

## Supplementary Information for

### **Ultra-fast detection and quantification of nucleic acids by amplification-free fluorescence assay**

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#### **This PDF file includes:**

General information on materials and methods, information on human samples and statistical analyses, HPLC, MALDI MS, qPCR and NGS data

Figs. S1 to S9

Tables S1 and S2

References for SI reference citations

## I. General Information and Materials

**General.** Reagents obtained from commercial suppliers were used as received. LNA phosphoramidite reagents and Eva Green dye (20X in water (20  $\mu$ M)) were obtained from Exiqon and Biotium, respectively. Unmodified and biotinylated DNA were purchased from IDT and used after HPLC purification.

**Oligonucleotide synthesis** was carried out on Äkta Oligopilot DNA synthesizer (GE Healthcare Life Sciences) in 250 nmol scale using manufacturer's standard protocols (DMT-off setting), and 500 Å controlled pore glass (CPG) support (ATDBio). LNA modifications were incorporated automatically (1*H*-tetrazole activator; 10 min coupling time). The coupling efficiencies of standard DNA phosphoramidites and LNA based on the absorbance of the dimethoxytrityl cation released after each coupling varied between 97% and 100%. CPG was dried in vacuo for 5 min and removed from the column.

**Cleavage from solid support and removal of nucleobase protecting groups** was performed using 32% aqueous ammonia and methylamine 1:1, v/v, for 4 h at room temperature (rt).

**IE HPLC** was performed using the Merck Hitachi LaChrom instrument equipped with a Dionex DNAPac Pa-100 column (250 mm  $\times$  4 mm). Elution was performed starting with an isocratic hold of A- and C-buffers for 2 min followed by a linear gradient to 60% B-buffer over 28 min at a flow rate of 1.0 mL/min (A-buffer: MQ water; B-buffer: 1M NaClO<sub>4</sub>, C-buffer: 25mM Tris-Cl, pH 8.0).

**MALDI-TOF** mass-spectrometry analysis was performed using a MALDI-LIFT system on the Ultraflex II TOF/TOF instrument from Bruker and using HPA-matrix (10 mg 3-hydroxypicolinic acid, 50 mM ammonium citrate in 70% aqueous acetonitrile).

## Human samples

Human samples were obtained from Odense University Hospital (OUH), Denmark, Statens Serum Institute (SSI, Denmark) Rigshospitalet (Denmark) and Radboud University Medical Center (RU, Netherlands). For the ctDNA study, three cancer patient samples were selected from the sample collection at the Rigshospital. The samples were characterized by clinical data, common serological biomarkers, treatment intensity and disease activity. Besides these disease-stated samples, 5 healthy controls from OUH were included in the ctDNA study.

For the miRNA study, the samples were acquired from the following: 50 healthy controls (serum; OUH+SSI), 8 SLE (serum; SSI), 33 RA (plasma (n=28) and serum(n=5); (RU) and 13 RA (serum; SSI). 4 CTR (miRNA from healthy individual) plasma (RU). The diagnoses were made by certified physicians based standard diagnostic criteria.<sup>1</sup>

For the aforementioned samples from OUH and SSI, written approval by The Danish Data Protection Agency was obtained in April 2016. The Committee of Information Safety of the Region Southern Denmark at the Danish Data Protection Agency specifically approved this study (permission signed by Pernille Winther Christensen). The methods were carried out in accordance with the relevant guidelines and regulations as stated in the Act on Processing of Personal Data adopted by the Danish Data Protection Agency on June 2<sup>nd</sup> 2000. Personal identifying data of patients was not used in this work. Therefore, the informed consent from the individuals was not needed.

## II. miRNA assay

### miRNA enrichment probes

Sequences of hsa-miR-223-3p enrichment probe and hsa-miR-486-1-5p enrichment probe:

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<sup>1</sup> SLE: Sebastiani, G. D. *et al. Isr Med Assoc J* **18**, 212–215 (2016). RA: Combe, B. *et al. Ann Rheum Dis* **76**, 948–959 (2017).

hsa-miR-223-3p enrichment probe; 5'-ACT G+AC +ACT CTA CCA CAT +GG+A GT-biotin-3'  
 hsa-miR-486-1-5p enrichment probe; 5'-GCT C+AG TA+C AGG A+TG C-biotin-3'  
 LNAs are marked by a "+" preceding the modified nucleotide. The enrichment probes were obtained from Qiagen.

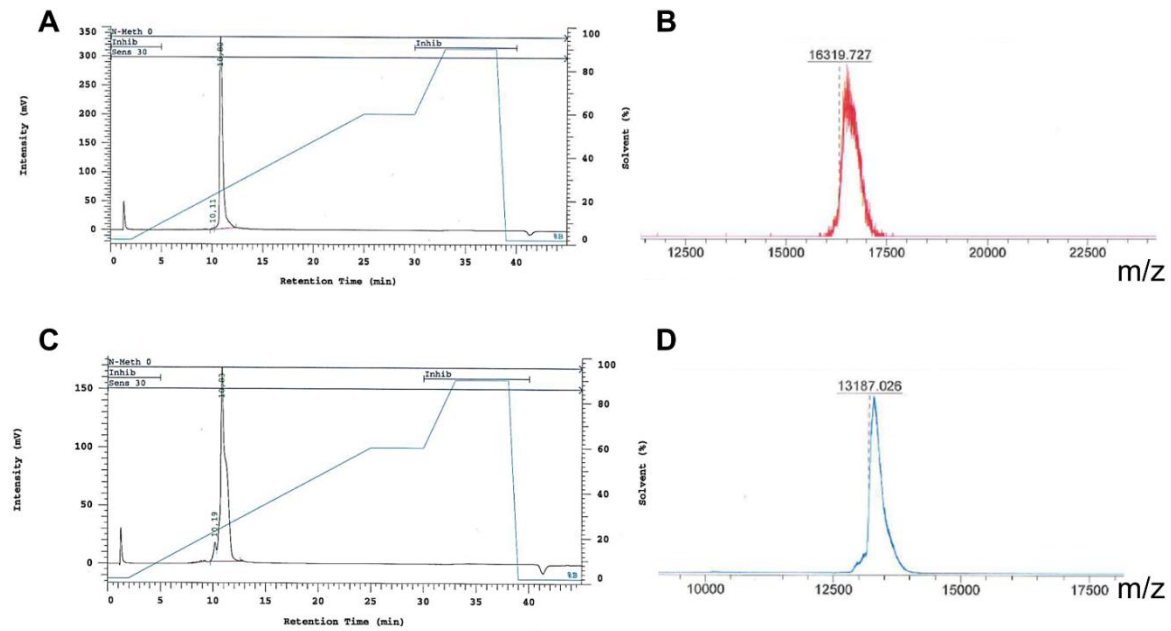
### miRNA Linking probes: design, MALDI-MS & IE HPLC

Sequences of hsa-miR-223-3p Linking probe and hsa-miR-486-5p Linking probe:

hsa-miR-223-3p; 5'-ATT +CGT AAA +CAA GCA +GTA +GTA ATT CTT TTG TGC +CG+C  
 ACT TGG GGT A+T T+T GAC-3'

hsa-miR-486-1-5p; 5'-ATT +CGT AAA +CAA GCA +GTA +GTA ATT CTT TTA AG+G GCC  
 TC+G GGG-3'

LNA's are marked by a "+" preceding the modified nucleotide.



**Supplementary Figure S1.** MALDI-TOF and IE HPLC of miRNA detection probes: hsa-miR-486-1-5p (A, B), and hsa-miR-223-3p (C, D).

### qPCR of RA samples

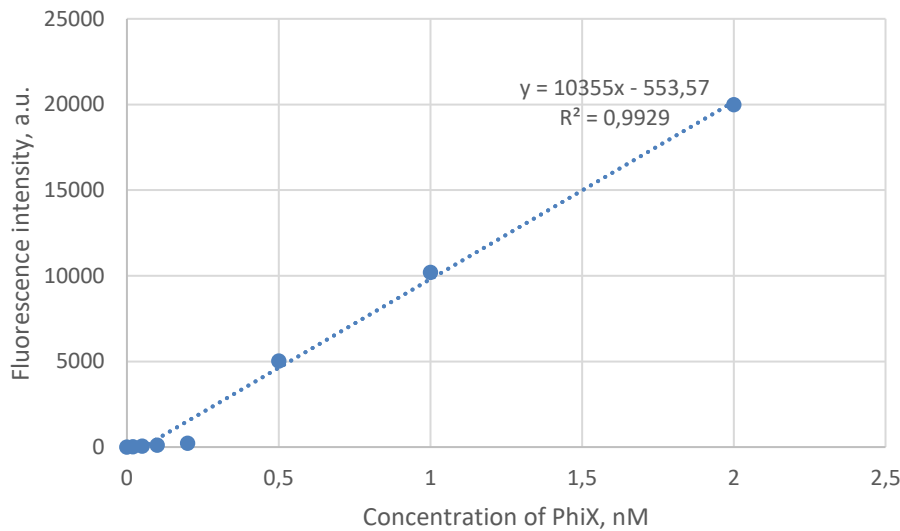
The TaqMan miRNA detection was carried out using commercial reagents (Thermo Fisher, assay ID 241474\_mat, cat no. 4440886; assay ID 001278, cat. no. 4427975), following described procedures.<sup>2</sup> The experiments were conducted on Light Cycler in 386 well microplate format (LightCycler® 480 Instrument II from Roche). The obtained Ct values were converted to concentrations by a relative method using internal calibrant of a known concentration.

### miRNA hybridisation assay

Dynabeads (100  $\mu$ L, 10 mg/mL) were placed in a magnetic separation rack to remove and discard the clear supernatant and then washed with 1 $\times$ PBS, pH 7.2 (3  $\times$  100 $\mu$ L). Hereafter, the Dynabeads were resuspended in 1 $\times$ PBS (20  $\mu$ L) and incubated with the miRNA specific enrichment probe (20  $\mu$ L, 1  $\mu$ mol) for 1 hour with rotation. When the incubation was finished the solution was washed

<sup>2</sup> Chen et al. Nucleic Acids Res. 2005; 33(20): e179.

with PBS (2 × 100 μL, 30 °C), resuspended in PBS (20 μL) and serum/plasma sample (20 μL). The resulting mixture was heated for 40 min at 65 °C with repetitive vortexing every 10 min, and subsequently cooled to room temperature over 30 min followed by a wash with PBS (2×100 μL, 30 °C). Afterwards we added the PhiX signal boosting complex (5,000 μg), The complementary Linking probe (15 μL or 132.34 μmol for hsa-miR-223-3p; 20 μL or 98.64 μmol for hsa-miR-486-5p), and 2×PBS (25 μL) was added to the solution. The mixture was, heated for 10 min at 85 °C, and cooled to room temperature over 20 min followed by a wash with 2×PBS (4×100 μL, 30 °C). The mixture was resuspended with 2×PBS (10 μL), heated 5 min at 92 °C and the supernatant was removed. The supernatant was placed in a solution with EvaGreen (3 μL) and 2×PBS (10 μL). The resulting mixture was vortexed for 7 min, followed by 5 min at 92 °C and finally cooled to room temperature over 60 min.



**Supplementary Figure S2. Calibration curve for PhiX:Eva Green complex used in miRNA study.**

**Supplementary Table S1. Demographic, and clinical features of RA patients used in this study, and results of miRNA analyses.<sup>1</sup>**

Sample No.	RF	Anti CCP	DAS	CRP	ESR	TD	G	F (486-1-5p), nM	F (223-3p), nM	qPCR 486-1-5p, nM	qPCR 223-3p, nM
1	1	1	3.98	1	9	11	1	0.89	1.43	1.32	0.24
2	1	0	2.03	2	14	8	1	1.83	0.32	1.16	0.76
3	1	1	1.55	1	5	25	1	0.75	0.75	0.99	0.52
4	1	0	3.88	50	37	15	0	1.64	1.36	1.23	1.12
5	1	0	2.09	3	5	27	1	1.14	1.22	2.34	1.03
6	1	0	1.97	1	16	1	1	1.24	0.18	3.17	0.54
7	0	1	3.71	9	20	6	1	3.58	2.70	4.67	1.71
8	1	1	1.92	2	14	18	1	3.56	0.55	5.11	0.21
9*	0	1	20.8	2	12	15	1	1.26	1.86	2.22	0.78
10	0	1	2.35	1	2	5	1	1.37	1.01	1.53	1.21
11	1	1	5.77	31	93	16	0	2.60	1.58	2.23	1.03
12	1	1	5.22	6	27	3	1	0.47	0.98	1.2	0.22

13	0	0	0.56	4	2	7	0	3.00	0.34	3.7	0.07
14	0	0	1.35	6	5	8	0	1.36	0.76	1.22	0.43
15	0	0	1.20	0	5	5	1	2.09	0.33	1.98	0.19
16*	0	1	20.8	2	12	15	1	0.83	0.77	0.45	0.56
17	1	1	4.79	25	57	3	0	1.37	0.56	1.12	0.43
18	0	0	5.43	4	9	7	1	0.57	0.37	1.43	0.22
19	1	1	2.23	3	14	19	1	3.56	0.73	5.15	0.13
20*	0	1	22	4	11	15	1	1.31	2.06	3.22	1.78
21	0	1	4	1	5	5	1	2.12	0.82	2.98	1.23
22	1	1	7	31	99	16	0	3.10	1.89	4.53	1.41
23	1	1	5	5	39	5	1	0.47	1.08	1.76	1.70
24	0	0	0.8	5	3	8	0	3.20	0.35	5.43	0.04
25	0	0	1.4	3	4	9	0	1.45	0.95	0.45	0.45
26	0	0	1.2	1	5	3	1	2.77	0.34	1.43	0.89
27*	0	1	22.3	2	11	11	1	0.94	0.95	0.04	0.30
28	1	1	5.6	28	66	7	0	1.67	0.56	0.87	0.34
29	0	0	6.1	3	14	10	0	0.37	0.36	1.23	0.04
30	1	1	4.11	2	11	9	1	0.88	1.43	2.76	0.76
31	1	0	2	3	17	6	1	1.82	0.32	4.30	0.12
32	1	1	1.77	2	6	20	1	0.70	0.76	1.65	0.05
33	1	0	3.2	44	41	14	1	1.63	1.27	2.67	1.18
34	1	0	1.89	5	6	22	1	1.20	1.17	2.21	0.67
35	1	0	2.11	2	19	2	0	1.19	0.18	1.03	0.34
36	0	1	3.17	7	22	3	1	3.31	2.97	6.70	3.56
37	1	1	1.55	3	18	21	1	3.19	0.37	3.23	1.12
38*	0	1	21.3	4	15	14	1	4.88	2.12	7.65	2.01
39	0	1	2.56	2	3	5	1	1.39	0.82	1.04	0.87
40	1	1	6.60	28	77	11	0	2.73	1.20	0.78	1.05
41	1	1	6.89	26	89	15	0	2.39	1.89	2.11	1.14

<sup>1</sup> 486-5p and 223-3p are hsa-miR-486-1-5p and hsa-miR-223-3p. RF = rheumatoid factor; Anti-CCP is antibody to cyclic citrullinated peptide. For RF and anti CCP, 1 is positive and 0 is negative. CRP is C reactive protein. ESR is erythrocyte sedimentation rate. TD is treatment duration. G is gender (1=female, 0=male). F = fluorometry. Numbers for outliers according to statistical analyses (described below), are indicated with \*.

### III. Statistical analysis of miRNA levels

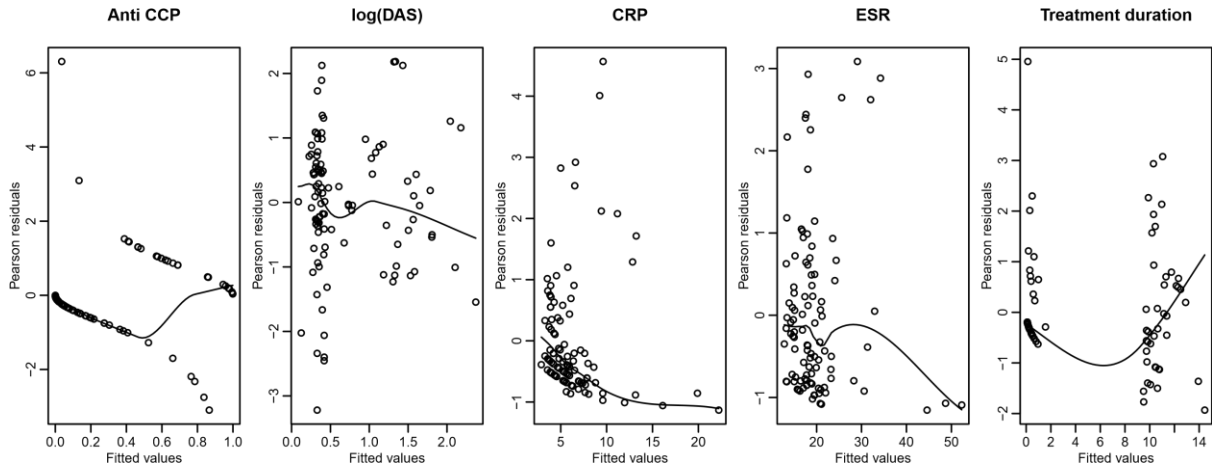
Initially, all groups were tested by one-way ANOVA in R. Next, the data was analysed by multivariate model using the following variables: anti-cyclic citrullinated peptide (Anti-CCP), disease activity score (DAS), C reactive protein (CRP), erythrocyte sedimentation rates (ESR), and treatment duration served as response variables. Hsa-miR-223-3p, hsa-mir-486-5p, and group served as covariates for all response variables.

*Wald chi-square tests of fixed effects*

**Supplementary Table S2.** Univariate Wald chi-square test of fixed effects. Response variable: AntiCCP, DAS, CRP, ERS and treatment duration.

<b>Anti-CCP</b>	<b>Df</b>	<b><math>\chi^2</math></b>	<b>p-value</b>
Group	1	0.9588	0.3275
hsa-miR-223-3p	1	2.7064	0.0999
hsa-miR-486-1-5p	1	1.4191	0.2335
group: hsa-miR-223-3p	1	1.2381	0.2658
group: hsa-miR-486-1-5p	1	1.8270	0.1765
<b>Log(DAS)</b>	<b>Df</b>	<b><math>\chi^2</math></b>	<b>p-value</b>
Group	1	3.2217	0.0727
hsa-miR-223-3p	1	0.1181	0.7312
hsa-miR-486-1-5p	1	0.3398	0.5600
group: hsa-miR-223-3p	1	10.6624	0.0011
group: hsa-miR-486-1-5p	1	1.0588	0.3035
<b>CRP</b>	<b>Df</b>	<b><math>\chi^2</math></b>	<b>p-value</b>
Group	1	0.1396	0.7087
hsa-miR-223-3p	1	0.9694	0.3248
hsa-miR-486-1-5p	1	0.2790	0.5974
group: hsa-miR-223-3p	1	1.0639	0.3023
group: hsa-miR-486-1-5p	1	0.3927	0.5209
<b>ESR</b>	<b>Df</b>	<b><math>\chi^2</math></b>	<b>p-value</b>
Group	1	0.8910	0.3452
hsa-miR-223-3p	1	1.0465	0.3063
hsa-miR-486-1-5p	1	2.7872	0.0950
group: hsa-miR-223-3p	1	5.3118	0.0212
group: hsa-miR-486-1-5p	1	0.3402	0.5597
<b>Treatment duration</b>	<b>Df</b>	<b><math>\chi^2</math></b>	<b>p-value</b>
Group	1	30.9338	< 0.001
hsa-miR-223-3p	1	4.4608	0.0347
hsa-miR-486-1-5p	1	0.0680	0.7943
group:miR-223	1	2.7691	0.0961
group: hsa-miR-486-1-5p	1	0.0563	0.8125

## Residuals



**Supplementary Figure S3.** Residuals of Anti-CCP, log(DAS), CRP, ESR and treatment duration.

## IV. ctDNA assay

### ctDNA enrichment probe

We designed the ctDNA enrichment probes to hybridise the *KRAS* and *BRAF* genes, but not overlap the specific regions for *BRAF* V600E, *KRAS* G12D and *KRAS* G13D mutations. Sequences of ctDNA enrichment probes were: (*BRAF*); 5'-AGC AAG CAT TAT GAA GAG TTT AGG TAA GAG ATC TAA TTT CTA TAA TTC TGT AAT ATA ATA TTC TTT AAA ACA TAG TAC TTC ATC TTT CCT CTT AGA GTC AAT AAG TAT GTC TAA AAC AAT-biotin-3' (*KRAS*); 5'-TGA AAG TTA AGT TAT CTG AAA TGT ACC TTG GGT TTC AAG TTA TAT GTA ACC ATT AAT ATG GGA ACT TTA CTT TCC TTG GGA GTA TGT CAG GGT CCA TGA TGT TCA CTC TCT GTG CAT TTT-biotin-3'

### Azide and alkyne modified precursors, IE HPLC & MALDI-TOF

We designed the amino-labelled LNA-DNA mutation specific probes with LNA positioned opposite to the SNP (marked with red) in the corresponding ctDNA target. Amino-labeled LNA-DNA oligonucleotides were:

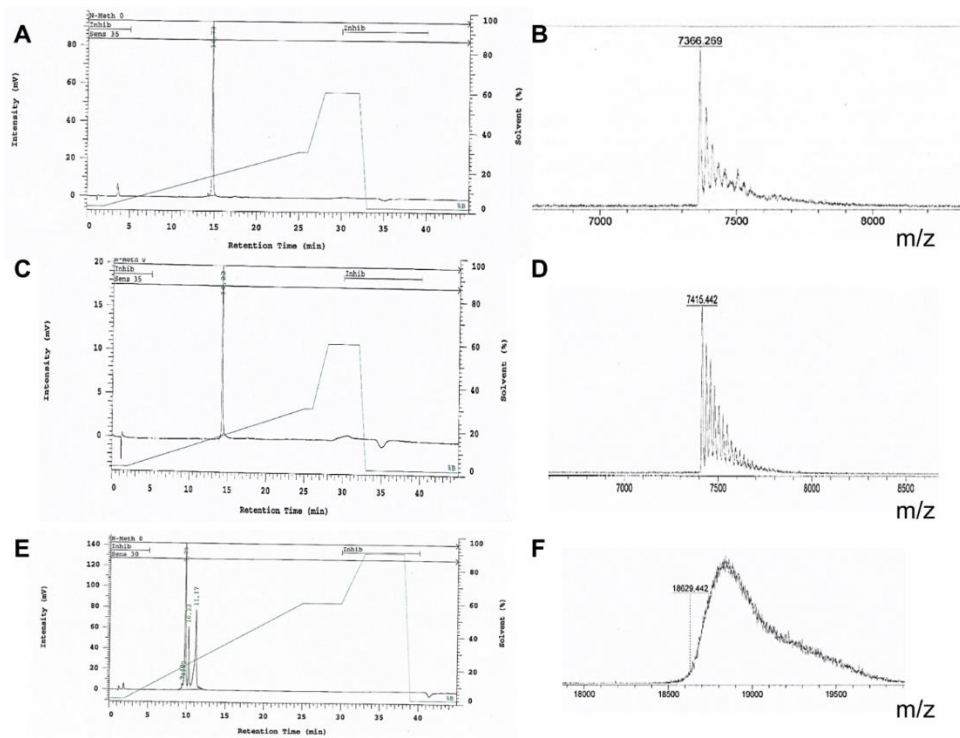
*BRAF* V600E mutation, complementary probe; /5AmMC6/TT TTT TTT TTG ATT T+C+T +CTG TAG-3'

*KRAS* G12D mutation, complementary probe; /5AmMC6/TT TTT TTT TTG AGC T+G+A +TGG CGT-3'

*KRAS* G13D mutation, complementary probe; /5AmMC6/TT TTT TTT TTG AG+C TGG T+G+A +CGT-3'

LNAs are marked as a "+" preceding the modified nucleotide.

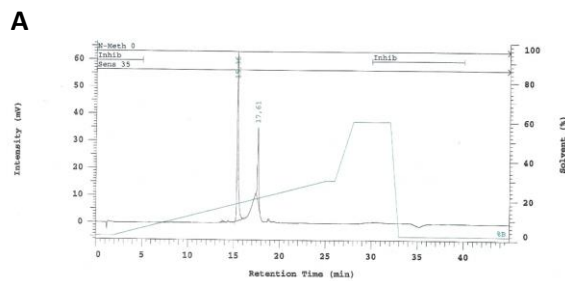
The three probes were alkyne functionalized using pentynoic acid sulfotetrafluorophenyl ester (PNHS). The probes(20 mmol) were dissolved in sodium bicarbonate Buffer(20  $\mu$ L, 0.5 M, 8.31 pH). Hereafter, a solution of PNHS (3 mg, 9.2  $\mu$ mol in 500  $\mu$ L dimethylformamide (DMF)) were prepared. Subsequent, the PNHS solution (7.5  $\mu$ L, 200 mmol) were added to the solution of the probes. The reaction mixture stirred overnight at rt. The probes were precipitated using cold acetone and used without further purification.



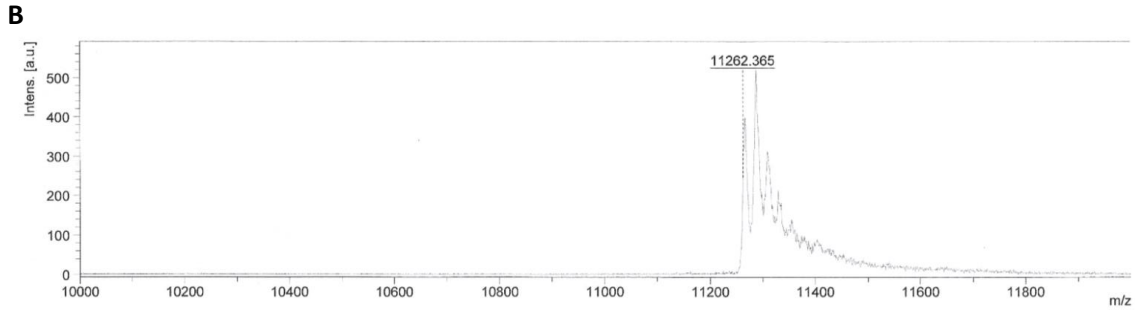
**Supplementary Figure S4** Representative IE HPLC and MALDI TOF of ctDNA detection probes: precursors (A-D) and conjugated products (E,F; *BRAF* V600E probe).

We designed the DNA/LNA PhiX linker probe to be complementary to PhiX. Furthermore, the 3'-end were amino-labelled for further functionalization. The PhiX linker probe were: 5'-CTT+CG+ATTTAATT+CGTAAA+CAAGCA+GTA+GTAATTC/3AmMO/-3'

The PhiX linker probe were functionalized with azide using azidobutyric acid *N*-Hydroxysuccinimide(NHS) ester (ANHS). The PhiX linker probe(20 mmol) were dissolved in sodium bicarbonate Buffer(20  $\mu$ L, 0.5 M, 8.31 pH). To the reaction mixture were a solution of ANHS(6.25 $\mu$ L, 200 mmol, 6 mg in 500 $\mu$ L DMF) and MQ water(3.75  $\mu$ L) added. The reaction mixture stirred overnight at rt. The probes were precipitated using cold acetone and used without further purification.



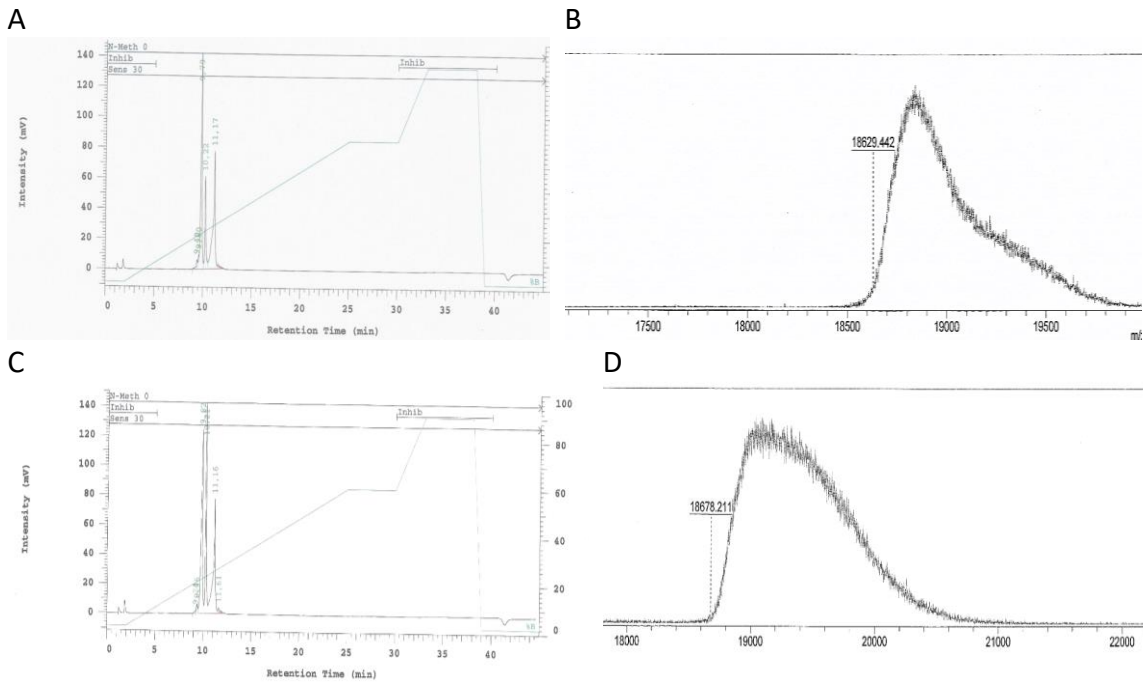


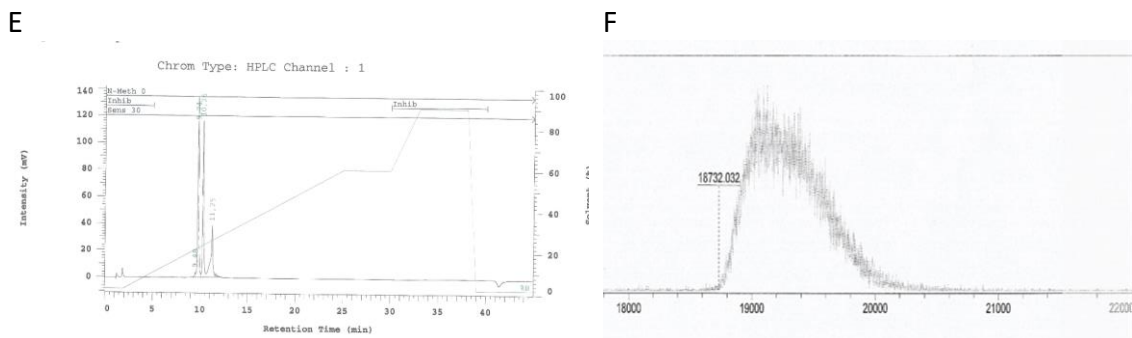


**Supplementary Figure S5.** Representative IE HPLC and MALDI TOF of the azide functionalized PhiX linker probe.

The alkyne functionalized ctDNA detection probes were “clicked” together with the azide functionalized PhiX linker probe following the general procedure:

The alkyne functionalized ctDNA detection probes(8.8  $\mu$ L, 56mg/L, 66.87 pmol) and the azide functionalized PhiX linker probe(12.3  $\mu$ L, 66mg/L, 72.10 pmol) were diluted in MQ water(20  $\mu$ L), dimethyl sulfoxide(15  $\mu$ L), triethylammonium acetate(5  $\mu$ L, 1 M, 7.0 pH) and copper(II) Tris(benzyltriazolylmethyl)amine(5  $\mu$ L, 10 mM) in a 1 mL reaction tube. The reaction mixture were degassed under argon and freshly prepared ascorbic acid were added (5  $\mu$ L 50 mM). Next, the reaction mixture were microwaved (30 min, 50  $^{\circ}$ C) and stirred overnight at rt. The probes were precipitated using cold acetone and used without further purification.





**Supplementary Figure S6** Representative IE HPLC and MALDI TOF of clicked ctDNA detection probes: PhiX clicked *BRAF* V600E mutation probe A-B. PhiX clicked *KRAS* G12D mutation probe C-D. PhiX clicked *KRAS* G13D mutation probe E-F.

### Cell line DNA

We used the following cell lines as positive controls:

1. LS411N (ATCC, product no. ATCC® CRL-2159™), Organism: Homo sapiens, human, Tissue: Cecum, Disease: Dukes' Type B, Colorectal Carcinoma, 66% *BRAF* V600E
2. HT-29 (ATCC® HTB-38™), Organism: Homo sapiens, human, Tissue: Colon, Disease: Colorectal Carcinoma, 25% *BRAF* V600E
3. HCT-15 (ATCC® CCL-225™), Organism: Homo sapiens, human, Tissue: Colon, Disease: Dukes' Type B, Colorectal Carcinoma, 100% *KRAS* G13D
4. LS 180 (ATCC® CL-187™), Organism: Homo sapiens, human, Tissue: Colon, Disease: Dukes' Type B, Colorectal Carcinoma, 100% *KRAS* G12D
5. HMEC-1 (ATCC® CRL-3243™), Organism: Homo sapiens, human, Tissue: Dermal Endothelium, Disease: none, wild type.

The protocols for cell line DNA purification and droplet digital PCR (ddPCR) were adapted from previous work.<sup>3</sup> ddPCR was performed using BioRad QX200 Droplet Digital PCR System and supermix reagents obtained from BioRad (assay ID dHsaCP2000028, **dMmuCPE5099897**). Each measurement was carried out in duplicate.

Fluorometry assay was done using Glomax-multi reader, Promega.

In each experiment, 5 ng of total DNA was used. Concentrations were calculated as described below.

The results of fluorometry/ddPCR analyses for the presence of target mutations (copy nr/ $\mu$ L in cell line no. 1-5) are given below:

<sup>3</sup> Miotke, L. et al. Anal Chem. **86**(5): 2618–2624 (2014).

**BRAF V600E**

(1),  $6 \times 10^7 / 6.7 \times 10^7$ ; (2),  $2.2 \times 10^7 / 1.3 \times 10^7$ ; (3), 0/0; (4),  $2 \times 10^2 / 4.2 \times 10^3$ ; (5), 0/0

**KRAS G12D**

(1),  $230 / 3.2 \times 10^3$ ; (2), 0/0; (3),  $9.3 \times 10^7 / 7.9 \times 10^7$ ; (4),  $2.8 \times 10^2 / 5.2 \times 10^3$ ; (5), 0/0

**KRAS G13D**

(1), 0/0; (2), 0/0; (3), 0/1150; (4)  $8.2 \times 10^7 / 7.0 \times 10^5$ ; (5), 0/0

Fluorometry results for **spike in experiments** (total 5 ng DNA, LS411N DNA + HMEC-1), % LS411N/determined copy No. ( $\times 10^7$ ): 100/6; 75/4.3; 50/3.0; 25/1.48; 15/0.92; 5/0.27; 3/0.13; 1/0.02

**Calculation of DNA concentration**

We chose copy number of the mutant sequence per 1  $\mu$ L of analyte as a measure of target concentration in cell line and ctDNA. For converting concentrations to copy numbers we used online resource <http://cels.uri.edu/gsc/cndna.html>.

For cell line study, we used 0.8-5 ng total genomic DNA. According to agarose gel electrophoresis, the average length of the genomic DNA fragments after digestion with EcoRI (described by Miotke *et al. PLoS ONE* (2015)), was approximately 5000 base pairs. Using the aforementioned on-line converter, the copy number for 5 ng DNA is  $9 \times 10^8$  copies. In the hybridization assay, genomic DNA concentration was kept at  $1.8 \times 10^6$  copies/ $\mu$ L, or 5 pM.

Total amount of purified ctDNA used per assay was 5 ng ctDNA. The length of ctDNA is only 300 nt, giving a copy number  $1.54 \times 10^{10}$ .

**Pre-enrichment of BRAF/KRAS gene fragment** was carried out using 120mer enrichment probe labeled with biotin (sequences given above), and streptavidin-coated magnetic beads (Dynabeads-M270, LifeTechnologies). The genomic DNA was pre-digested with EcoRI and annealed to biotinylated 120mer probe (40 min at 60 °C), followed by attachment to magnetic beads, multiple washing steps and detachment by heat as suggested by the supplier (IDT; heating to 92 °C for 5 min). As a result, single stranded DNA attached to the bead was obtained.

**Detecting ctDNA by solid-support hybridization assay.** Solid support containing corresponding capture probe and target DNA (1 eq.) were placed into 1.5 mL eppendorf tube containing 100  $\mu$ L of 1 $\times$ PBS buffer. The resulting mixture was heated for 10 min at 85 °C and subsequently cooled to room temperature over 20 min. The support was centrifuged at 11.000 rpm for 10 min, and after removing the supernatant it was washed 2 times with 100  $\mu$ L PBS at 37 °C. Afterwards PhiX was annealed (10 min at 85 °C and subsequently cooled to room temperature over 20 min), followed by 4x washing with cold PBS. The resulting beads were resuspended in 20  $\mu$ L 1 $\times$ PBS, heated to 90 °C and the PhiX-probe complex was detached from the beads. At the final step, EvaGreen dye (0.06–0.6X) was added and the samples were subjected to fluorometry detection.

**ctDNA preparation: Qiagen QIASymphony platform and Illumina NGS**

Qiagen QIASymphony automated platform was used at Rigshospitalet, Denmark, following manufacturer's instructions and using complementary reagents.

NGS was carried out on HiSeq2500 Illumina machine using the kits and protocols provided by manufacturer. NGS of ctDNA extracts confirmed *BRAF* V600E mutation in P1, *KRAS* G13D in P2 and *KRAS* G12D in P3. All healthy controls were tested negative for these three mutations.

Following Danish data protection regulations, personal data of the patients has not been disclosed for this work.

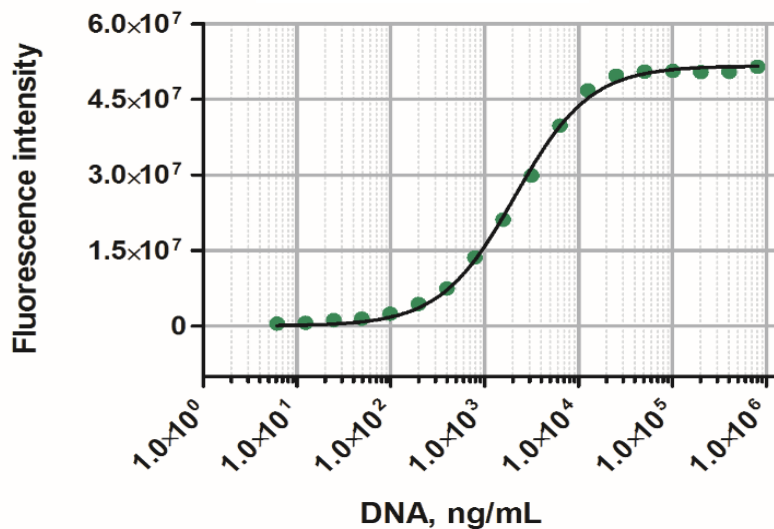
### Fluorometry – ctDNA from patient blood

Each sample containing detached DNA-probe-dye complexes from the analysis of blood samples was slightly diluted (12.2%) in order to adjust to measurement conditions, (final measurement conditions: 2×PBS (20 mM Na<sub>2</sub>HPO<sub>4</sub>, 3.6 mM KH<sub>2</sub>PO<sub>4</sub>, 274 mM NaCl and 5.4 mM KCl) and 780 nM EvaGreen). Immediately hereafter, fluorescence spectra were measured at a temperature of 21 °C, (excitation: 500 nm, emission 510-600 nm, FluoroMax-3 fluorometer, Jobin Yvon, Horiba). Three consecutive measurements were performed for each diluted sample (instrumental repetitions) and mean fluorescence values at 530 nm were converted to DNA concentrations by subtracting background fluorescence intensity at 530 nm ( $F_{ba}$ ) followed by comparison with the calibration curve (Figure S7). The background fluorescence intensity calculated from negative control samples without DNA ( $9.1 (\pm 0.5) \times 10^5$ ) was subtracted from all samples and the average fluorescence intensity at 530 nm was extracted. To be able to convert the measured fluorescence intensity into concentration of DNA, we prepared a calibration curve (Figure S7). The average value for  $F_{ba}$  of  $9.1 (\pm 0.5) \times 10^5$  was determined from negative control samples without any DNA. A calibration curve was prepared by plotting measured fluorescence intensity of control samples with known PhiX DNA concentrations ( $0-0.8 \times 10^6$  ng mL<sup>-1</sup>) (Figure S7). The plotted data were fitted to a one-site specific binding model:

$$F = B_{\max} \frac{([DNA] \cdot A)}{k_d + ([DNA] \cdot A)} \quad (1)$$

Here  $F$  is the fluorescence intensity (after  $F_{ba}$  subtraction),  $B_{\max}$  is the maximal possible fluorescence intensity when all EvaGreen is bound (given by  $5.2 (\pm 0.04) \times 10^7$ ),  $[DNA]$  is the DNA concentration in ng mL<sup>-1</sup>,  $A$  is the factor of dilution from the original sample (1.122) and  $k_d$  is the DNA concentration that binds half of the EvaGreen ( $2111 (\pm 81.3)$  ng mL<sup>-1</sup>). This model assumes that EvaGreen binds uncooperatively to specific sites in the DNA.

Note that, all samples with a measured fluorescence intensity in the raw data lower than the limit of detection (LOD) ( $\sim 10.6 \times 10^5$  fluorescence intensity) were regarded as negative. We defined LOD as  $F_{ba} + 3$  times the standard deviation.



**Supplementary Figure S7.** Calibration curve and limit of detection for fluorometry based detection of ctDNA. LOD with signal to noise ratio over 3,<sup>4</sup> has been determined as 780 ng/mL  $\pm$  11 ng/mL, or 10 pM  $\pm$  2.3 M.

#### **Effect of probe purity on the detection of genomic DNA**

Probe purity was evaluated by IE HPLC as described above. Sensitivity was calculated as a ratio of the detected copy number to the theoretical DNA abundance. In each experiment, 5 ng total DNA was used.

For *BRAF* V600E in LS411N cell line DNA, 84% pure probe resulted in 87% sensitivity of detection. Increasing probe purity to 93% resulted in 98% sensitivity.

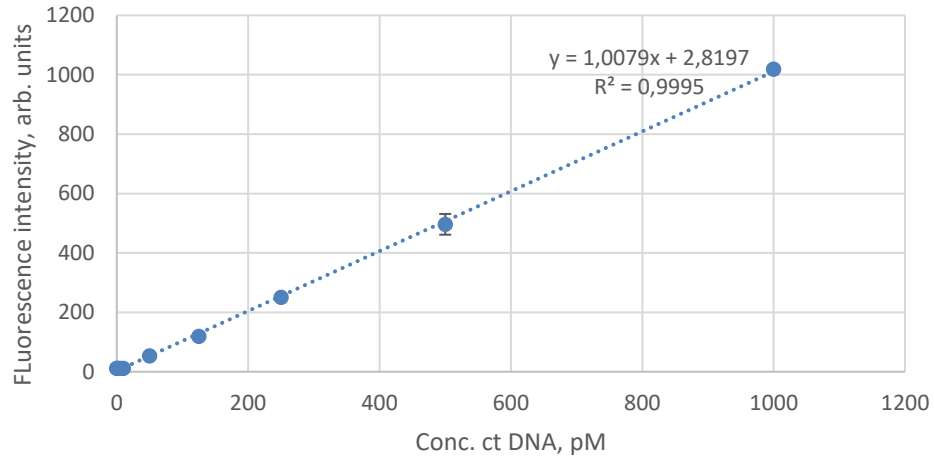
For *KRAS* G13D in HCT-15 cell line DNA, 85% pure probe resulted in 82% sensitivity of detection. Increasing probe purity to 92% resulted in 93% sensitivity.

#### **Control experiments with calf thymus DNA (CTD) signal booster**

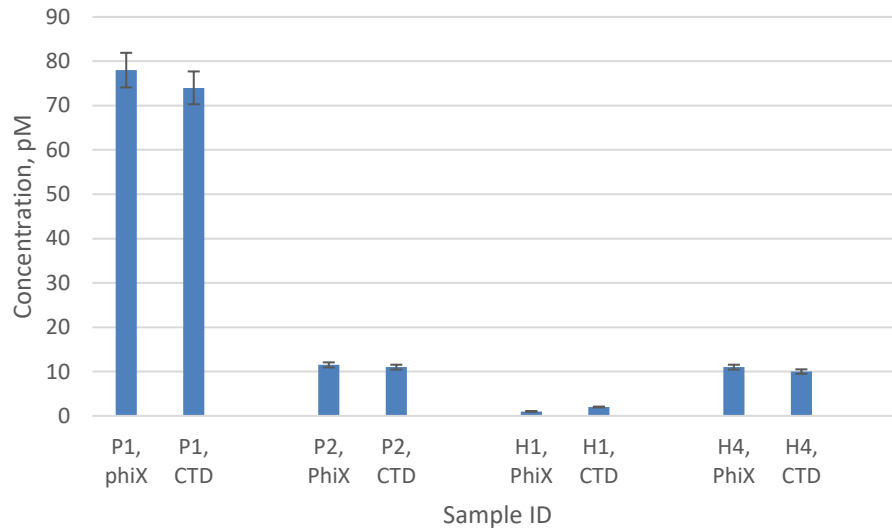
Ct DNA (CTD) genome accession code used for the linker sequence design was NC\_037330.1, GI: 1378962611.

For the assay, CTD Sigma reagent no. D1501 has been used.

<sup>4</sup> McNaught, Alan D., and Alan D. McNaught. Compendium of chemical terminology. Vol. 1669. Oxford: Blackwell Science, 1997.



**Supplementary Figure S8.** Calibration curve for CTD:Eva Green complex.



**Supplementary Figure S9.** Comparison data for PhiX and CTD signal boosters in *BRAF* V600E detection from patients P1,P2, and healthy controls H1,H4.