Supporting Information

Quantification of mRNA Expression Using Single-Molecule Nanopore Sensing

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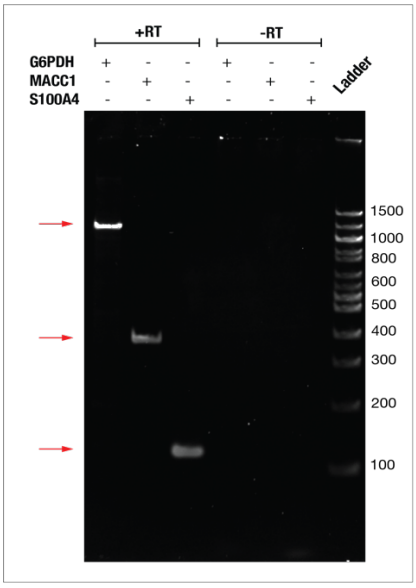
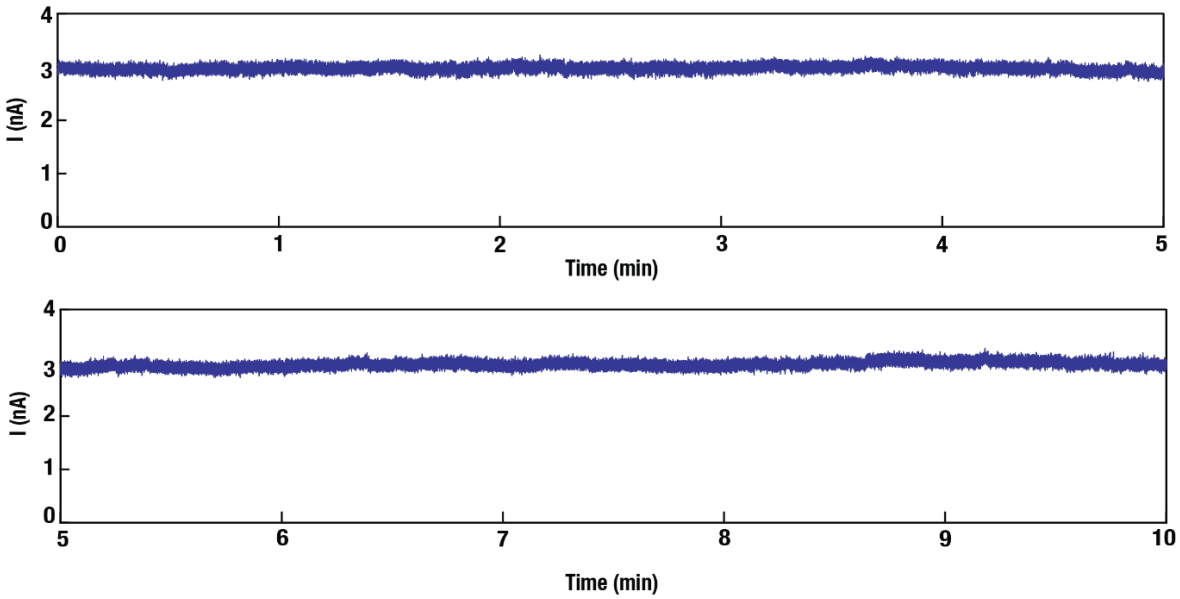
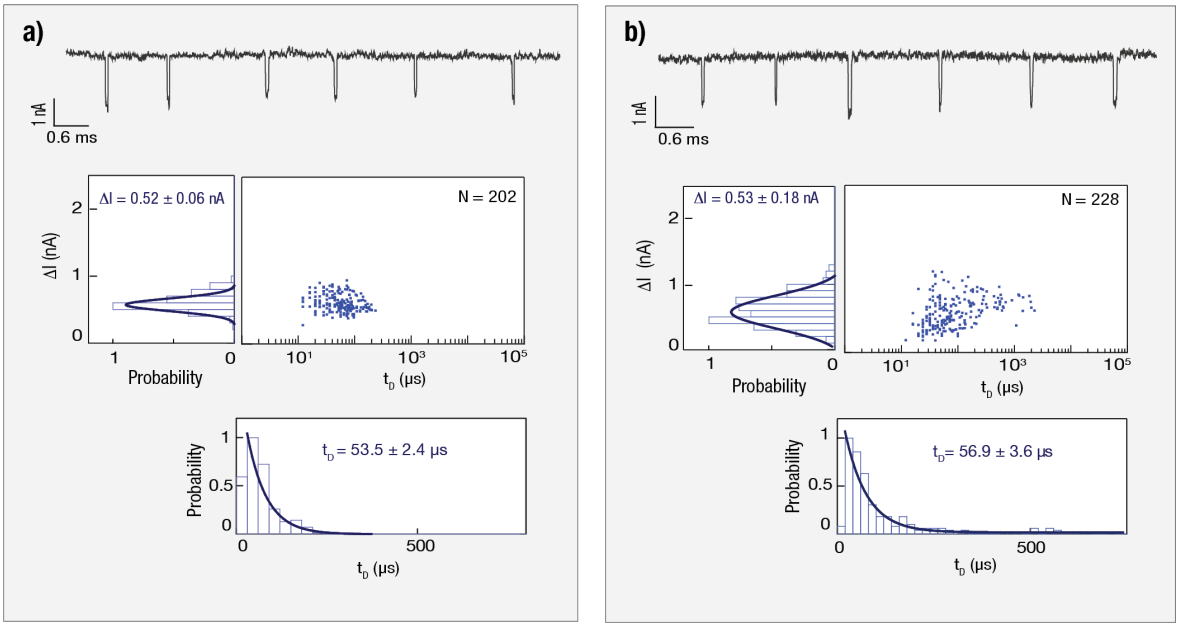
I. Supplementary Figures



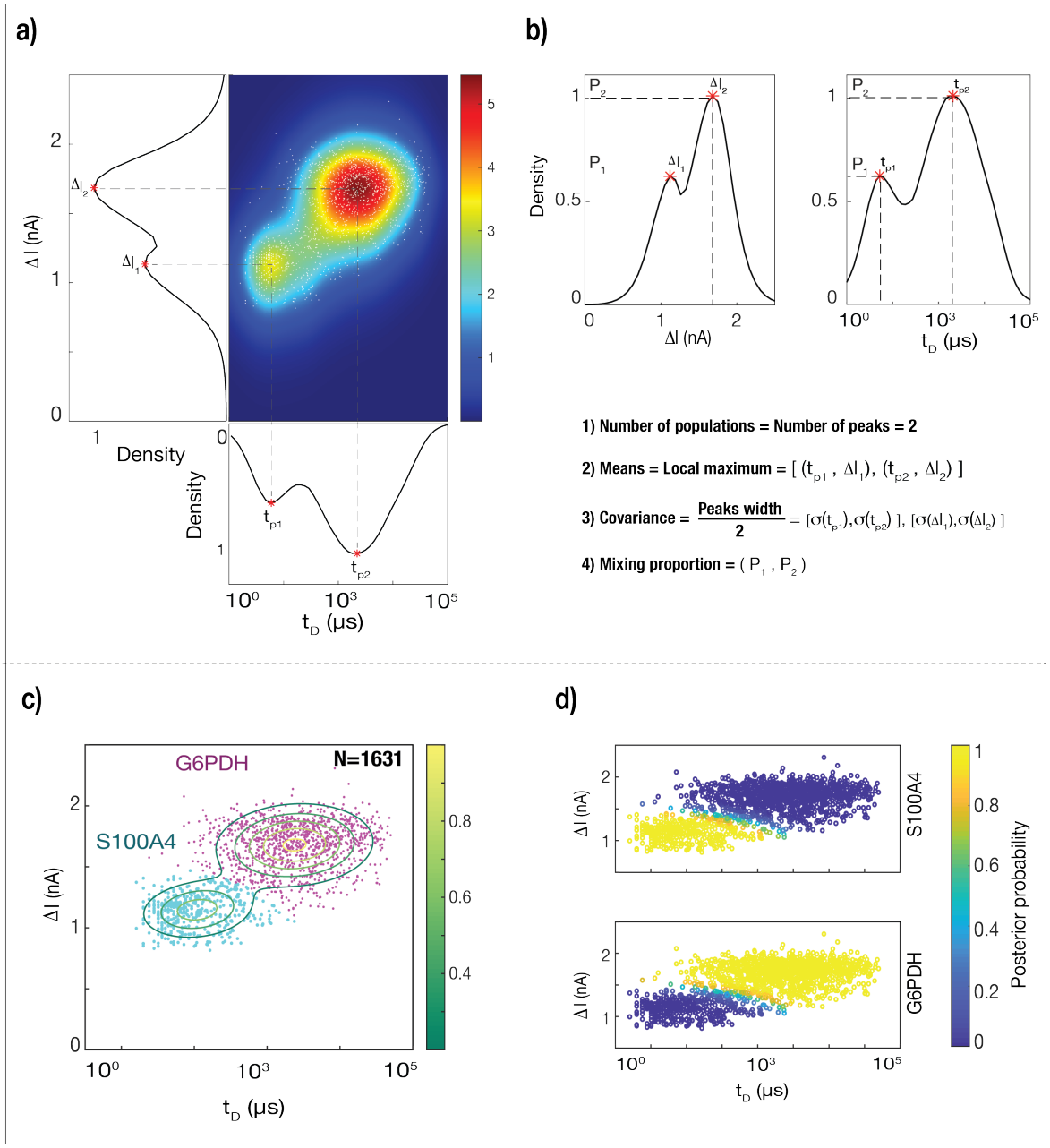
SI Figure 1. Gel electrophoresis of purified MACC1, S100A4, and G6PDH amplicons. Purified cDNA fragments for each gene were prepared using 50 ng of total RNA derived from SW620 cells, and treated with DNase I. In each case, the samples underwent gene-specific cDNA synthesis, were amplified by PCR and treated with RNase I. Then, the samples were further purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. cDNA fragments of expected sizes for each gene were obtained (1231 bp for G6PDH, 360 bp for MACC1 and 123 bp for S100A4). Negative control samples (-RT) were subjected to the same preparation procedure without the RT enzyme. The cDNA samples were separated in 4% PAGE, stained with SYBR Gold, and imaged by GelDoc EZ (BioRad).



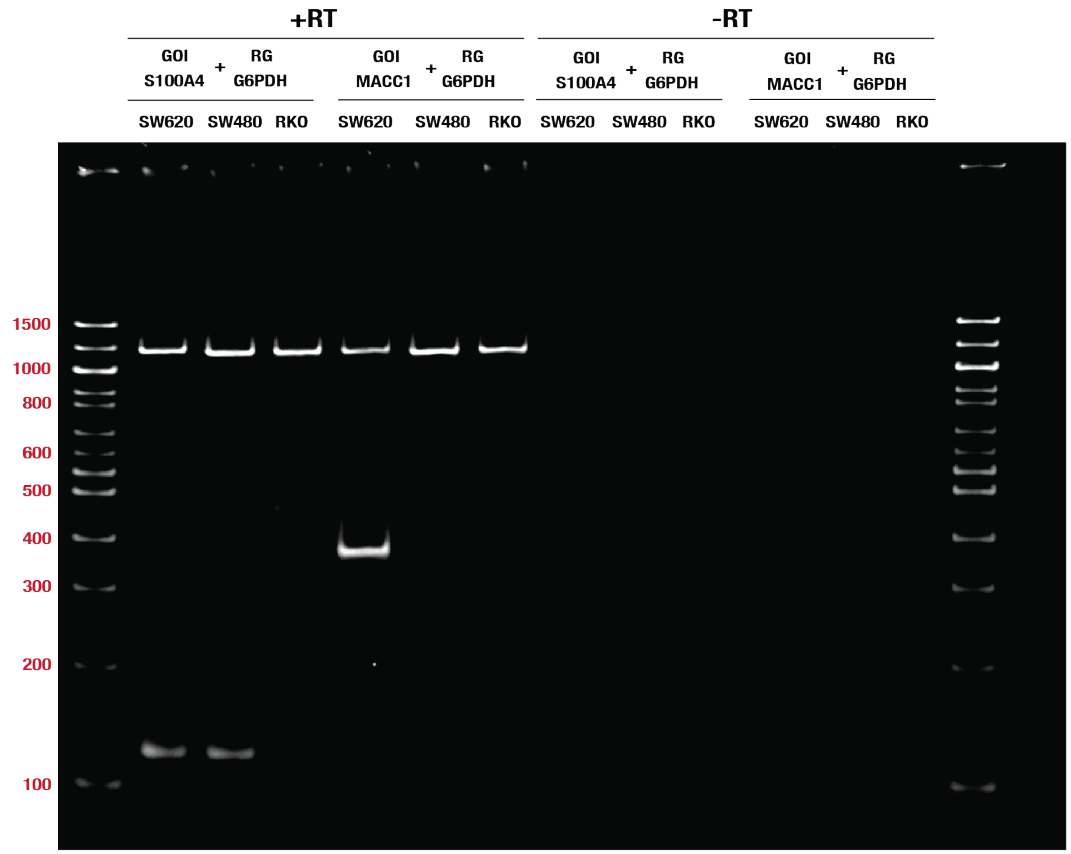
SI Figure 2. Ionic current after addition of a "-RT" sample to the nanopore. 10 minutes of continuous recording at 300 mV bias, after addition of a "-RT" sample (see SI Figure 1), shows that no non-specific translocation events occur in the absence of cDNA synthesis. The nanopore diameter was ~4 nm.



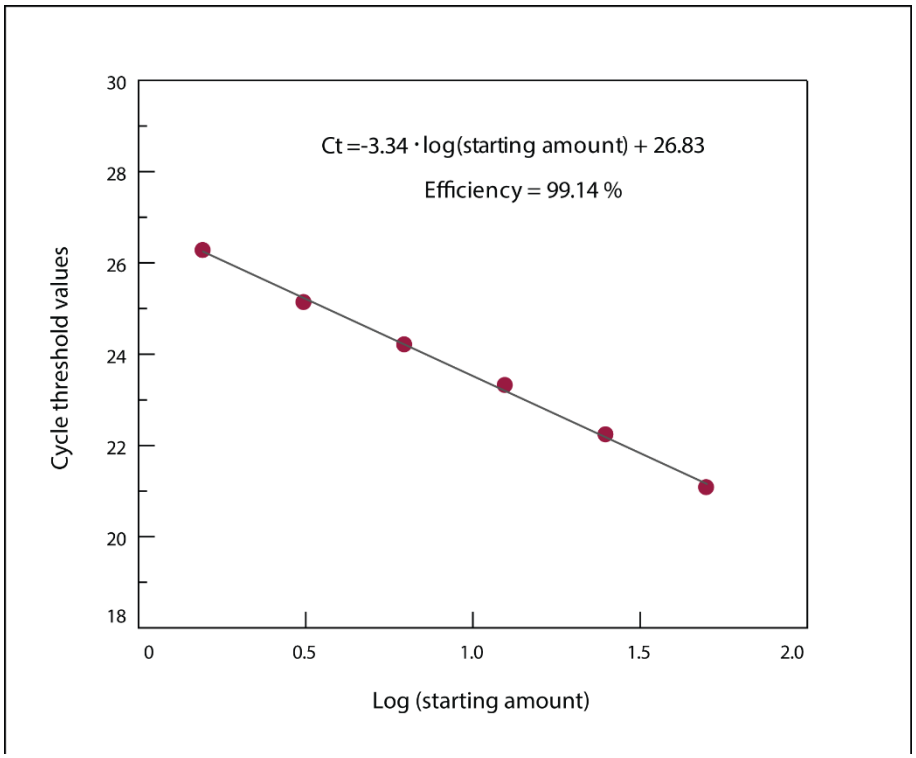
SI Figure 3. Comparison of DNA translocation events with and without purification. MACC1 cDNA samples were prepared as described in SI 1, and amplified by 15 PCR cycles. Samples were either subjected to enzymatic digestion following the purification-free method and then diluted 1000-fold **(a)**, or purified using a PCR clean-up kit **(b)**. Each sample was introduced into a separate nanopore at 300 mV bias (conductance ~9 nS, corresponding to ~4 nm diameter). Typical concatenated events are shown at the top. An event diagram and a histogram of the blockage amplitude are shown in the middle (individual events marked in blue dots), and a histogram of the event dwell times is shown at the bottom.



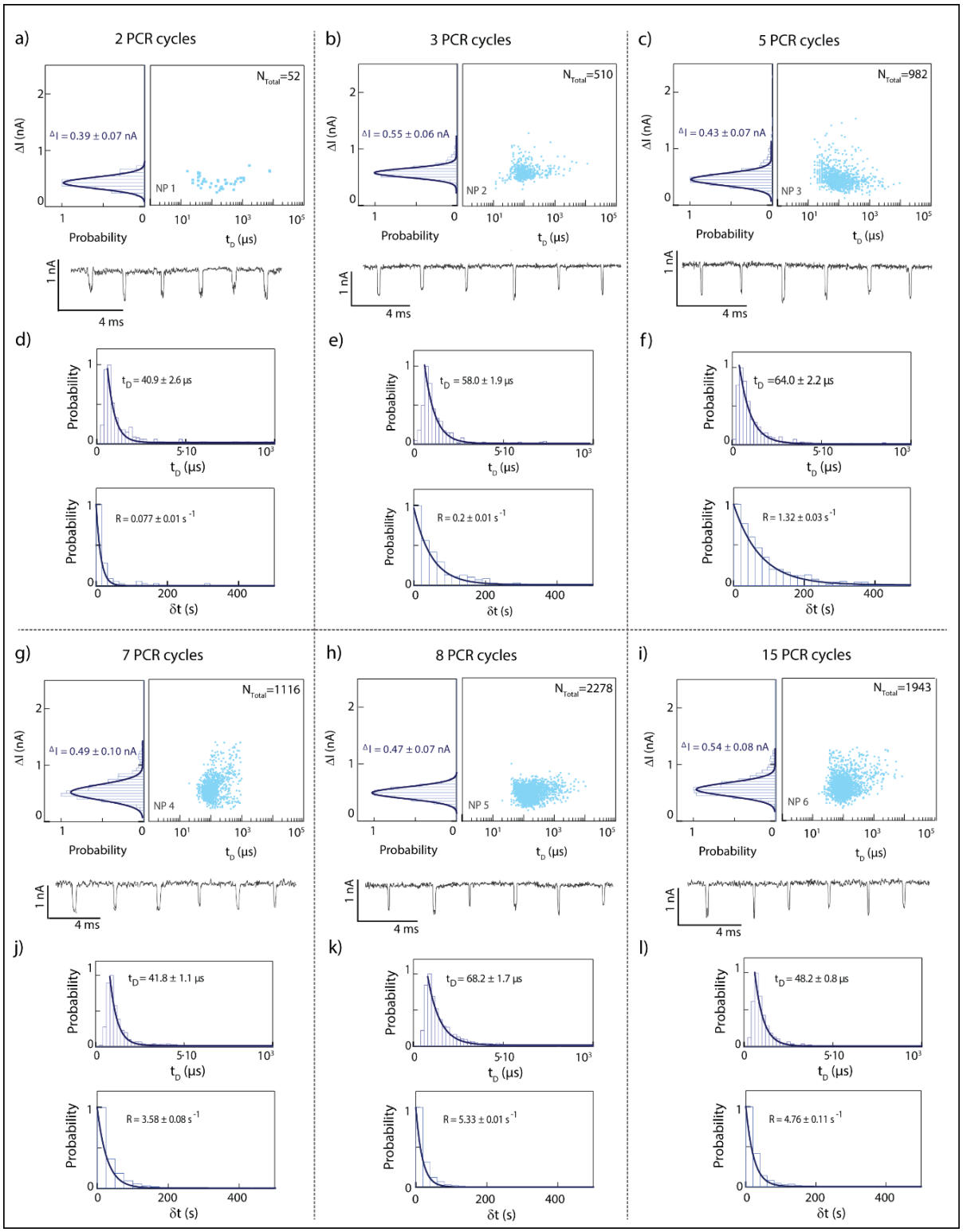
SI Figure 4. Data analysis procedure for separating populations of events based on a Gaussian mixed model (GMM). a) 2D density plots and their corresponding density histograms are generated to determine the initial conditions for the GMM (x: log-scale dwell time; y: blockage amplitude). **b)** The local maxima, marked by red asterisks, are used as initial estimations of the means in the GMM. The covariance matrix is then calculated for both of the event parameters (blockage amplitude and dwell time). Finally, the mixing proportions are estimated as the ratio between the maxima. **c)** Scatter plots represent the two populations recognized by the GMM algorithm, corresponding to the GOI (S100A4, cyan) and the RG (G6PDH, magenta). The GMM distribution is shown as a contour plot. **d)** Color maps show the separation of events based on their posterior probability of belonging to the GOI or RG population.



SI Figure 5. Gel electrophoresis of cDNA fragments prepared from CRC cell lines. 50 ng DNase-treated total RNA was obtained from RKO cells or from the SW480 (non-metastasizing) or from the SW620 (metastasizing) cell lines. The cDNA fragments were then prepared for the GOI (123 bp S100A4 or 360 bp MACC1) and the RG (1231 bp G6PDH) using our purification-free method. Negative control samples (-RT) were subjected to the same preparation procedure without the RT enzyme. The samples were separated using 4% PAGE after 30 cycles of PCR amplification and imaged using the GelDoc EZ (BioRad) after SYBR Gold staining.

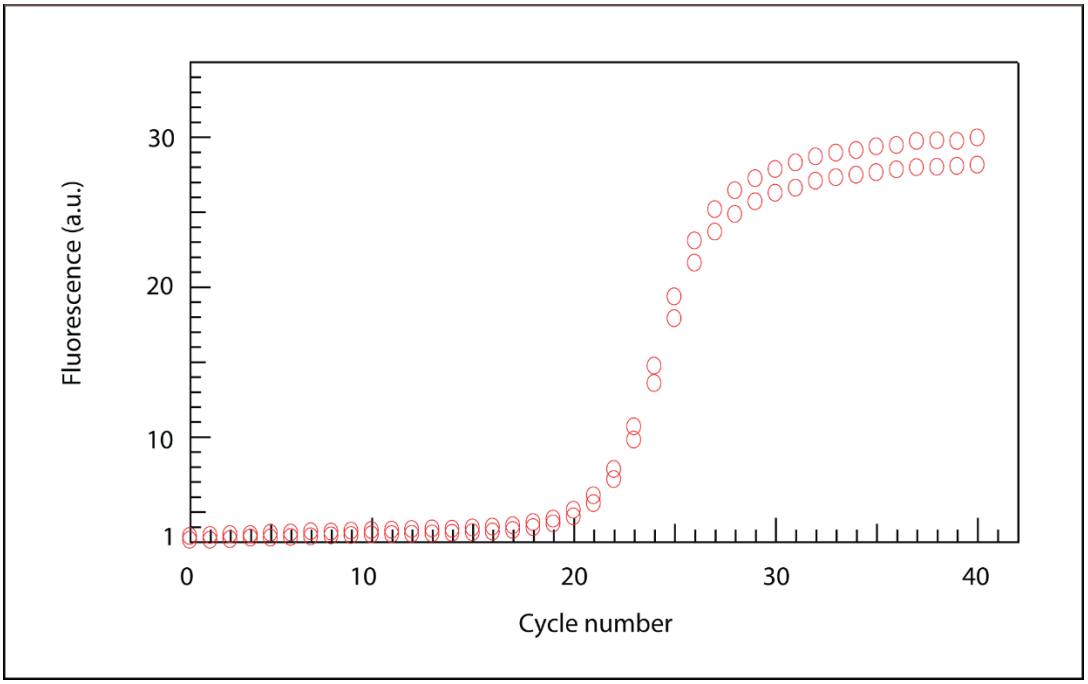


Sl Figure 6. Calibration curve of MACC1 for the CRC cell lines samples, obtained by RT-qPCR. A serial dilution of starting amount of total RNA extracted from SW620 metastasizing cell line, between 50 ng to 1.56 ng, were used to form the calibration curve.

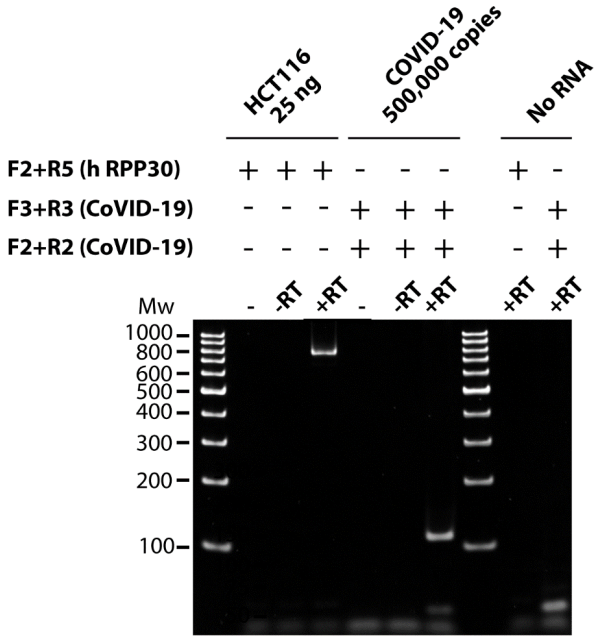


SI Figure 7. RT-qNP results for MACC1 cDNA samples after optional PCR amplification. Nanopore results after PCR amplification of 2 **(a, d)**, 3 **(b, e)**, 5 **(c, f)**, 7 **(g, j)**, 8 **(h, k)** and 15 **(i, l)** cycles, following our purification-free protocol. Each sample was measured using a different nanopore. In the first PCR cycle, no amplification occurs, only the second complementary strand is synthesized. The top panel of **(a-c)** and **(g-i)** shows a scatter plot of the translocation events with the corresponding blockage amplitude histograms on the y-axis, as well as the concatenated current traces of representative translocation events. **(d-f)** and **(j-l)** show the dwell time histograms (top) and event rate histograms (bottom) from which the

capture rate was calculated. Nanopore measurements were taken at 300 mV bias, and the typical conductance of pores was ~8-10 nS, corresponding to a pore diameter of ~4.5-5.2 nm.

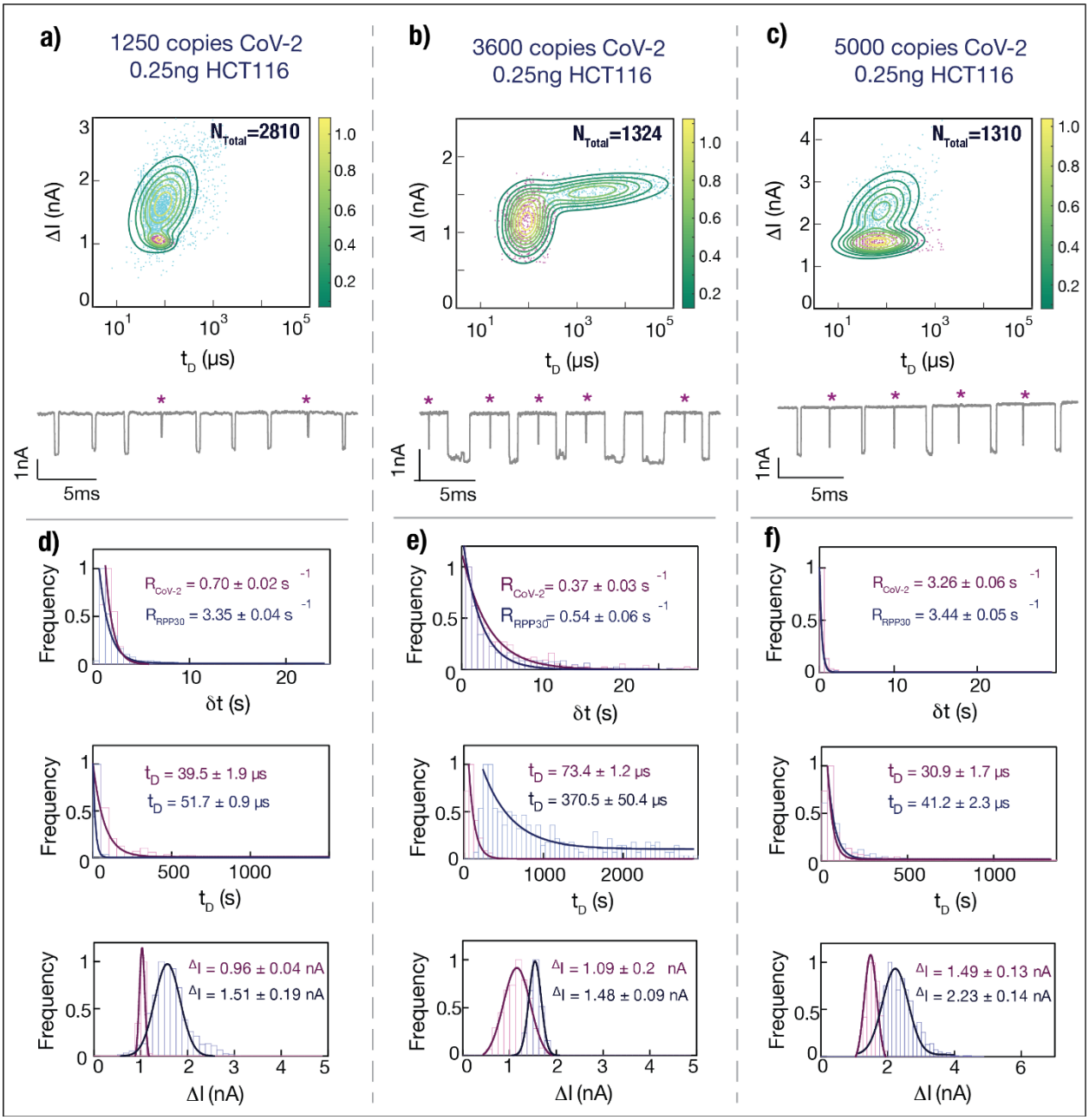


SI Figure 8. Amplification curves for MACC1 obtained by RT-qPCR using hybridization probes. Duplicate RT-qPCR amplification curves for MACC1 cDNA, obtained from 50 ng total RNA extracted from the SW620 metastasizing cell line. The cycle threshold (C_t) was found to be 20.



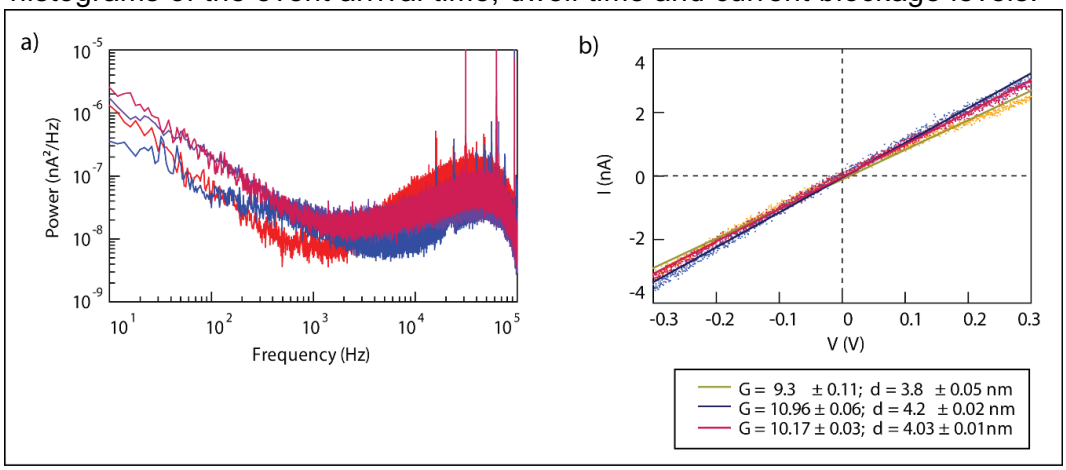
SI Figure 9. Gel electrophoresis of cDNA fragments obtained from SARS-CoV-2 RNA. cDNA fragments for each gene were prepared from 500,000 copies of either synthetic SARS-CoV-2 RNA or 25 ng of DNase-treated total RNA from HCT116 cells. The samples were processed using our purification-free method, followed by 30 cycles of PCR amplification for visualization purposes. Negative control samples (-RT) underwent the same procedure but

without the RTx and Bst 2.0 enzymes. PCR negative control samples (-) contained water instead of RT template in the PCR reaction. The 'No RNA + RT' samples contained the RTx and the Bst 2.0 enzymes but contained water instead of RNA in the RT step. Hence, these can be considered as "0 copies" or "0 ng" +RT samples. The samples were separated using 4% PAGE, stained with SYBR Gold, and imaged by GelDoc EZ (BioRad).

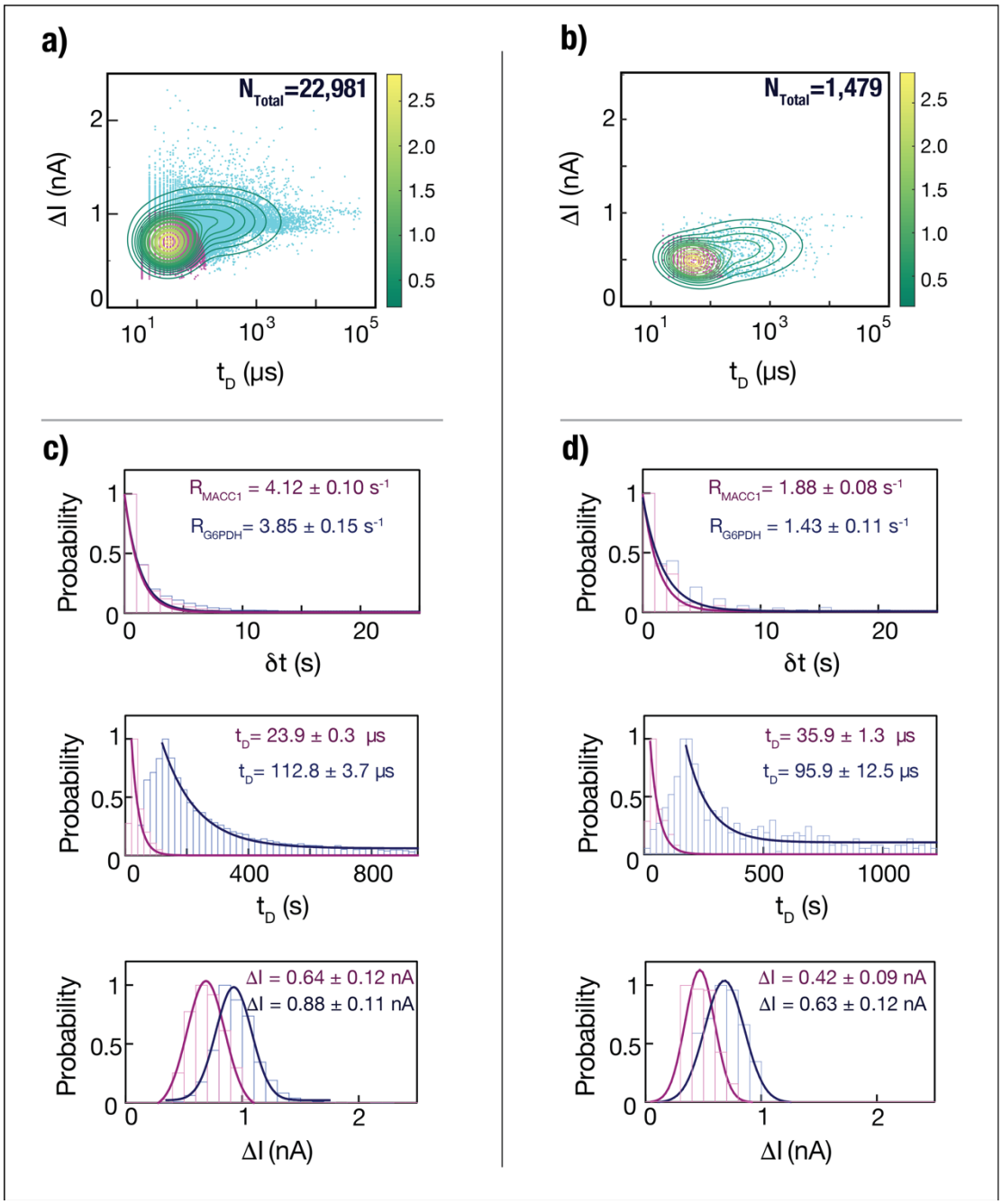


SI Figure 10. RT-qNP quantification of SARS-CoV-2 RNA against the human reference gene RPP30 at three concentration ratios. The composition of each sample is shown at the

top. Each sample contained the same amount of RPP30, reverse transcribed from 0.25 ng of total RNA from HCT116 cells. The top panels **(a-c)** show the event diagram with representative events. Purple asterisks denote the shorter translocations, which are associated with cDNA synthesized from SARS-CoV-2 RNA. The bottom panels **(d-e)** show histograms of the event arrival time, dwell time and current blockage levels.



SI Figure 11. Noise spectra and current-voltage (IV) curves of a nanopore. a) Representative noise power spectral density (PSD) *versus* frequency at an applied bias of 300mV. **b)** Representative open pore ionic current *versus* voltage. A linear I-V curve indicates a symmetric nanopore. All ionic currents and noise spectra were measured in a buffer containing 1M KCl and 10 mM Tris (pH 7.5).



SI Figure 12. RT-qNP quantification repeats of the SW480 cell line. 50 ng of total RNA underwent cDNA synthesis followed by 5 PCR cycles amplification. Left column and right column represent two separate experiments using two nanopores (conductivities 10 nS and 12 nS, respectively). The top panels (a-b) show the event diagram. The bottom panels (c-d) show histograms of the event arrival time, dwell time and current blockages.

II. Supplementary Tables

SI Table 1. Gene-specific primers used in nanopore sensing experiments and for validation of cDNA targets by sequencing.

No	Gene	Oligo Name	Sequence (5'---3')
1	G6PDH	hG6PDH For	ATCGACCACTACCTGGGCAA
2		hG6PDH Rev RT-qNP*	TCCTCCTTCCTTCTGTTGGG
3	MACC1	hMACC1 For RT-qNP*	GCACATGCCCATCAGAGTAT
4		hMACC1_Rev RT-qNP*	GCTCACAGTGCACGAAAGAT
5	S100A4	hS100A4 For RT-qNP	ACTCCTCTGATGTGGTTGGG
6		hS100A4 Rev RT-qNP*	ATCTTTATTGAACTTGCTCAGCATC

*The reverse primers of each PCR primer pair were also used as the primers in each of the gene-specific RT reactions.

SI Table 2. Gene-specific primers used in RT-qPCR experiments.

No	Gene	Oligo Name	Sequence (5'---3')
1	G6PDH	hG6PDH For	ATCGACCACTACCTGGGCAA
2		hG6PDH_Rev	TTCTGCATCACGTCCCGGA
3	MACC1	hMACC1 For	TTCTTTTGATTCCTCCGGTGA
4		hMACC1 Rev	ACTCTGATGGGCATGTGCTG
5		FITC-probe	GCAGACTTCCTCAAGAAATTCTGGAAGATCTA
6		LCRed640-probe	AGTGTTTCAGAACTTCTGGACATTTTAGACGA
7	S100A4	hS100A4 For	GAGCTGCCCAGCTTCTTG
8		hS100A4 Rev	TGCAGGACAGGAAGACACAG

SI Table 3. Gene-specific primers used in SARS-CoV-2 experiments.

No	Gene	Oligo Name	Sequence (5'---3')
1	Human RPP30	hRPP30 For	AGATTTGGACCTGCGAGC
2		hRPP30 Rev	GACAATCTTCATCTCCTTCTGAT
3	Viral SARS-CoV-2 RdRp	vRdRp For1	GGTAACTGGTATGATTTCGGT
4		vRdRp Rev1	CTGGTCAAGGTTAATATAGGCATT
5	Viral SARS-CoV-2 RdRp	vRdRp For2	CTCATCAGGAGATGCCAC
6		vRdRp Rev2	GCAATTTTGTTACCATCAGTAGAT

III. Supplementary Notes

SI 1: Sample preparation.

Sample preparation for RT-qPCR of cancer metastasis biomarkers

Total RNA was isolated using GeneJet RNA Purification Kit (Thermo Fisher scientific), treated with DNase I (NEB) and further purified according to the manufacturer's instructions. 50 ng of DNaseI-treated RNA was reverse transcribed with random hexamers in a reaction mix (10 mM $MgCl_2$, 1x RT buffer, 250 µM pooled dNTPs, 1 U/µl RNAse inhibitor, and 2.5 U/µl Moloney Murine Leukemia Virus reverse transcriptase; all from Thermo Fisher Scientific) using the following procedure: incubation at 23°C for 15 min, cDNA synthesis at 42°C for 45 min, denaturation at 95°C for 5 min, and cooling at 4°C. The cDNA was amplified by qPCR using SYBR Green dye (PerfeCTa SYBR Green Fastmix, Quanta Biosciences) chemistry or hybridization probes chemistry (FITC-probe and LCRed-640 probe within the amplicon, see SI Table 2) with specific primer-sets for MACC1 or S100A4 as described in SI Table 2. PCR was performed under the following conditions: 95°C for 2 min followed by 40 cycles of 95°C for 7 s, 60°C for 15 s and 72°C for 20 s.

Sample preparation for RT-qNP analysis of cancer metastasis biomarkers

50 ng total RNA was extracted from SW480 or SW620 cell lines, and reverse transcribed with specific primers (SI Table 1) for each gene separately or with a combination of desired primers in a reaction mix (10 mM $MgCl_2$, 15 pmol of each specific primer, 1x RT buffer, 500 µM pooled dNTPs, and 200 U/µl Maxima H Minus Reverse transcriptase, Thermo Fisher Scientific) at 60°C for 30 min. The reaction was terminated at 85°C for 5 min. Subsequently, cDNA was amplified to the specified PCR cycle using Kapa HiFi polymerase (Roche). The amplified cDNA was treated with 2.5 U RNase I (Thermo Fisher Scientific) at 37°C for 30 min. Next, the sample was treated with 0.2 U ProK (Thermo Fisher Scientific) and 0.2% SDS at 37°C for 30 min. The sample was diluted twice with the nanopore buffer.

Sample preparation for RT-qNP analysis of SARS-CoV-2 RNA and hRPP30

Either DNaseI-treated RNA, extracted from HCT116 cells, or synthetic SARS-CoV-2 RNA (control 2; MN908947.3; Twist Biosciences) were reversed transcribed with specific primers (SI Table 3) for human RPP30 cDNA or for two amplicons within the RdRp open reading frame of SARS-CoV-2. The reaction contained 1x isothermal buffer, 6 mM $MgSO_4$, 1.4 mM dNTPs, 0.2 µM of each primer, 3 U WarmStart® RTx (NEB) and 6 U Bst 2.0 WarmStart™ DNA polymerase (NEB). The reaction was carried out at 62°C for 30 min. cDNA samples were treated with 20 U of Exonuclease I (NEB) at 37°C for 15 min, followed by 2.5 U RNase I (Thermo Fisher Scientific) at 37°C for 30 min, and finally 0.2 U of ProK (NEB) in 0.2% SDS for 30 min. The sample was diluted twice with the nanopore buffer.

SI 2: Optimizing conditions for synthesis and multiplex detection of cDNA targets.

We performed extensive optimization to achieve high specificity and yield of cDNA products for all target genes. G6PDH was selected as a reference gene for both GOIs, based on previous studies.[1–3] The optimal primer sequences for each of the three genes are summarized in **Error! Reference source not found.**. The corresponding cDNA products were 123 bp S100A4, 360 bp MACC1 and 1231 bp G6PDH (SI Figure 1). The following optimal annealing temperatures were chosen for each cDNA: 58-70°C for MACC1, <68°C for S100A4 and <70°C for G6PDH. We confirmed the sequence of the cDNA fragments by Sanger sequencing.

SI 3: Negative control of purification-free assay and nanopore sensing.

To control for translocation events arising from contaminating background molecules, such as enzymes and RNA, we performed nanopore sensing experiments on "-RT" samples. These samples underwent the whole process but did not contain the RT enzyme. As demonstrated in SI Figure 2, the current trace displayed negligible or no translocation events over extended periods of time, showing that the purification-free assay is suitable for detection of cDNA reverse transcripts by nanopore sensing.

SI 4: Controls and characteristics of the CRC cell-line sample using multiplex sensing.

In all the prepared multiplexed samples from the RKO cell line, no bands were observed for either of the GOIs (S100A4 or MACC1), as expected (see SI Figure 5). Hence, the RKO cell line was used as a negative control for both GOI genes, while the RG band was readily obtained, controlling for the sample preparation method.

SI 5: Nanopore fabrication using CBD and characterization

The solid-state nanopores fabricated in localized thin regions using controlled dielectric breakdown (CBD) as previously described[4] and were Weibull-distributed. The membrane was ≤10 nm thick in the area where the pore was formed. Representative noise power spectral density (PSD) and current-voltage curves are shown in SI Figure 11. The I-V curve (SI Figure 11b) shows a symmetric and linear relationship between the current and voltage, supporting the formation of symmetric pores.

IV. Supplementary References

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(3) Juneja, M.; Kobelt, D.; Walther, W.; Voss, C.; Smith, J.; Specker, E.; Neuenschwander, M.; Gohlke, B. O.; Dahlmann, M.; Radetzki, S.; Preissner, R.; von Kries, J. P.; Schlag, P. M.; Stein, U. Statin and Rottlerin Small-Molecule Inhibitors Restrict Colon Cancer Progression and Metastasis *via* MACC1. *PLoS Biol.* **2017**, *15*, e2000784.

(4) Zrehen, A.; Gilboa, T.; Meller, A. Real-Time Visualization and Sub-Diffraction Limit Localization of Nanometer-Scale Pore Formation by Dielectric Breakdown. *Nanoscale* **2017**, *9*, 16437–16445.