SUPPORTING INFORMATION

Dual detection system for cancer-associated point mutations assisted by a multiplexed LNA-based amperometric bioplatform coupled with rolling circle amplification

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Material and methods

Chemicals and apparatus

T4 DNA ligase, phi29 DNA polymerase, Fast Digest MspI enzyme, casein blocking buffer (CBB, sold as Blocker™ Casein) and streptavidin-peroxidase polymer (SPP, sold as poly-HRP streptavidin) were from Thermo Fisher Scientific (USA), Sera-Mag™ SpeedBead carboxylate modified magnetic particles (MPs, average size of 1 μm) were from Cytiva (USA), biotin-16-dUTP (biotin-dUTP) was from Jena Bioscience (Germany), dNTP mix was from Serva (Germany), hydroquinone (HQ) was from Sigma-Aldrich (USA), TwistAmp® Basic Kit for RPA reaction was from TwistDx Limited (UK), agarose for gel electrophoresis was from The Carl Roth GmbH (Germany) and GelRed nucleic acid gel stain was from Biotium (USA). All other chemicals were of analytical grade and all solutions were prepared in deionized water. DNA oligonucleotides (synthetic targets, primers, and padlock probes) were synthesized by Generi Biotech (Czech Republic) and LNA-containing capture probes (sold as Affinity Plus single-stranded DNA) were synthesized by Integrated DNA Technologies (USA). The corresponding sequences are given in Table S1.

The multipotentiostat/galvanostat μStat 8000 (Metrohm DropSens, Spain) was connected to an electrochemical array of eight cells, each in a three-electrode setup (Metrohm DropSens, DRP-8X110) controlled by a DropView software. The three-electrode setup consisted of carbon working and auxiliary electrodes and a pseudo-reference silver electrode. Appropriate-sized magnetic support was placed beneath the array. Horizontal gel electrophoresis system (Sub Cell GT Cell and Mini-Sub Cell GT Cell) was from Bio-Rad (USA).

RCA protocol

First, a target BRAF duplex DNA (made of equimolar amounts of wt target + wt complement, or mut target + mut complement, see Table S1) was mixed with 100 nM padlock probe (PP, either wtPP or mutPP) and with 100 nM RCA primer in PBS. The whole DNA mixture was denatured at 95°C for 5 minutes and immediately put on ice. Then, a ligation reaction was set up to covalently close a gap between 5'-end and 3'-end of the PPs upon binding with the target. The ligation mixture contained the whole DNA mixture from previous step, 2 units of T4 ligase enzyme and 1× ligation buffer (supplied as a 10× stock solution) and was incubated for 30

minutes at 30°C, followed by an enzyme inactivation at 65°C for 10 minutes. Next, the RCA reaction was performed by mixing 10 µL of the ligation product, 1× RCA reaction buffer (supplied as a 10× stock solution), 0.4 mg mL^{-1} BSA, 0.2 mM dNTPs mix, 12.5 µM biotin dUTP, and 3 units of phi29 polymerase in 40 µL total reaction volume. The mixture was incubated for 60 minutes at 30°C. Amplified RCA products were then digested by MspI enzyme (using 1.5 µL of undiluted enzyme) in 1× Fast Digest buffer at 37°C for 30 minutes. The digested RCA products were visualized with gel electrophoresis (using 1.5% agarose gel and GelRed stain) and transferred to LNA-modified magnetic particles, prepared as detailed in the following section.

Protocol with magnetic particles

We tested several protocols for coupling carboxylic magnetic particles with NH_2-modified LNA capture probes, including one-step (EDC without NHS) and two-step protocols (with EDC/NHS), as well as various washing and blocking steps. The best results were obtained using a one-step protocol as follows. 20 µL of magnetic particles were washed three times with 190 µL 25 mM MES buffer (pH 5.0), with each washing step performed under agitation for 10 min at room temperature (RT), and then were resuspended in the same 25 mM MES buffer. Afterwards, 20 µL of 100 µM LNA capture probes (2 nmol) were added to MPs and incubated for 30 min at RT, without a washing step. Thereafter, 30 µL of 100 mg mL^{-1} EDC dissolved in MES buffer was added to the unwashed MP mixture and incubated for 2 h at 4°C. The LNA-modified MPs were washed three times with 190 µL of PBS, followed by the addition of 200 µL of 0.02% sodium azide (in PBS), covered with parafilm and stored at 4°C for future use. All incubation steps were performed with a rotator (20 rpm).

After RCA reaction, 2 µL of LNA-modified MPs were washed three times with 100 µL of a washing buffer (1 M NaCl, 0.5 mM EDTA, 5 mM Tris-HCl, pH 7.5), and incubated in 0.3 M sodium chloride containing 3 µL RCA digested product at RT (or at 50°C) for 30 minutes (depending on the experiment). Thereafter, the mixture was washed three times with CBB and incubated with 25 µL of streptavidin poly-HRP (diluted 2,000× in CBB) for 30 minutes at RT. The resulting MPs mixture was washed three times with 0.1 M phosphate buffer (pH 6.0) and resuspended in 15 µL of the same phosphate buffer for the amperometric measurements.

Amperometric measurements

15 µL of resuspended MPs were loaded on the surface of a working electrode of the screen-printed carbon electrode chip (Metrohm DropSens, DRP-8X110), with the commercial magnetic support (Metrohm DropSens, MAGNET8X) placed beneath the chip. Then, the MPs were covered with 50 µL of a peroxidase substrate, i.e., a solution of 50 mM H_2O_2 and 10 mM HQ in 0.1 M phosphate buffer (pH 6.0). Waiting time was set to 3 s and chronoamperometry was then performed at −0.3 V (vs the pseudo-reference silver electrode) for 90 seconds to monitor the cathodic current arising from the enzymatic reduction of H_2O_2 mediated by HQ.

Cell lines and patient samples

Human colorectal adenocarcinoma cell line HT-29, lung carcinoma cell line A549 and malignant melanoma cell line A375 were maintained in Dulbecco's modified Eagle's medium, complemented with 1% pyruvate, penicillin-streptomycin (Biosera, France) and 10% FBS (Fetal Bovine Serum from Gibco, USA). All cells were grown at 37°C under a humidified atmosphere of 5% CO_2. For DNA extraction, we used Tissue DNA Preparation Column Kit (Jena Bioscience, Germany) according to the manufacturer's instructions.

The study was approved by the Ethical Committee of Masaryk Memorial Cancer Institute (2020/1471/MOU) and informed consent was received from all patients enrolled in the study. Patient samples were collected in a time period 2019-2021 at Masaryk Memorial Cancer Institute. DNA extraction was performed from formalin-fixed paraffin-embedded tissues using QIAamp DNA FFPE Tissue Kit (Qiagen, Germany), following manufacturer instructions. DNA concentration was measured on Qubit Fluorometer 3.0 with Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, USA). The sequencing was performed on MiSeq instrument (Illumina, USA), and evaluation of the mutation status was assessed using the NextGENe software (SoftGenetics, USA). The minimum allele frequency was set to 5% and the minimum coverage of sequenced regions (300×) was checked in IGV (Broad Institute) and/or NextGENe.

RPA protocol

The recombinase polymerase reaction (RPA) was used to pre-amplify genomic DNA from cancer cells and from patient samples to obtain shorter DNA fragments. We used TwistAmp® Basic Kit (TwistDx Ltd, UK) with a supplied 96-well plate according to the manufacturer

recommendations. Briefly, lyophilized reagents in a well were reconstituted (in this order) with 29.5 µL of rehydration buffer, 2.4 µL of 10 µM forward RPA primer, 2.4 µL of 10 µM reverse RPA primer, 100 ng of cell DNA and, lastly, with 2.5 µL of 280 mM magnesium acetate to initiate the reaction. The mixture was incubated in a thermoblock for 20 min at 39°C, visualized with gel electrophoresis and subsequently used as a template DNA in the RCA protocol (see section "Rolling circle amplification protocol").

High resolution melting analysis

Melting temperatures (T_m) of LNA capture probes/DNA target duplexes were assessed by high resolution melting (HRM) analysis, using QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, USA) and MeltDoctor HRM Master Mix (Thermo Fisher Scientific, USA). Melting curves were plotted as a derivative of emitted fluorescence.

Optimization of the assay

To obtain satisfactory sensitivity, mismatch recognition and reproducibility, numerous working variables of the assay were optimized. A complete list of all the tested variables as well as the selected values are given in Table S3. Moreover, data regarding several optimization experiments such as the PP concentration (Fig. S3), time of ligation (Fig. S4) and RCA reaction (Fig. S5), temperature of RCA (Fig. S6), volume of used MPs (Fig. S7), and SPP dilution factor (Fig. S8) are shown below. All variables were optimized for the wtPP and wtLNA CP attached to carboxyl MPs. In that setup, the wt target DNA served as a positive control and the mut target DNA as a negative control (using 25 nM input concentration for both). In the main text, we show that this optimized protocol (with slight modifications) worked well also in a reverse setup, i.e., using mutated padlock and mutation-specific LNA probes.

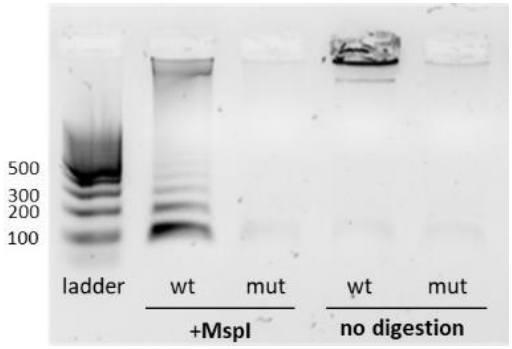

Fig. S1. Gel electrophoresis of wt and mut template samples after RCA amplification using wtPP. Samples were either cleaved with MspI enzyme (left) or left intact ("no digestion", right). As expected, only wt samples were successfully amplified. Size of DNA ladder bands are included.

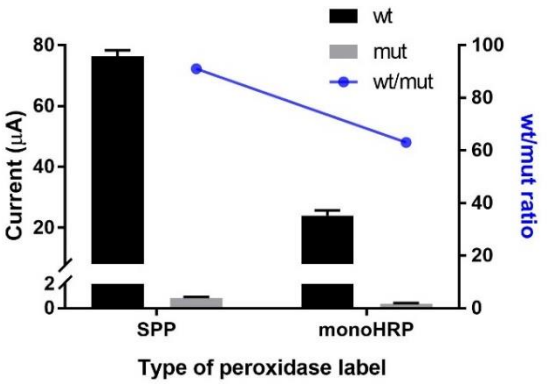

Fig. S2. Comparison of peroxidase labels, i.e., streptavidin-peroxidase polymer (SPP) and streptavidin-peroxidase monomer (monoHRP) using wt template (black) and mut template (grey) samples and wtPP. Dilution of labels was 1/2,000 in CBB. Right axis: blue dots represent ratio of signals between wt and mut sample, i.e., signal/noise ratio.

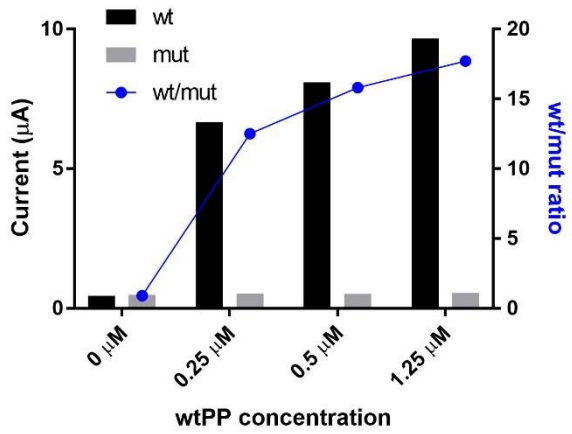

Fig. S3. Dependence of the amperometric responses on the wtPP concentration, shown for wt template (black) and mut template (grey) sample. Right axis: blue dots represent ratio of signals between wt and mut sample, i.e., signal/noise ratio.

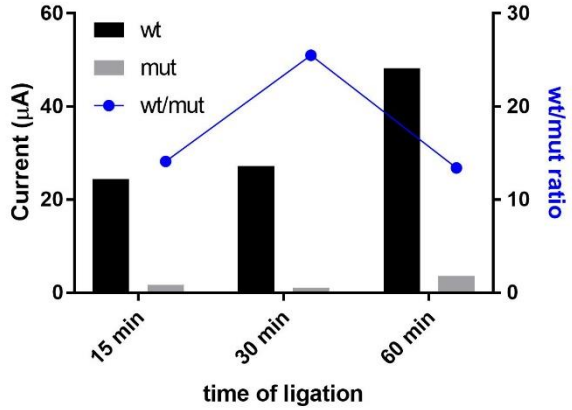

Fig. S4. Dependence of the amperometric responses on the time of ligation reaction, shown for wt template (black) and mut template (grey) sample and using wtPP and wtLNA capture probes. Right axis: blue dots represent ratio of signals between wt and mut sample, i.e., signal/noise ratio.

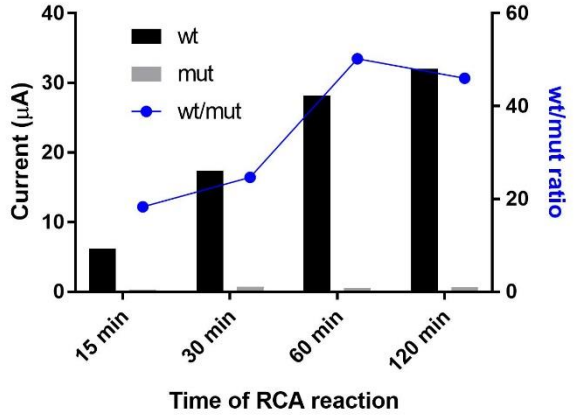

Fig. S5. Dependence of the amperometric responses on the incubation time of RCA reaction, shown for wt template (black) and mut template (grey) sample and using wtPP and wtLNA capture probes. Right axis: blue dots represent ratio of signals between wt and mut sample, i.e., signal/noise ratio.

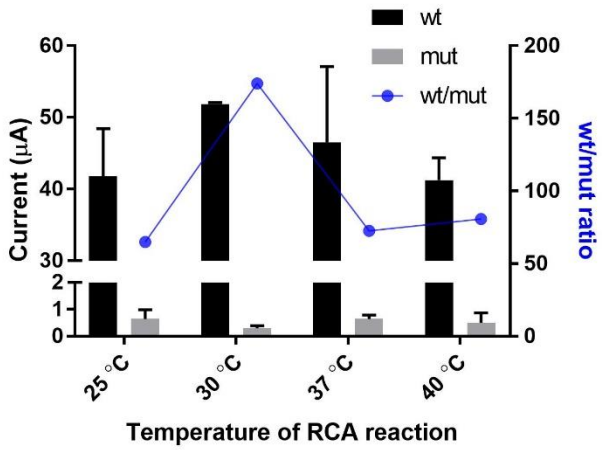

Fig. S6. Dependence of the amperometric responses on the temperature of RCA reaction, shown for wt template (black) and mut template (grey) sample and using wtPP and wtLNA capture probes. Right axis: blue dots represent ratio of signals between wt and mut sample, i.e., signal/noise ratio.

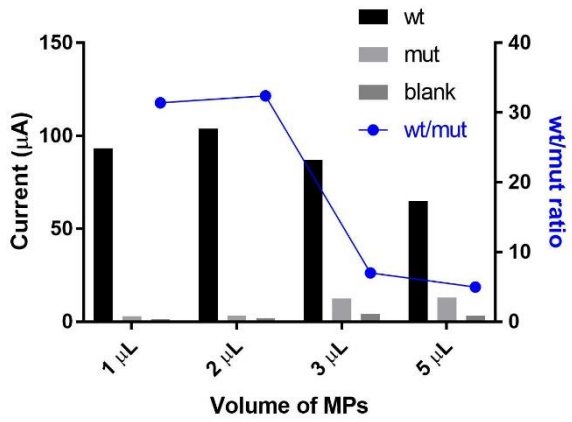

Fig. S7. Dependence of the amperometric responses on the volume of carboxylated MPs modified with wtLNA capture probe, shown for wt template (black), mut template (grey) sample and without template (blank) using wtPP. Right axis: blue dots represent ratio of signals between wt and mut sample, i.e., signal/noise ratio.

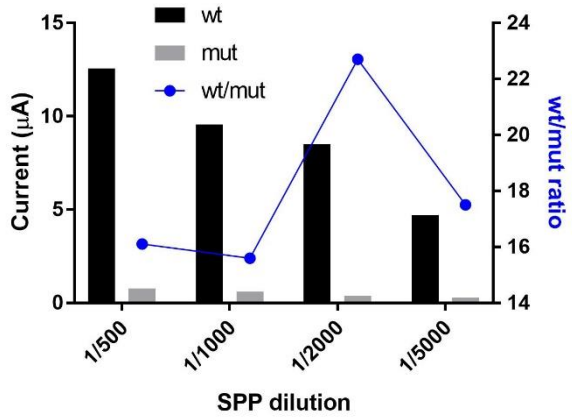

Fig. S8. Dependence of the amperometric responses on the SPP dilution (diluted in CBB), shown for wt template (black) and mut template (grey) sample and using wtPP and wtLNA capture probes. Right axis: blue dots represent ratio of signals between wt and mut sample, i.e., signal/noise ratio.

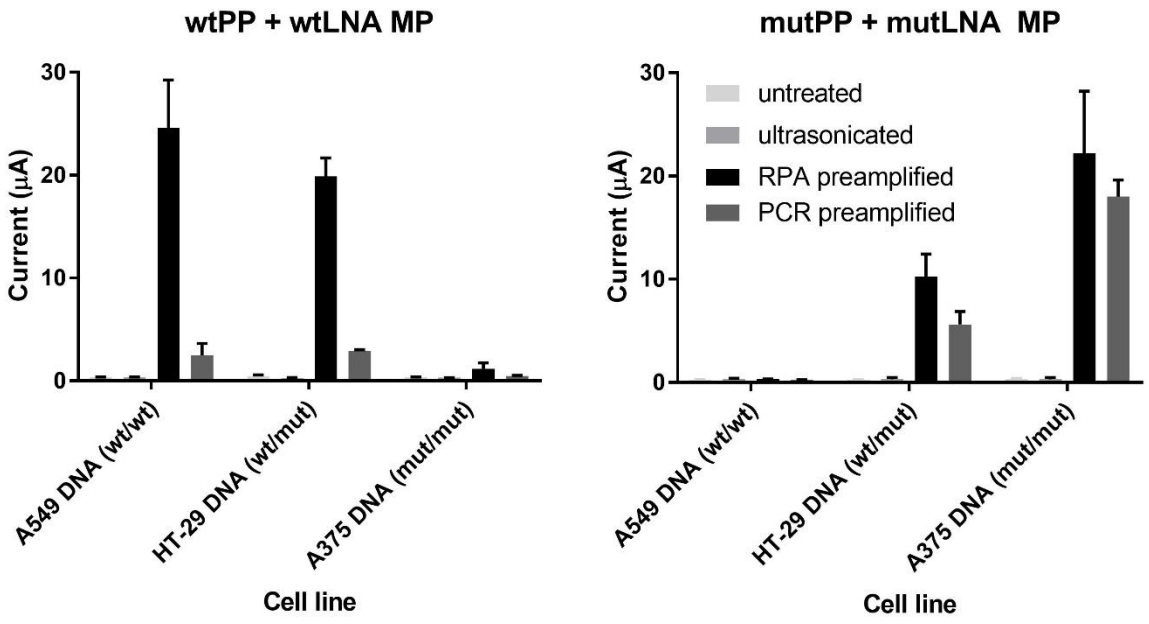

Fig. S9. Pre-treatment of DNA from cell lines. Comparison of untreated DNA with DNA treated by Covaris ultrasonication or enzymatically with RPA or PCR to generate shorter template fragments for subsequent RCA. EC signals from three cell lines are shown after the whole RCA protocol, using either wtPP or mutPP. wtPP/mutPP – padlock probe; wtLNA/mutLNA – capture probe with LNA internal modification.

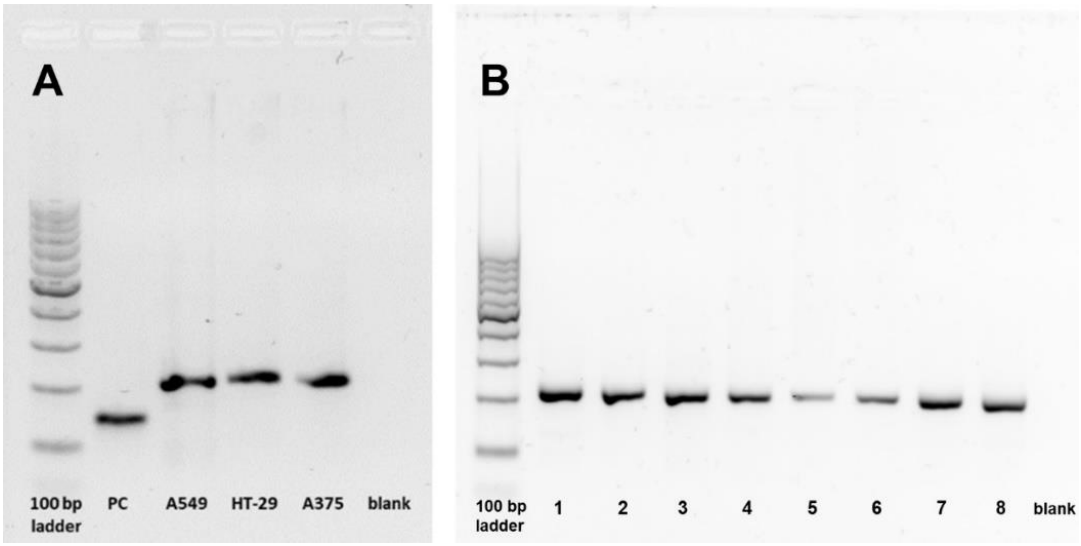

Fig. S10. Gel electrophoresis of RPA products amplified from (A) DNA of cancer cell lines and (B) eight clinical samples from patients. Approx. size of RPA products is 200 bp and were further used as DNA targets in RCA reaction. PC - positive control of RPA reaction itself as supplied by vendor (not used in RCA).

Table S1. List of oligonucleotide sequences used in the study.

Oligonucleotide	Sequence (5'-3') [a]
RCA primer	TCAGCACTAACGTCAACGCGTGCTGAAGTCAAGTT
wt padlock probe (wtPP)	phosphate-CTGTAGCTAGACAGAGCGCATGAATCCGTAGTAACTTGACTTCAGCACGCGTGAGG*CCGG*TACATCTCCCCACTCCATCGAGATTTC**A**
mut padlock probe (mutPP)	phosphate-CTGTAGCTAGACAGAGCGCATGAATCCGTAGTAACTTGACTTCAGCACGCGTGAGG*CCGG*TACATCTCCCCACTCCATCGAGATTTC**T**
wt target	CTTTACTTACTACACCTCAGATATATTTCTTCATGAAGACCTCACAGTAAAAATAGGTGATTTTGGTCTAGCTACAG**T**GAAATCTCGATGGAGTGGG
wt complement	CCCACTCCATCGAGATTTC**A**CTGTAGCTAGACCAAAATCACCTATTTTTACTGTGAGGTCTTCATGAAGAAATATATCTGAGGTGTAGTAAGTAAAG
mut target	CTTTACTTACTACACCTCAGATATATTTCTTCATGAAGACCTCACAGTAAAAATAGGTGATTTTGGTCTAGCTACAG**A**GAAATCTCGATGGAGTGGG
mut complement	CCCACTCCATCGAGATTTC**T**CTGTAGCTAGACCAAAATCACCTATTTTTACTGTGAGGTCTTCATGAAGAAATATATCTGAGGTGTAGTAAGTAAAG
wtLNA capture probe (CP)	NH_2-C6-AGATTTC<u>**A**C</u>TGTAGC
mutLNA CP	NH_2-C6-AGATTTC<u>**T**C</u>TGTAGC
wtDNA CP	AGATTTC**A**CTGTAGC
mutDNA CP	AGATTTC**T**CTGTAGC
RPA forward primer	CTGATAGGAAAATGAGATCTACTG
RPA reverse primer	GGCCAAAAATTTAATCAGTGGA

[a] Mutation site (and corresponding complementary base) is shown in bold, restriction site CCGG is in italics, and LNA triplets are underlined.

Table S2. High resolution melting analysis for estimation of melting temperature values (T_m) of LNA CPs/DNA target duplexes and DNA CPs/DNA target duplexes. HRM was performed at two different NaCl concentrations and for all combinations of CPs and targets. Each sample was run in triplicate. The largest T_m values were obtained for fully complementary LNA/DNA duplexes (samples 1, 4, 9, 12), while fully complementary DNA/DNA duplexes (5, 8, 13, 16) gave lower T_m values. All mismatched samples, as expected, yielded lower T_m values than their fully complementary pairs. The ΔT_m values were calculated as a difference between T_m values of wt and mut targets.

ID	[NaCl], mM	Type	Duplex	Average T_m [°C]	ΔT_m [°C]
1	0	LNA/DNA	wtLNA CP + wt target	62.020	**11.390**
2			wtLNA CP + mut target	50.630	$T_m(1) - T_m(2)$
3			mutLNA CP + wt target	51.820	**9.490**
4			mutLNA CP + mut target	61.310	$T_m(4) - T_m(3)$
5		DNA/DNA	wtDNA CP + wt target	54.797	**7.410**
6			wtDNA CP + mut target	47.387	$T_m(5) - T_m(6)$
7			mutDNA CP + wt target	47.703	**6.103**
8			mutDNA CP + mut target	53.807	$T_m(8) - T_m(7)$
9	300	LNA/DNA	wtLNA CP + wt target	65.930	**12.690**
10			wtLNA CP + mut target	53.240	$T_m(9) - T_m(10)$
11			mutLNA CP + wt target	55.730	**9.140**
12			mutLNA CP + mut target	64.870	$T_m(12) - T_m(11)$
13		DNA/DNA	wtDNA CP + wt target	58.500	**8.427**
14			wtDNA CP + mut target	50.073	$T_m(13) - T_m(14)$
15			mutDNA CP + wt target	50.470	**7.237**
16			mutDNA CP + mut target	57.707	$T_m(16) - T_m(15)$

Table S3. List of tested variables along with their selected values

Parameter	Tested values	Selected
Primer concentration	0, 0.25, 0.5, 1.25 (μM)	0.5 μM
Padlock probe concentration	0, 0.25, 0.5, 1.25 (μM)	0.5 μM
Time of ligation	15, 30, 60 (min)	30 min
Time of RCA reaction	15, 30, 60, 120 (min)	60 min
Volume of biotin-dUTP	0.25, 0.5, 1 (μL)	0.5 μL
Volume of magnetic particles	1, 2, 3, 5 (μL)	2 μL
Volume of RCA product	1, 2, 3, 5, 10 (μL)	3 μL
Incubation time of RCA product	15, 30, 45, 60, 120 (min)	30 min
SPP dilution	1/500, 1/1000, 1/2000, 1/5000	1/2000

Table S4. Patient sample information, including type of cancer, percentage of tumor cells present in a biopsy tissue, and mutation status of the *BRAF* gene.

Patient number	Type of cancer	% of tumor cells in biopsy	% of mutated sequences in biopsy	*BRAF* mutation status
1	melanoma	90	0	wt/wt
2	melanoma	90	0	wt/wt
3	colorectal cancer	75	0	wt/wt
4	colorectal cancer	50	26	wt/V600E
5	melanoma	75	37	wt/V600E
6	melanoma	90	46	wt/V600E
7	colorectal cancer	50	50	V600E/V600E
8	melanoma	90	81	V600E/V600E

Table S5. Comparison of EC-based assays using RCA reaction for the determination of DNA point mutations.

Gene	Strategy	Limit of detection	Real samples	Dual detection	Assay time	Ref.
TP53	ECL-based strategy at streptavidin MPs	0.1 pM	No	No	6 h	[1]
KRAS	RCA coupled to mutS protein and radical polymerization	3.09 aM	Spiking in human serum	No	8 h 45 min	[2]
KRAS	CRISPR/Cas12a system coupled to hyperbranched RCA	10 aM	No	No	3 h 10 min	[3]
ADRB3	Label-free EIS-based assay	100 pM	No	No	4 h 30 min	[4]
TP53	Glucose meter-based assay with nicking enzyme strategy	0.36 pM	Spiking in human serum	No	13 h	[5]
TP53	Dual-amplified nuclease-assisted target recycling assay	0.25 fM	Spiking in human serum	No	6 h 15 min	[6]
BRAF	RPA-RCA reaction coupled with LNA-modified MPs	55 pM	Patient samples (FFPE tissues)	Yes	3 h 5 min	This work

References

[1] Q. Su, D. Xing, X. Zhou, Magnetic beads based rolling circle amplification–electrochemiluminescence assay for highly sensitive detection of point mutation, Biosens. Bioelectron., 25(2010) 1615-21, https://doi.org/10.1016/j.bios.2009.11.025.
[2] S. Lee, J. You, I. Baek, H. Park, K. Jang, C. Park, et al., Synergistic enhanced rolling circle amplification based on MutS and radical polymerization for single-point mutation DNA detection, Biosens. Bioelectron., 210(2022) 114295, https://doi.org/10.1016/j.bios.2022.114295.
[3] J. You, H. Park, H. Lee, K. Jang, J. Park, S. Na, Sensitive and selective DNA detecting electrochemical sensor via double cleaving CRISPR Cas12a and dual polymerization on hyperbranched rolling circle amplification, Biosens. Bioelectron., 224(2023) 115078, https://doi.org/10.1016/j.bios.2023.115078.
[4] S. Zhang, Z. Wu, G. Shen, R. Yu, A label-free strategy for SNP detection with high fidelity and sensitivity based on ligation-rolling circle amplification and intercalating of methylene blue, Biosens. Bioelectron., 24(2009) 3201-7, https://doi.org/10.1016/j.bios.2009.03.012.
[5] Y. Jia, F. Sun, N. Na, J. Ouyang, Detection of p53 DNA using commercially available personal glucose meters based on rolling circle amplification coupled with nicking enzyme signal amplification, Anal. Chim. Acta, 1060(2019) 64-70, https://doi.org/10.1016/j.aca.2019.01.061.
[6] Q. Wang, C. Yang, Y. Xiang, R. Yuan, Y. Chai, Dual amplified and ultrasensitive electrochemical detection of mutant DNA biomarkers based on nuclease-assisted target recycling and rolling circle amplifications, Biosens. Bioelectron., 55(2014) 266-71, https://doi.org/10.1016/j.bios.2013.12.034.