Supporting Information for:

Solid-State Nanopore Counting of Amplicons from Recombinase Polymerase Isothermal Amplification

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Section S0: RPA Reaction Scheme

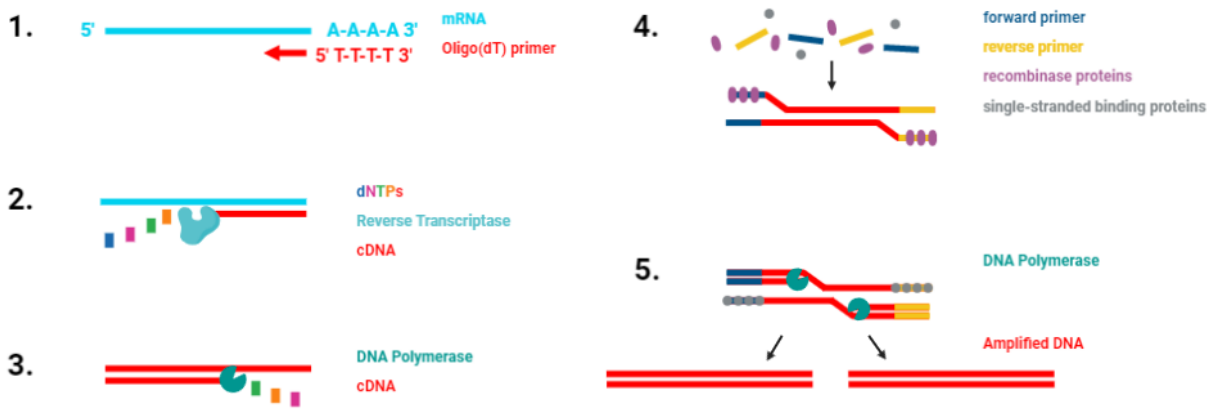

***Figure S0**. Schematic overview of RT-RPA. 1) mRNA is transcribed into ssDNA using an oligo(dT) primer. 2) Reverse transcriptase binds to the primer and synthesizes single-stranded cDNA by adding dNTPs to the RNA template, which forms a cDNA-mRNA hybrid strand. The RNA half of the strand is then degraded by the reverse transcriptase. 3) The ss-cDNA strand is used as a template to generate ds-cDNA by DNA polymerase. 4) Recombinase and RPA primer complexes form and target homogenous dsDNA strands. The strands are separated as the polymerase initiates synthesis. 5) The parental dsDNA strands separate completely, and synthesis continues until two duplexes are formed (until DNA is amplified).*

Section S1: Method of Primer Design

Multiple primers were designed for the nucleocapsid region of the Sars-Cov-2 genome, based on the approved primers from the CDC [1]. As a control, the yellow (P1) and dark blue (P3) CDC primer regions were used exactly as is to form a 466 bp amplicon.

[1] Lu X, Wang L, Sakthivel SK, Whitaker B, Murray J, Kamili S, et al. US CDC Real-Time Reverse Transcription PCR Panel for Detection of Severe Acute Respiratory Syndrome Coronavirus 2. Emerg Infect Dis. 2020;26(8):1654-1665. https://doi.org/10.3201/eid2608.201246

Excerpt of nucleocapsid region with CDC primer regions highlighted in color:

ATAATGGACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCTCAGATTCAACTGGC
AG
TAACCAGAATGGAGAACGCAGTGGGGCGCGATCAAAACAACGTCGGCCCCAAGGTTTACCCAATAAT
ACT
GCGTCTTGGTTCACCGCTCTCACTCAACATGGCAAGGAAGACCTTAAATTCCCTCGAGGACAAGGCGT
TC
CAATTAACACCAATAGCAGTCCAGATGACCAAATTGGCTACTACCGAAGAGCTACCAGACGAATTCGT
GG
TGGTGACGGTAAAATGAAAGATCTCAGTCCAAGATGGTATTTCTACTACCTAGGAACTGGGCCAGAAG
CT
GGACTTCCCTATGGTGCTAACAAAGACGGCATCATATGGGTTGCAACTGAGGGAGCCTTGAATACAC
CAA
AAGATCACATTGGCACCCGCAATCCTGCTAACAATGCTGCAATCGTGCTACAACTTCCTCAAGGAACA
AC
ATTGCCAAAAGGCTTCTACGCAGAAGGGAGCAGAGGCGGCAGTCAAGCCTCTTCTCGTTCCTCATCAC
GT
AGTCGCAACAGTTCAAGAAATTCAACTCCAGGCAGCAGTAGGGGAACTTCTCCTGCTAGAATGGCTGG
CA
ATGGCGGTGATGCTGCTCTTGCTTTGCTGCTGCTTGACAGATTGAACCAGCTTGAGAGCAAAATGTCTG
G
TAAAGGCCAACAACAACAAGGCCAAACTGTCACTAAGAAATCTGCTGCTGAGGCTTCTAAGAAGCCTC
GG
CAAAAACGTACTGCCACTAAAGCATACAATGTAACACAAGCTTTCGGCAGACGTGGTCCAGAACAAA
CCC
AAGGAAATTTTGGGGACCAGGAACTAATCAGACAAGGAACTGATTACAAACATTGGCCGCAAATTGC
ACA
ATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGTCACACCTTCGGGAAC
G

Full nucleocapsid region used for primer design (highlighted in yellow):

N – nucleocapsid phosphoprotein
location: 28274 – 29533
protein ID: YP_009724397.2

28261 cgaacaaact aaaatgtctg ataatggacc ccaaaatcag cgaaatgcac cccgcattac
28321 gtttggtgga ccctcagatt caactggcag taaccagaat ggagaacgca gtggggcgcg
28381 atcaaaacaa cgtcggcccc aaggtttacc caataatact gcgtcttggt tcaccgctct
28441 cactcaacat ggcaaggaag accttaaatt ccctcgagga caaggcgttc caattaacac
28501 caatagcagt ccagatgacc aaattggcta ctaccgaaga gctaccagac gaattcgtgg
28561 tggtgacggt aaaatgaaag atctcagtcc aagatggtat ttctactacc taggaactgg
28621 gccagaagct ggacttccct atggtgctaa caaagacggc atcatatggg ttgcaactga
28681 gggagccttg aatacaccaa aagatcacat tggcacccgc aatcctgcta acaatgctgc

28741 aatcgtgcta caacttcctc aaggaacaac attgccaaaa ggcttctacg cagaagggag
28801 cagaggcggc agtcaagcct cttctcgttc ctcatcacgt agtcgcaaca gttcaagaaa
28861 ttcaactcca ggcagcagta ggggaacttc tcctgctaga atggctggca atggcggtga
28921 tgctgctctt gctttgctgc tgcttgacag attgaaccag cttgagagca aaatgtctgg
28981 taaaggccaa caacaacaag gccaaactgt cactaagaaa tctgctgctg aggcttctaa
29041 gaagcctcgg caaaaacgta ctgccactaa agcatacaat gtaacacaag ctttcggcag
29101 acgtggtcca gaacaaaccc aaggaaattt tggggaccag gaactaatca gacaaggaac
29161 tgattacaaa cattggccgc aaattgcaca atttgccccc agcgcttcag cgttcttcgg
29221 aatgtcgcgc attggcatgg aagtcacacc ttcgggaacg tggttgacct acacaggtgc
29281 catcaaattg gatgacaaag atccaaattt caaagatcaa gtcattttgc tgaataagca
29341 tattgacgca tacaaaacat tcccaccaac agagcctaaa aaggacaaaa agaagaaggc
29401 tgatgaaact caagccttac cgcagagaca gaagaaacag caaactgtga ctcttcttcc
29461 tgctgcagat ttggatgatt tctccaaaca attgcaacaa tccatgagca gtgctgactc
29521 aactcaggcc taaactcatg cagaccacac aaggcagatg ggctatataa acgtttcgc

Final amplicon from best primer set (**p7BF**+**p7BR**)- amplicon 363:

GAACTAATCAGACAAGGAACTGATTACAAACATTGGCCGCAAATTGCACAATTTGCCCCCAGCGCTT
CAGCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGTCACACCTTCGGGAACGTGGTTGACCTACACA
GGTGCCATCAAATTGGATGACAAAGATCCAAATTTCAAAGATCAAGTCATTTTGCTGAATAAGCATAT
TGACGCATACAAAACATTCCCACCAACAGAGCCTAAAAAGGACAAAAAGAAGAAGGCTGATGAAACT
CAAGCCTTACCGCAGAGACAGAAGAAACAGCAAACTGTGACTCTTCTTCCTGCTGCAGATTTG**GATGA**
TTTCTCCAAACAATTGCAACAATCC

Table S1: Primer Sequence Information

Name	**Sequence**
P10F	GAGCCTTGAATACACCAAAAGATCACATTG
P10R	TTTGTAATCAGTTCCTTGTCTGATTAGTTC
P7BF	GAACTAATCAGACAAGGAACTGATTACAAA
P7BR	GGATTGTTGCAATTGTTTGGAGAAATCATC
NF1A	TCAGCGAAATGCACCCCGCATTACGTTTGG
NF1B	TGGACCCTCAGATTCAACTGGCAGTAACCAGA
NR3A	AAGCCTTTTGGCAATGTTGTTCCTTGAGGAAGT
P10FA	GCACCCGCAATCCTGCTAACAATGCTGCAATC
P10FB	GTGCTACAACTTCCTCAAGGAACAACATTGCC
P7BRA	CAAATCTGCAGCAGGAAGAAGAGTCACAGT
P7BRB	TTGCTGTTTCTTCTGTCTCTGCGGTAAGGC
P1	GACCCCAAAATCAGCGAAAT
P3	TGTAGCACGATTGCAGCATTG

Table S2: Primer pairs and target amplicon sizes

Primer Set (PS)	**Primer A**	**Primer B**	**Size of Amplicon**
1	p10F	p10R	488
2	p7BF	p7BR	363
3	p7BF	p7BRA	333
4	p7BF	p7BRB	303
5	p10FA	p10R	485
6	p10FB	p10R	426
7	NF1A	NR3A	489
8	NF1B	NR3A	459
C	P1	P3	466

Section S2: Gel Analysis of Control Reactions and Primer Screen

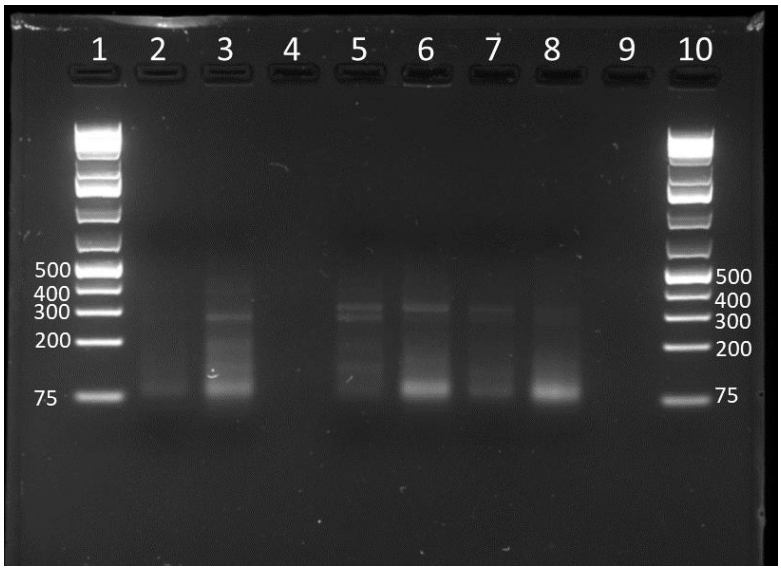

Figure S1: *TwistDx control DNA RPA reactions. The RPA reactions were carried out according to the manufacturer instructions for "Performing positive control reactions"[2] (twistdx.co.uk). Lanes: 1, 10: Gene Ruler 1 kb Plus DNA ladder (Thermo Scientific™ GeneRuler 1 kb Plus DNA Ladder | Fisher Scientific); 2: control reaction (no DNA template), column purified; 3: control reaction (no DNA template), direct RPA product; 5,7: positive reaction with DNA template, column purified; 6,8: positive reaction with DNA template, direct RPA product. Having used the DNA template and primers provided by the kit, the expected amplicon was 289 basepairs.*

[2] https://www.twistdx.co.uk/wp-content/uploads/2021/04/twistamp-liquid-combined-manual_revb_v2-web.pdf

Results: This experiment revealed that RPA produces non-specific amplification, even in the presence of the target DNA that in some cases is relatively abundant to the desired amplicon. Comparison of commercially available PCR clean-up column purified reactions versus direct RPA product run on agarose gel and stained using GelRed suggest that non-specific amplicons are not efficiently removed. Though the intensity of non-specific amplicon bands is decreased, so is the intensity of the desired amplicon. The kit manual indicates that non-specific amplification may occur at lower copy numbers and that either a PCR clean up kit, or both a clean-up kit and gel electrophoresis may be required to isolate the product. The presence of non-specific amplicon is particularly challenging for nanopore analysis, as products between 75-300 pb are challenging to resolve.

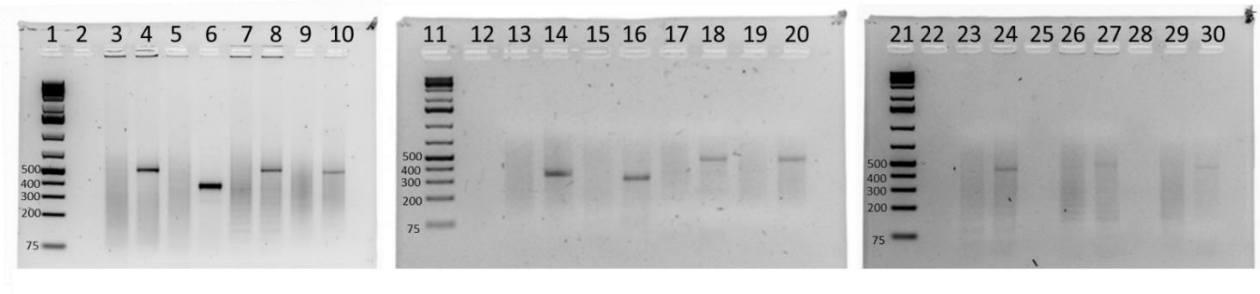

Figure S2: *Primer Screen with RNA Template. The primer pairs were screened against the RNA template according to the manufacturer's instructions for "Primer Screen Set Up" [2]. Lanes: 1, 11, 21: 1kb Generuler DNA ladder; 3: PS1 control; 4: PS1 + RNA; 5: PS2 control; 6: PS2 + RNA; 7: PS7 control; 8: PS7 + RNA; 9,19,29: PSC control; 10,20,30: PSC + RNA; 13: PS3 control; 14: PS3 + RNA; 15: PS4 control; 16: PS4 + RNA; 17: PS5 control; 18: PS5 + RNA; 23: PS6 control; 24: PS6 + RNA; 26: PS8 control; 27: PS8 + RNA. Expected amplicon sizes were: PS1 – 488 bp, PS2 – 363 bp, PS3 – 333 bp, PS4 – 485 bp, PS5 – 485 bp, PS6 – 426, PS7 – 489 bp, PS8 – 459, PSC – 466 bp. Abbreviations: primer set (PS), base pairs (bp), reactions with non-template control (control), reactions with RNA template (RNA)*

Results: The best primer set was determined to be that which showed minimal non-specific amplification in both the non-template control reaction and the RNA template positive reaction. Additionally, amplification efficiency, meaning the ratio of positive amplicon to non-specific amplicon in the positive reaction was also considered. Since the primer screen was completed over multiple days, primer set C was used to compare between reaction sets. The best primer set was determined to be PS2. Compared to all other sets, it had the most efficient amplification in the positive reaction and showed minimal non-specific amplification in the control reaction.

Section S3: RPA Reaction Optimization

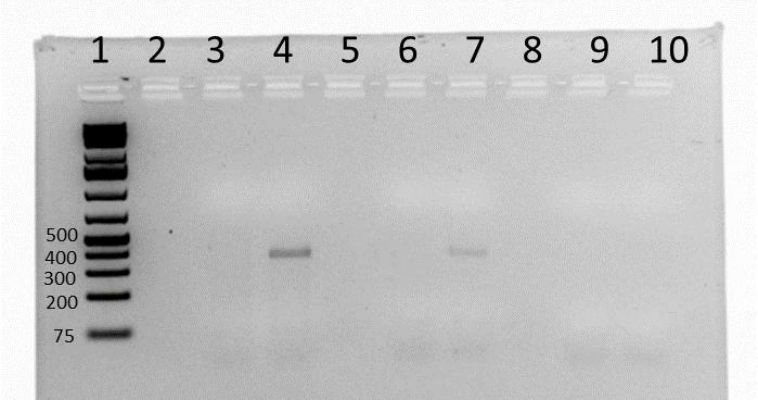

Figure S3*: Optimization of RPA Reaction Temperature using Primer Set 2. Lanes: 1: Generuler 1kb Plus DNA Ladder, 3: 42°C NTC; 4: 42°C + RNA; 6: 35°C NTC; 7: 35°C + RNA; 9: 42°C for 5 min, then RT for 15 min NTC; 10: 42°C for 5 min, then RT for 15 min + RNA. Abbreviations: non-template control (NTC), room temperature (RT).*

Results: the temperatures evaluated were chosen since 42°C is the ideal functional temperature for reverse transcriptase, and 35°C was chosen as it is equivalent to being held in hand (in lieu of a hot plate). Amplification was observed at both 42°C and 35°C, albeit less efficient for the latter. When the reaction was held at 42°C for 5 minutes then left at RT without RNA template (i.e. NTC), amplification was not observed. Therefore, 42°C was chosen as the ideal reaction temperature.

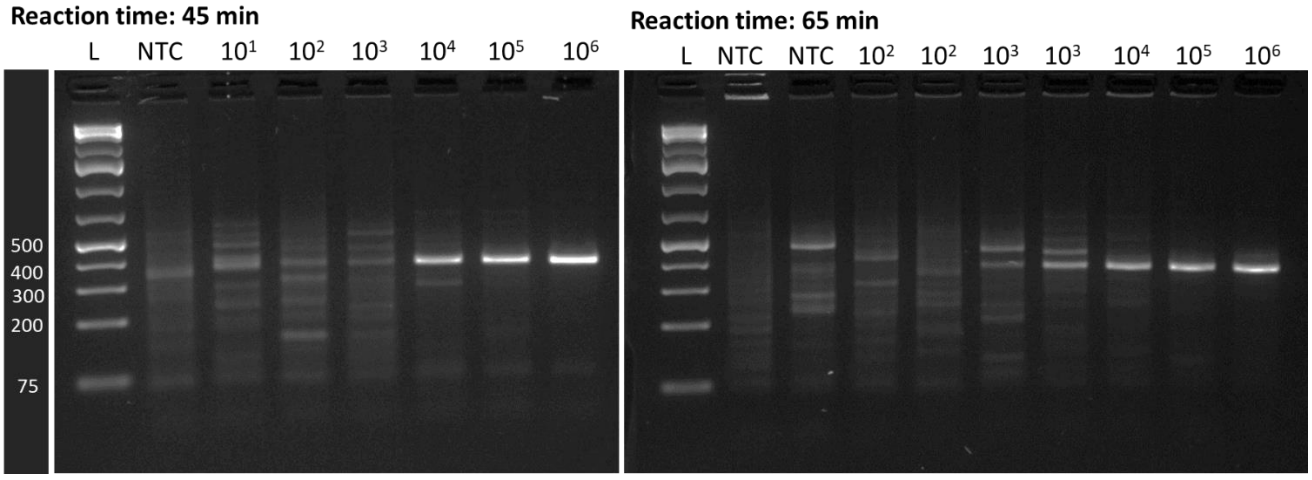

Figure S4: *RPA Reaction Sensitivity at 42°C. The reaction sensitivity was determined at two reaction times, 45 and 65 minutes. In both cases, 10^4 copies could be detected reliably. A challenge of RPA is that reproducibility is inconsistent. In the optimization work, clear target amplicon bands were observed at as low as 10^2 copies (not shown), but it was not replicated. Here amplicon band is observed in the 45 min trial, but not the 65 min trials.*

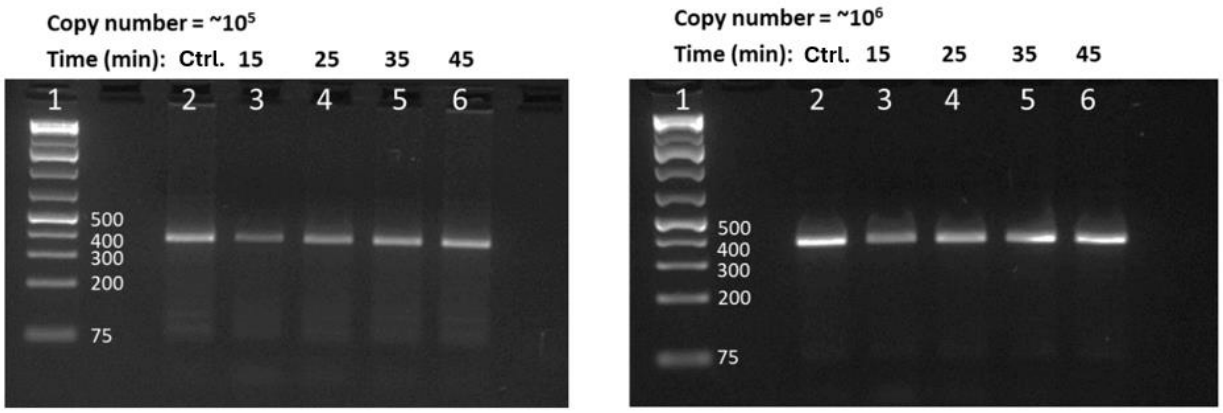

Figure S5: *Optimization of RPA reaction time using Primer Set 2. Amplicons were compared to lane 1 containing Generuler 1kb plus DNA ladder. The ideal reaction time was determined by assessing the RPA reaction efficiency with a constant copy number at different time points ranging from 15-45 minutes. With ~ 10^5 or 10^6 copy numbers, chosen for reproducibility, efficient amplification with minimal non-specific amplification was observed as early as 15 minutes.*

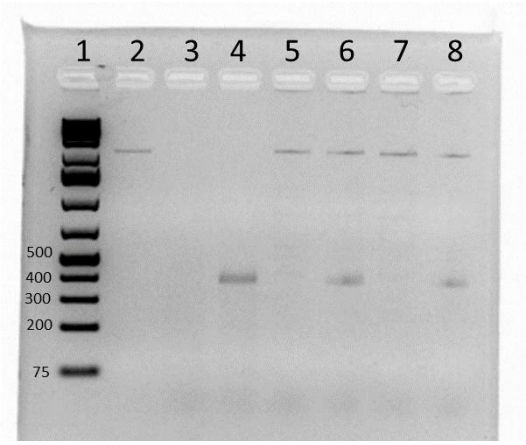

Figure S6: *RPA reaction in presence of 2kb DNA using Primer Set 2 run at 42°C for 20 min. Lanes: 1: Generuler 1 kb plus DNA ladder; 2: 2kb dsDNA fragment control; 3: NTC; 4: positive RPA reaction; 5: NTC + 2kb fragment; 6: RNA + 2kb fragment; 7: NTC + 2kb fragment replicate; 8: RNA + 2kb fragment replicate.*

Results: it was important to determine that the 2 kbp fragment would not interfere with the RPA reaction. From this experiment, it was clear that the 2 kbp fragment was not producing non-specific amplicons.

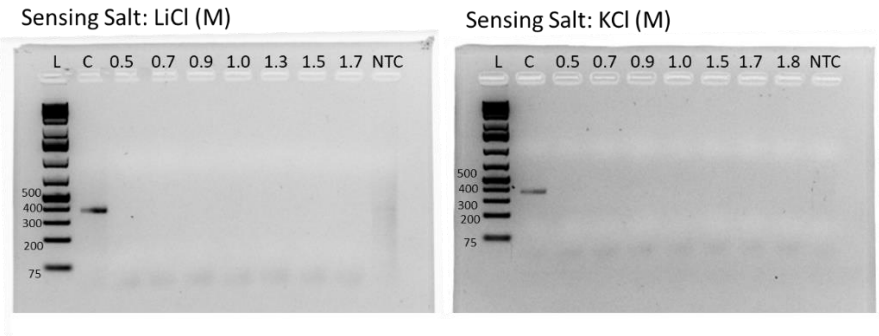

Figure S7: *RPA efficiency in the presence of metal salts, LiCl or KCl. Various salt concentrations ranging from 0.5 to 1.8 M were added to the RPA reaction mixtures. The reactions were run for 30 min at 42°C. No amplification was observed in the presence of either salt, indicating the high salt concentration was prohibitive. Therefore, it was necessary to complete detection of the amplicon as a two-step process, RPA reaction then sensing, rather than a simultaneous process with RPA and nanopore sensing at the same time.*

B. Nanopore Sensing and Data Analysis

Section S4: Classification of Amplicon Events

To classify the events produced by amplicon translocation, we compared short dsDNA fragments to the signals produced by the RPA products. From Figure S8, we can determine that 400 bp dsDNA translocations have mean current deviations of ~19 μs and dwell times of ~2200 pA for the operating conditions used in this study. We use the DNA fragment event histogram peaks as a benchmark for the expected translocation characteristics of the amplicons.

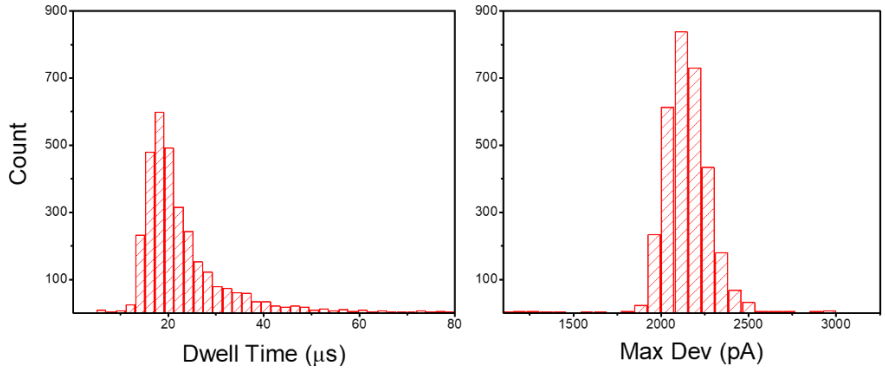

Figure S8: *Histograms of dwell time (left) and maximum current deviation (right) of translocation events with 400 bp dsDNA on a 5.1 nm pore.*

Using this information, we fitted a Gaussian peak to this expected amplicon distribution (Figure S9). From this fit, we extracted the mean and standard deviation to be used in our signal classification.

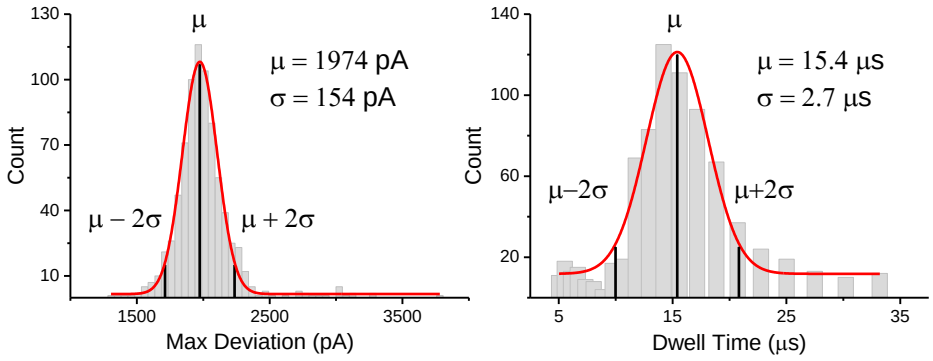

Figure S9: *Histograms and fitted Gaussian curves of maximum current deviation (left) and dwell time (right) of translocation events with isothermal amplification from 10^6 RNA template copies on a 4.8 nm pore.*

The signal classification uses an asymmetric elliptical selection filter centered on the mean values of dwell time and maximum deviation for each dataset. The filter spans 2σ on either side of the mean maximum current deviation, while also spanning 2σ to the left and 4σ to the right of the mean dwell time, selecting longer amplicon translocation events. The asymmetry of the filter along the translocation time

axis helps to offset the impact of background signals on the quantification of amplicon products as it selectively filters out some of the attenuated background noise. Figure S10 shows an example of the filter applied to a sample amplified from 10^6 initial RNA template copies.

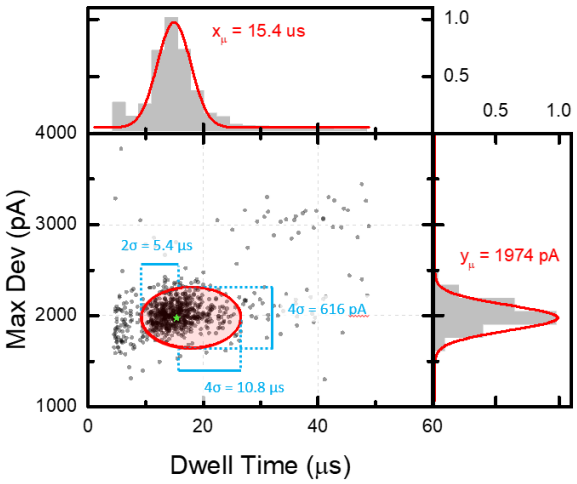

Figure S10: *Signal classification filter applied to sample amplified from 10^6 initial RNA template copies. The elliptical filter is centered at the mean obtained above (15.4 µs, 1974 pA, visible as a green star) with major axes spanning ± 2SD (308 pA) on either side of y_μ. The horizontal axis spans -2SD (5.42 µs) and +4SD (10.84 µs) around x_μ.*

To facilitate applying this filter to the data, we artificially shifted the mean translocation time +1σ and applied an elliptical filter spanning ±3σ of the mean translocation time, as seen in Equation 1 in the main text.

$$1 > \left(\frac{\left(\tau - (\bar{\tau} + \sigma_\tau)\right)^2}{9\sigma_\tau^2} + \frac{(\Delta G - \overline{\Delta G})^2}{4\sigma_{\Delta G}^2} \right) \quad (1)$$

Section S5: Optimization of Nanopore Sensing Conditions

The nanopore sensing conditions used in this study were selected to maximize pore longevity and minimize background noise from non-specific amplification products. Samples were run on ~5 nm pores at 200mV in pH 8 3.6M LiCl, and some samples were filtered using a PCR-purification kit to minimize pore clogging from remnant proteins.

Figure S11 presents the nanopore detection of unfiltered RPA products alongside 5 kbp dsDNA fragments on a 7.1 ± 0.5 nm pore. This pore size was too large and allowed passage of many amplification proteins that completely obstructed the amplicon signal. Smaller pores (< 4 nm) were prone to clogging immediately. Consequently, ~5 nm pores were targeted for sensing.

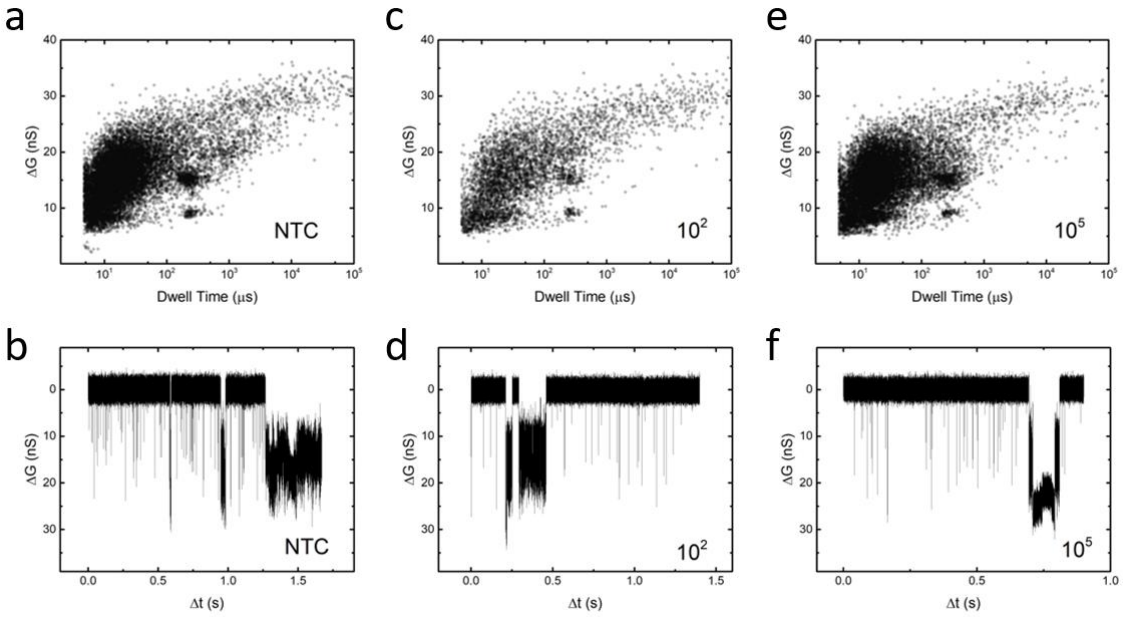

Figure S11. *Nanopore detection of unfiltered RPA products and 3.1 nM 5 kbp DNA fragments. (a) Conductance blockage of an event versus its passage time from NT-control. (b) Representative NT-control current trace over ~2s, with pore clogging shown at the end. (c) Conductance blockage of an event versus its passage time for RPA products from amplification using 10^2 initial RNA template copies. (d) ~2s current trace for 10^2. (e) Conductance blockage of an event versus its passage time for RPA products from amplification using 10^5 initial RNA template copies. (f) ~2s current trace for 10^5. Data was acquired on a 7.1 ± 0.5 nm nanopore at 200 mV in pH 8 3.6 M LiCl. Data was low pass filtered at 400 kHz.*

Figure S12 presents another experiment in which non-optimized sensing conditions were used. The RPA products were cleaned using a PCR-purification kit and sensed alongside 2 kbp dsDNA fragments in a 6.8 ± 0.7 nm pore at 300 mV. Although the purification kit filtered out most of the background noise, the large pore size and higher applied voltage caused the amplicon population to be completely indistinguishable from shorter-fragment background molecules, and a larger number of events being attenuated at < 10 μs.

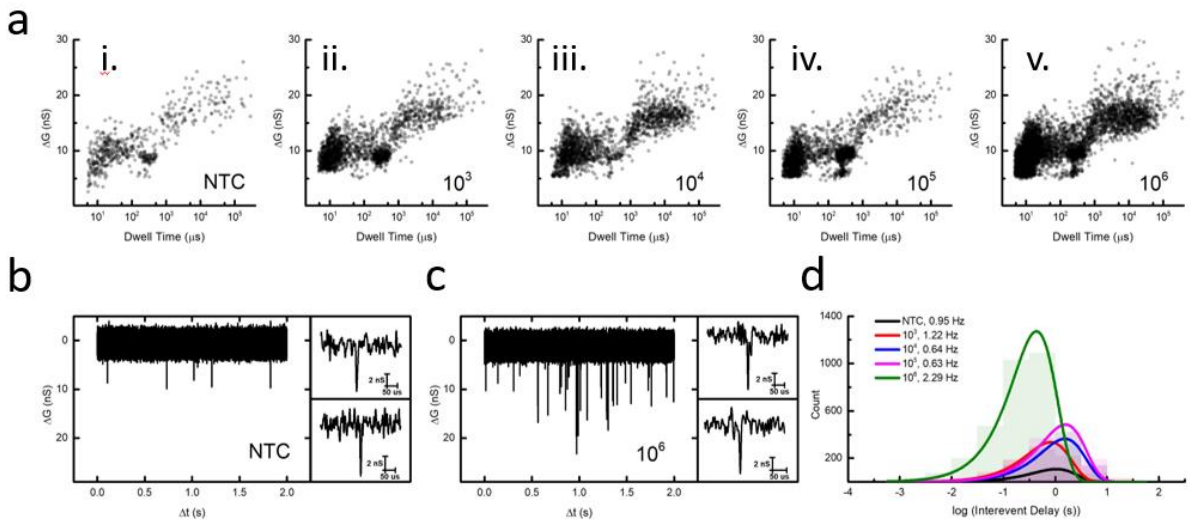

Figure S12. *Nanopore detection of RPA products in non-optimized sensing conditions. (a) Conductance blockage of an event versus its passage time for (i) NT-Control, (ii) 10^3 initial RNA template copies, (iii) 10^4 initial RNA template copies, (iv) 10^5 initial RNA template copies, (v) 10^6 initial RNA template copies. Amplicons were sensed alongside 7.7 nM 2kbp dsDNA fragments. (b-c) event and current traces for NT-Control and 10^6 samples illustrating the similarities in event shape and duration of both samples. (d) Distribution of the log of inter-event times for all events <100us from each sample. Data was acquired on a 6.8 ± 0.7 nm nanopore at 300 mV in pH 8 3.6M LiCl. Data was low pass filtered at 200 kHz.*

Under these sensing conditions, events cannot be classified effectively using the same elliptical filter and are indistinguishable from background noise.

As mentioned, some of the RPA products were filtered using a PCR-purification kit prior to sensing to minimize pore clogging. Figure S13 presents an experiment in which the pore clogged prior to running all controls.

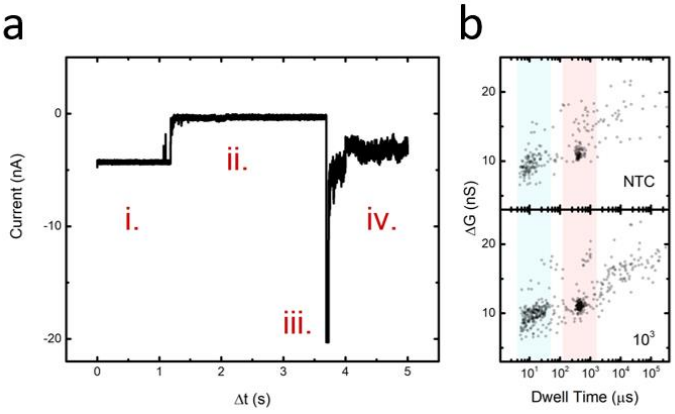

Figure S13. *Example of pore clogging prior to running all controls. (a) Current trace illustrating when the 10^3 sample clogged after ~500s. Four different segments of this trace are shown: (i) Stable open-pore current with a few translocation events, (ii) a large molecule clogs the pore and blocks almost 100% of the current, (iii) a 1V voltage 'zap' was applied to try and dislodge the molecule, (iv) the molecule is still stuck, the pore is now irreversibly clogged, which is shown by the resulting highly unstable current. (b) Scatterplots of the NT-control and 10^3 passage events. The amplicon events are highlighted in blue, and the 7kbp dsDNA passage events are highlighted in red. Data was acquired on a 4.5 ± 0.5 nm nanopore at 200 mV in 3.6 M LiCl 10 mM HEPES pH 8. Data was low-pass filtered at 400 kHz prior to analysis.*

Section S6: Template Concentration Sweep with 2 kbp Internal Control

Here, we present the full dataset from Figure 3 in the main text, including the 2 kbp dsDNA internal control. The dsDNA population was filtered out in the main text to 'zoom in' on the amplicon population to more clearly illustrate the capture rate increase at higher initial template copy numbers.

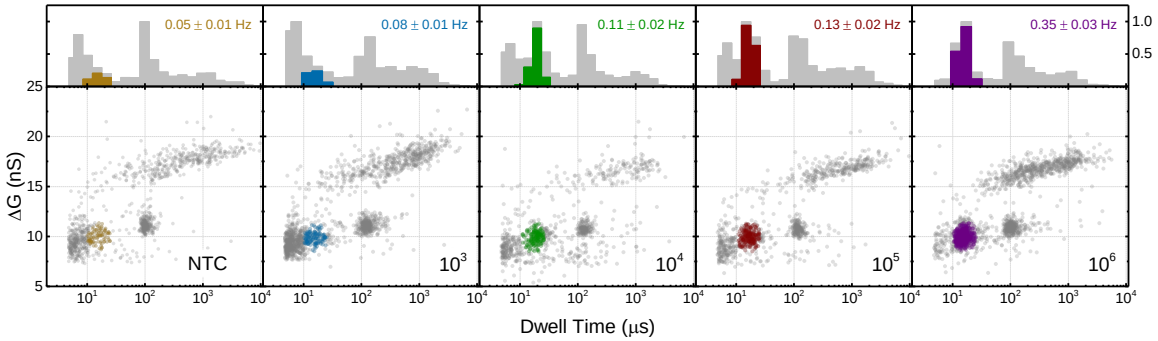

Figure S14. *Nanopore identification of RT-RPA amplicons after PCR-purification. The scatterplots show the conductance blockage (max deviation) vs. passage time for all passage events (grey) and amplicon-classified events (color) collected over 1800 seconds on the same nanopore for RT-RPA product samples (from left to right): non-template control (n = 95 classified events), amplification from 10^3 initial RNA copies - in 50 µL, equivalent to a ~30 aM initial concentration (n = 128 classified events), amplification from 10^4 initial RNA copies - in 50 µL, equivalent to a ~0.3 fM initial concentration (n = 179 classified events), amplification from 10^5 initial RNA copies - in 50 µL, equivalent to a ~3 fM initial concentration (n = 237 classified events), and amplification from 10^6 initial RNA copies- in 50 µL, equivalent to a 30 fM initial concentration (n = 581 classified events). The capture rate of each sample is shown in the top-right corner of each panel. Amplicons were run alongside 7.7 nM 2kbp dsDNA fragments, which can be seen as the grey population >100 µs. Data was acquired on a 5.3 ± 0.5 nm nanopore at 200 mV in 3.6 M LiCl 10 mM HEPES pH 8.*

Section S7: Primer Control Experiment

Here we display the results of a control experiment of translocating the RPA primers on a 5.5 ± 0.5 nm nanopore at 200 mV. The primer passage events are visible on the nanopore as short, fast events, and have a slight overlap with 300 bp dsDNA fragments. Thus, the leftover RPA primers that cannot be filtered out of solution may contribute to the background signals, especially in the samples amplified from lower initial template copies.

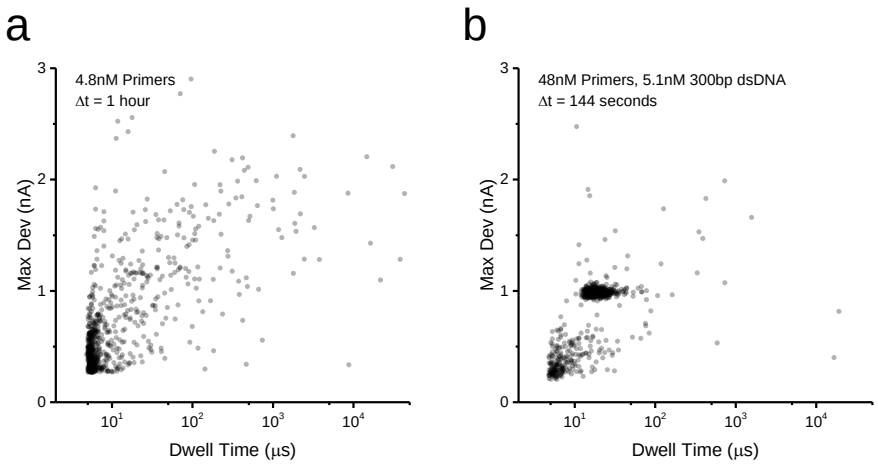

Figure S15. *Nanopore detection of RPA primers. (a) Scatterplot of 4.8 nM primer translocation events, acquired over 1 hour. (b) Scatterplot of 48 nM primer translocation events spiked with 5.1 nM 300 bp dsDNA fragments, acquired over 144 seconds. Data was acquired on a 5.5 ± 0.5 nm nanopore at 200 mV in 3.6 M LiCl 10 mM HEPES pH 8. Data was low pass filtered at 400 kHz prior to analysis.*