

Supplemental Information

Methods

Protocol: ssRPA-LFD.

Figure S1: Gels and full-length images of strips in main text Fig. 1d (sensitivity).

Figure S2: Quantification of spike-in RNA.

Figure S3: Full-length images of LFD strips in main text Fig. 1e (extended sensitivity).

Figure S4: Full-length images of LFD strips in main text Fig. 1f (specificity).

Figure S5: Gels and full-length images of LFD strips in main text Fig. 1g (clinical samples).

Figure S6: Demonstration of efficacy of extraction protocol.

Figure S7: Relative quantification of clinical sample RNA.

Methods. Sample sources. Synthetic SARS-CoV-2 RNA (Twist Biosciences, 102019) was used in sample qPCR quantification and in the serial dilution experiment of Fig. 1d. Synthetic fragment RNA (IDT), constituting the 76 nt region beginning at the 5' end of the spike sequence ("5' S region"), was used in sample qPCR quantification and ordered as Ultramers at 20 nmol scale. All synthetic primers and probes (IDT) were chemically synthesized with any specified modifications, ordered at 100 or 250 nmol scale, desalted or PAGE-purified, and used as is. Viral genomic RNA (isolated from infected cells) from coronaviruses 229E (ATCC, VR-740D), MERS (BEI, NR-50549), SARS-CoV-1 (BEI, NR-52346), and NL63 (BEI, NR-44105), as well as influenza A (ATCC, VR-1736D), influenza B (ATCC, VR-1535D), respiratory syncytial virus (ATCC, VR-1580DQ), and rhinovirus (ATCC, VR-1663D), were used for the specificity experiment in Fig. 1e. Cultured and heat-inactivated SARS-CoV-2 viral RNA (BEI, NR-52286) was used in specificity (Fig. 1e) and in spiked saliva (Fig. 1f) experiments. Pooled human saliva from ≥ 3 de-identified donors (Lee Biosolutions, 991-05-P) was collected prior to November 2019 and used to prepare the contrived samples. All clinical samples (Fig. 1g) were purchased from BioCollections Worldwide, Inc. and heat-inactivated at 95 °C for 5 min prior to shipping.

Primer and probe design. Relatively clean RPA products are the result of multiple primer pairs designed *in silico* for the appropriate salt and temperature conditions (NUPACK.org) and extensively tested empirically, with the goal of minimizing primer dimers and other unintended reactions that might slow the targeted amplification. RPA primer sequences and LFD probes for SARS-CoV-2 5' spike target were as follows, where "*" denotes a phosphorothioate bond (for exonuclease protection), "/iFluorT/" denotes an internal FAM fluorophore (for nanoparticle capture), "/3ddC/" denotes 3' dideoxycytidine (to prevent 3' end extension), and "/3Bio/" denotes 3' biotin (for test line capture). Here, the probe sequences partly overlap with primer sequences, but not enough to bind significantly to those sequences in isolation. This overlap is not necessary. Also note that, for extended protection, an internal fluorescein with poly-phosphorothioate protection is noted here, though we have had similar results with a simple 5' /56-FAM/ moiety. Probes and primers for 5' spike were: Fwd primer: T*T*T*T*T*T*T*TAACATCACTAGGTTTCAAACCTTACTTGC; Rev primer: CCAACCTGAAGAAGAATCA CCAGGAGTCAA; FAM LFD probe: T*T*T*T*T*T*T* /iFluorT/ TTTTTTTTTTTTTT AGGAGTCAA ATAACCTT/3ddC/; Biotin LFD probe: T*T*T*T*T*T*T* TATGTAAG GCAAGTAAAG TTTTTTTTTTTTTT /3Bio/.

Sample preparation. SARS-CoV-2 samples were quantified in-house, comparing fluorometrically-quantified synthetic RNA fragments (IDT), Twist Biosciences qPCR RNA standards, and culture-derived BEI viral genome samples, all diluted in DNase/RNase-free water (See SI Fig. S2). Synthetic fragment (IDT) RNA segment was quantified by Nanodrop spectrophotometry by blanking with IDTE buffer (IDT) and then measuring absorbance at 260 nm with a 2 μ l sample volume. Ten-fold serial dilutions of the synthetic fragment (IDT) RNA segment were made from 10^7 copies/ μ l down to 10^3 copies/ μ l in DNase/RNase-free water, using low binding tips and tubes to avoid sample loss. Similarly, Twist synthetic SARS-CoV-2 RNA were serially diluted in ten-fold ($0.1\times$, $0.01\times$, $0.001\times$) for comparison. Amplification by qPCR (Bio-Rad, CFX Connect) was then performed with $4\times$ TaqPath 1-Step RT-qPCR master mix (Life Science, A15300), 5' spike primers (100 nM), and EvaGreen dye (ThermoFisher) at 20 μ l total volume with 1 μ l sample volume. A linear regression was performed on the C_t and expected dilution concentrations of the standard ($R^2 = 0.99745$), and in turn used to convert sample C_t to absolute quantities of 552000, 55400, and 7200 copies/ μ l, respectively. This quantified Twist sample was then used in a dilution series to quantify the culture-derived (BEI) RNA using Charite "E" primer and sequence-specific probe set (IDT). Here, qPCR was performed with 5 μ l of $4\times$ TaqPath 1-Step RT-qPCR master mix, 3 μ l of Charite "E" primers/probes, and 7 μ l of DNase/RNase-free water at 20 μ l total volume with 5 μ l input sample volume. Simple and contrived samples were prepared by further diluting quantified samples as necessary, spiking into DNase/RNase-free water or pooled human saliva at ~ 2 or more copies per 5 μ l, and used directly in RT-RPA reactions. For the specificity experiments, genomic RNA from 8 respiratory viruses were spiked in DNase/RNase-free water at 10^5 copies/ μ l, unless noted (in which case quantification was not supplied from source). As a positive control, heat inactivated SARS-CoV-2 virus was used at 100 copies/ μ l. All were diluted 1:50 (1 μ l input into 50 μ l total reaction volume) in the RT-RPA reaction mixture.

RT-RPA. A mixture of 2.5 μ l each of 10 μ M forward and reverse primers to the specified target, 29.5 μ l of TwistAmp Basic RPA rehydration buffer (TwistDx, TABAS03KIT), 5 μ l of DNase/RNase-free water, and 0.5 μ l of Protoscript II reverse transcriptase (NEB, M0368S) were vortexed briefly and added to the TwistAmp lyophilized powder mix. Then, 5 μ l of sample was added to the tube while 5 μ l of 280 mM Magnesium Acetate was added to the reaction tube lid. The 50 μ l mixture was vortexed briefly, spun down, vortexed again, spun down once more and immediately incubated at 42 °C for 5 min in a standard PCR machine (Applied Biosystems, 4484073) or heating block (Benchmark Scientific, BSH300).

Amplicon digestion. During amplification, a digestion and LFD buffer mixture was prepared with 64 μ l of LFD running buffer (Milenia, MGH1), 1 μ l of 10 μ M biotin probes, 1 μ l of 10 μ M FAM probes, 10 μ l of $10\times$ NEBuffer 4 (NEB, B7004S), and 4 μ l of T7 exonuclease (NEB, M0263S), in a total volume of 80 μ l. The mixture was vortexed briefly and added to a 2 mL lo-bind tube (Eppendorf). Once complete, 8 μ l of the RT-RPA reaction was subsequently mixed thoroughly and completely with a 10% Sodium Dodecyl Sulfate (SDS) at a ratio of 8 μ l sample:12 μ l SDS to inactivate enzymes. This 20 μ l mixture was then added to the 80 μ l digestion mixture above and incubated for 1 min at room temperature.

Electrophoresis. All gels (8 \times 8 cm) were denaturing PAGE at 15% polyacrylamide (Invitrogen, EC6885BOX), run in $1\times$ TBE buffer that was diluted from $10\times$ TBE (Promega, V4251) with filtered water, at 65 °C, 200V, for 30 min. Immediately after 5 min of RT-RPA, 5 μ l of products were directly mixed with 5 μ l of formamide dye consisted of 100% formamide (ThermoFisher) and Bromophenol blue dye (Sigma Aldrich). Gels were then removed from cassettes, stained in $1\times$ SybrGold (Life Technologies) for 3 min, and imaged with a Typhoon scanner (General Electric). Ladders shown are 25–766 nt DNA (NEB, #B7025).

Lateral flow assay. A standard HybriDetect LFD strip (Milenia Biotec, MGH1) was inserted into the 2 mL Eppendorf tube above, arrows pointing up/away from the mixture, with care taken not to handle the strip roughly. Note that strips are covered with a membrane that protects the nitrocellulose and supported by a semi-rigid backing card. The strip was incubated for 2 min or longer, as desired.

Protocol: ssRPA-LFD

Materials needed:

- TwistAmp Basic Kit (thawed at ambient temperature); this protocol is specifically written to use these dried powder kits (Basic Kit) and has yet to be verified for the TwistAmp Liquid Kit
- Forward primer 10 μ M (IDT)
- Reverse primer 10 μ M (IDT)
- RNA template
- Protoscript II reverse transcriptase (NEB, M0368S)
- DNase/RNase-free water
- SDS
- Lateral flow strips and buffer (Milenia, MGH1)

Quick RNA extraction protocol (adapted from Ladha et al, doi: 10.1101/2020.05.07.20055947)

- 0.1. Take 5 μ l of patient sample (whether VTM, water, or saliva)
- 0.2. Mix with 5 μ l of Lucigen DNA extraction buffer
- 0.3. Incubate at 95 $^{\circ}$ C for 2 minutes
- 0.4. Take out the tube and immediately keep on ice
- 0.5. Use 5 μ l (out of the total 10 μ l per sample) for ssRPA

ssRPA protocol

Step 1: Set up and run RPA (on PRE-AMPLIFICATION BENCH)

* Please have the heat block set up before setting up reaction to ensure reaction time.

- 1.1. Prepare (per reaction) in the following order at room temperature:

5 μ l	DNase/RNase-free water
29.5 μ l	Rehydration buffer (included in TwistDX kit)
2.5 μ l	10 μ M forward primer
2.5 μ l	10 μ M reverse primer
0.5 μ l	Protoscript II Reverse Transcriptase

Vortex ~ 3 seconds and spin briefly (~ 3 seconds).

If making a master mix, ensure that $\sim(n+1)\times$ master mix solution is mixed for n samples to ensure that all samples get the full, correct amount of master mix.

- 1.2. Add above reaction to a TwistAmp Basic reaction (dried powder included in TwistDX kit). Do not mix or pipette up and down. Then add 5 μ L of RNA template directly into the tube (or water for the negative control).

1.3. Add 5 μ L of 280 mM Magnesium Acetate (included in TwistDX kit) to tube lid (this way, MgOAc is kept separate in the tube lid prior to overall mixing). Carefully close tube lid without touching the inside of the tube lid (to avoid any contamination from previous amplicons or other sources), vortex briefly (~ 2 seconds), spin down briefly (~ 2 seconds on a micro-centrifuge), then vortex again ~ 2 seconds to start reaction. Spin briefly (~ 2 seconds) before the next step.

- 1.4. Immediately, incubate it at 42 $^{\circ}$ C for 5 minutes.*

Step 2: Set up LFD (on POST-AMPLIFICATION bench)

- 2.1. While RPA is running, prepare (per reaction) the following in a 2 mL lo-bind tube:

1 μ l	10 μ M FAM probe
1 μ l	10 μ M protected biotin probe
64 μ l	running buffer
10 μ l	NEB buffer 4

Vortex and spin briefly, then to each add:

4 μ l	T7 exonuclease
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Vortex and spin briefly.

2.2. Add 12 μ L of 10% SDS to lid of the tube. (Avoid long spin-down of 10% SDS solution, and make sure to store at room temperature prior to this addition.) Once RPA reaction is complete, immediately vortex RPA samples for 3-5 seconds. Then add 8 μ L of RPA sample to lid and pipette up and down 25-30 times quickly and vigorously until white bubbles appear to mix RPA sample with SDS. Immediately, spin solution down, vortex for 5 seconds, spin for 5 seconds.

- 2.3. Incubate at room temperature for 1 minute (for exonuclease digestion).

2.4. Place the lateral flow strip into tube, incubate for 2 minutes (but may wait up to an hour).

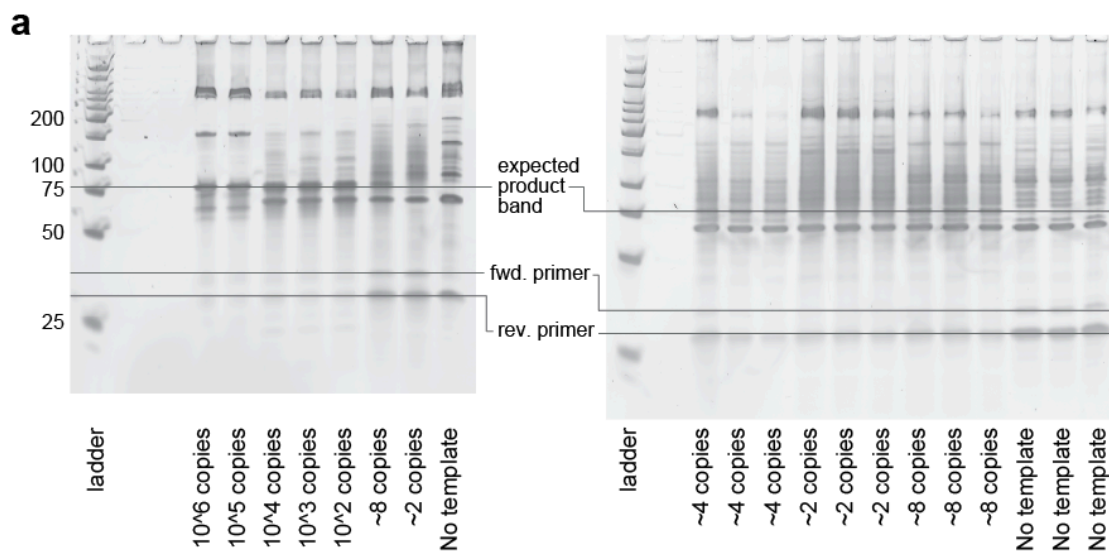
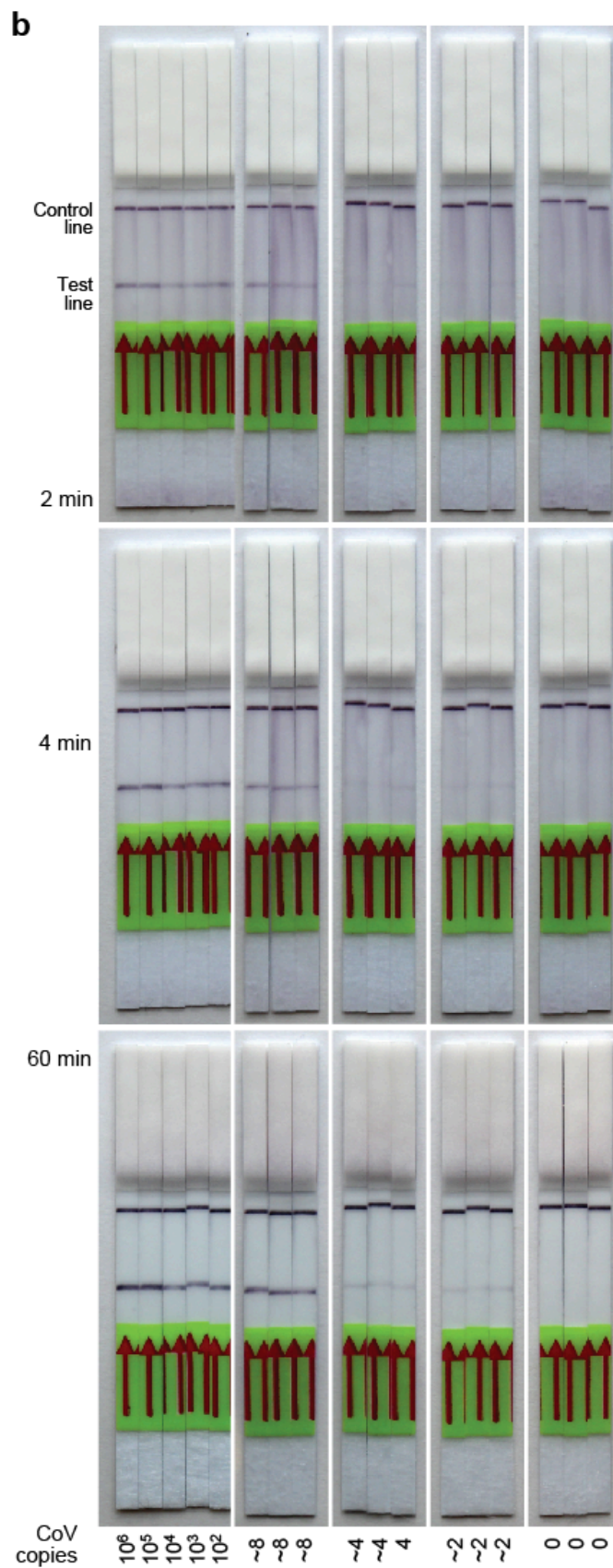


Figure S1: Gels and full-length images of strips in main text Figure 1d. (a) A denaturing PAGE gel showing the results of a 5 min RT-RPA for the series dilution of synthetic SARS-CoV-2 RNA shown in main text Figure 1d. Results show strong product bands over a large dynamic range of copy number. **(b, next page)** Full length LFD strips from main text Figure 1d, in which RT-RPA amplicons were subsequently treated via SDS addition, 1 minute T7 exonuclease digestion, and addition of LFD biotin and FAM probes.



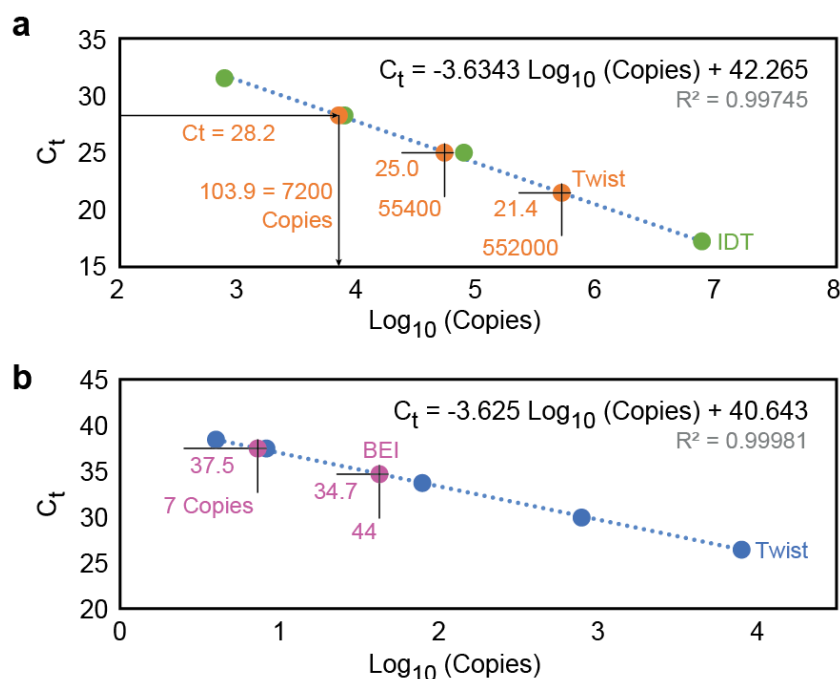


Figure S2: Quantification of spike-in RNA. Synthetic (IDT or Twist) and culture-derived (BEI) SARS-CoV-2 RNA were quantified by spectrophotometry and qPCR for spike-in experiments. **(a)** A 76 nt, 5' spike sequence RNA segment, synthesized by IDT and shipped at high (~ 100 μ M) concentration, was first quantified by Nanodrop (Thermo Fisher) spectrophotometry. The machine was calibrated and “blanked” with RNA-free buffer, and absorbance at 260 nm (A_{260}) was recorded for IDT RNA in the same buffer in triplicate. The average value (69.3 ± 0.15) was used in conjunction with the sequence-specific extinction coefficient (756800 /M/cm) to calculate a stock concentration of 91.6 μ M. This RNA was diluted and spiked at ~ 8E2 to 8E6 copies per 5 μ l into RT-qPCR reactions, with our ssRPA 5' spike primers and EvaGreen dye, and RT-qPCR was performed in triplicate as described in Methods. Values for C_t were recorded and a least squares fit was made between C_t and known copy number (shown), with $R^2 > 0.997$. In separate wells of the same run, 3 dilutions of Twist synthetic SARS-CoV-2 RNA were amplified in an identical manner, and those C_t values were converted to copy numbers by applying the described fit, yielding 7200 to 552,000 copies per 5 μ l. This quantified Twist sample was used in the spike-in buffer experiments of main text Fig. 1d. **(b)** This quantified Twist sample was then used in a dilution series to quantify the culture-derived (BEI) RNA at very low copy, to ensure that low-copy ssRPA spike-in experiments were accurate. As in (a), the Twist and BEI samples were both amplified by RT-qPCR, this time using Charite “E” primer and sequence-specific probe set (IDT) for maximum accuracy at low copy number. The Twist results were fit to the relation shown, in turn used to quantify BEI copy numbers to 44 and 7 per sample, as shown. These BEI dilutions were used in the spike-in saliva and specificity experiments of main text Fig. 1e and 1f.

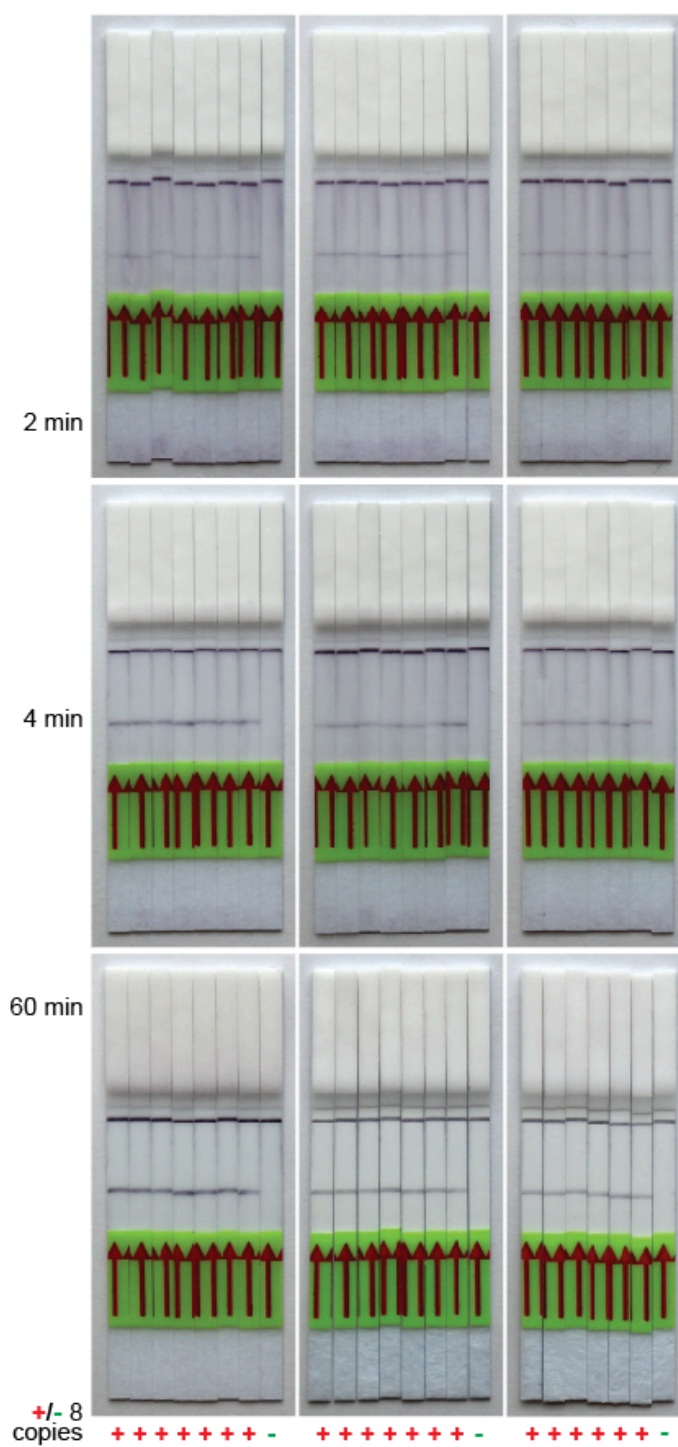


Figure S3: Full-length LFDs of main text Fig. 1e. LFDs were run as in main text, with 0 or 8 copies of cultured, heat inactivated SARS-CoV-2 virus (BEI) per reaction spiked in human pooled saliva, as the LFD incubation times indicated. LFD images were cropped and adjusted for contrast to appear as viewed by the naked eye.

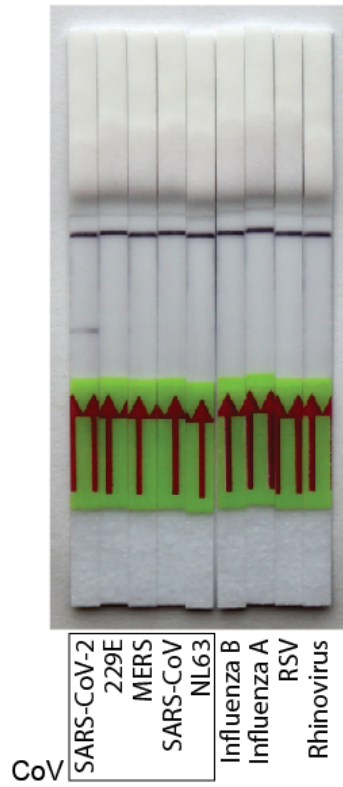


Figure S4: Full-length LFDs of main text Fig. 1f. LFDs were run as in main text, with high copy genomic RNA from the alternative diagnosis viruses shown. LFDs shown at 10 min to indicate lack of false positive bands. LFD images were cropped and adjusted for contrast to appear as viewed by the naked eye.

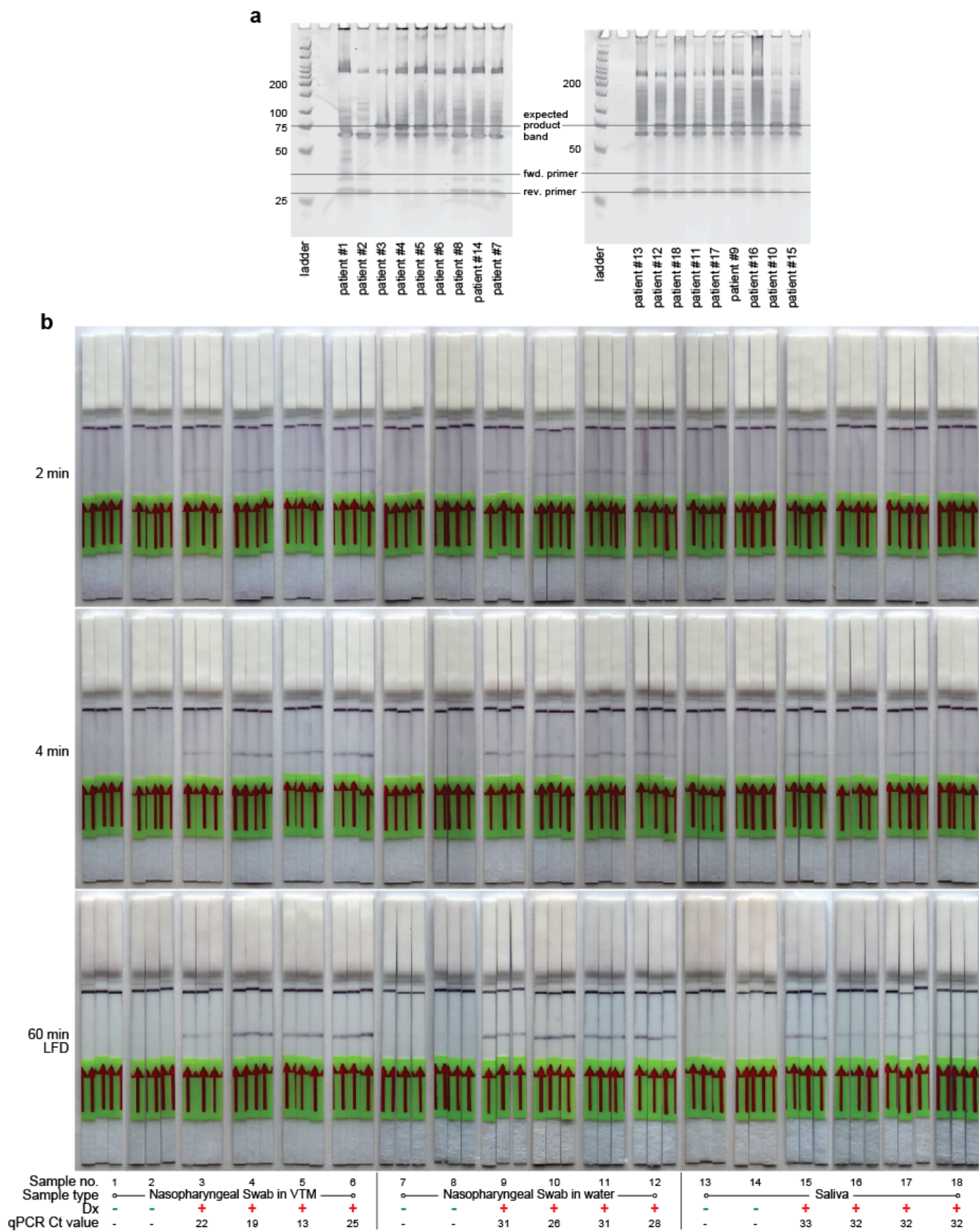


Figure S5: Gel and full-length LFDs of main text Fig. 1g. (a) A denaturing PAGE gel showing the results of a 5 min RT-RPA and subsequent SDS addition, 1 min T7 exonuclease digestions, and addition of LFD biotin and FAM probes, for the clinical samples shown in the first of each triplicate of main text Fig. 1g. **(b)** Full length LFD strips from main text Fig. 1g.

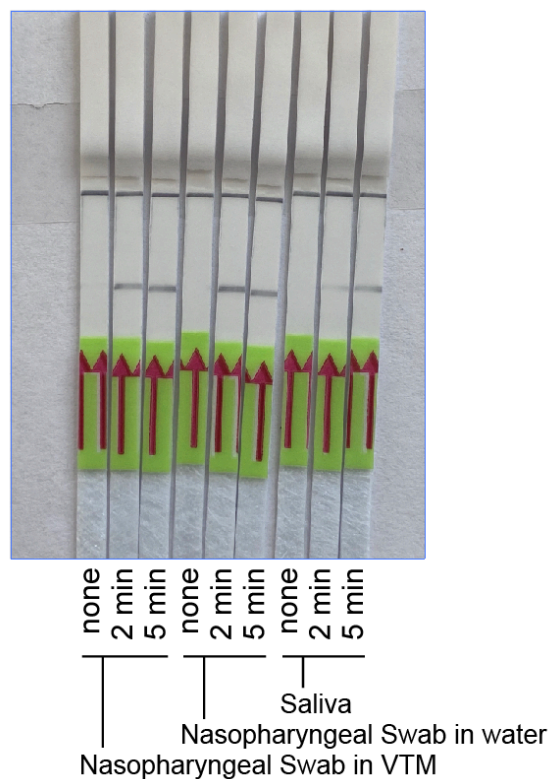


Figure S6: Demonstration of efficacy of extraction protocol. Clinical samples were received from vendor after heat inactivation ($95\text{ }^{\circ}\text{C} \times 5\text{ min}$) to protect researchers. The ssRPA protocol applied directly to shipped samples resulted in little or no signal, but the 2 min extraction protocol with Lucigen DNA extraction buffer (see Methods) was both necessary and sufficient to yield positive results. Strips were photographed at 60 min.

Sample No.	Sample type	vendor status	Ct	Ct	Ct	Average Ct
1	NP in VTM	negative	-	-	-	
2	NP in VTM	negative	-	-	-	
3	NP in VTM	positive	21.7	21.9	21.8	22
4	NP in VTM	positive	18.8	18.7	18.6	19
5	NP in VTM	positive	13.3	13.4	13.3	13
6	NP in VTM	positive	24.7	24.5	24.5	25
7	NP in water	negative	-	-	-	
8	NP in water	negative	-	-	n/a	
9	NP in water	positive	31.3	31.1	31.2	31
10	NP in water	positive	25.7	25.9	26.1	26
11	NP in water	positive	31.5	31.3	31.3	31
12	NP in water	positive	27.8	27.9	28.1	28
13	Saliva	negative	-	-	-	
14	Saliva	negative	-	-	-	
15	Saliva	positive	33.8	32.8	32.4	33
16	Saliva	positive	32.4	32.2	31.6	32
17	Saliva	positive	31.9	31.8	32.0	32
18	Saliva	positive	31.7	31.7	n/a	32

Figure S7: Clinical sample qPCR quantification. Clinical samples were received from vendor with assigned SARS-CoV-2 status based in vendor qPCR. We repeated qPCR quantification in a single experiment to compare relative C_t values across samples and to confirm RNA status. Volumes of 100 μ l of each raw clinical sample were extracted and buffer exchanged, per CDC qPCR recommendations, with the Qiagen QiaAMP viral RNA mini kit, eluting into 20 μ l. Column-extracted volumes of 5 μ l each were used in 20 μ l qPCR reactions with CDC N2 primers and probes and TaqPath 1-step RT-qPCR master mix, per Methods and CDC recommendations. Extracted samples were tested in triplicate when extraction volumes allowed (“n/a” indicates volumes were insufficient). Forty-four temperature cycles were applied, and those that did not cross the C_t threshold after 44 cycles are listed as “-”. Average C_t values are also shown and correspond to those in main text Fig. 1g. In other experiments not shown, it appeared that small column elution (extraction) volumes (meant to concentrate samples) led to a 5-fold or higher variation in column elution yield. Quantification C_t values shown are therefore only accurate to $\sim \pm 2$ cycles. Ranges nevertheless suggest relatively high RNA concentrations for NP swabs in VTM compared to NP swabs in water or in raw saliva.