arthritis, polymyositis, and systemic sclerosis *(2)*.