SUPPLEMENTARY DATA FOR

Multiplexed biomarkers dynamically detect heterogeneous residual neuroblastoma cell clone activity in the bone marrow niche

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TABLES

Table S1. Clinical characteristics of neuroblastoma study cohort.

Patient number	Sex	Age at diagnosis (in days)	Age at death (in days)	Tumor stage*	Site of primary tumor	Metastasis	Cytomorphological bone marrow infiltration	Relapse	HSCT
1	F	1368	alive	IV	retroperitoneal	renal, osseous	yes	no	yes
2	M	665	alive	IV	adrenal gland (left)	pleural, thoracal	yes	no	yes
3	F	1095	2190	IV	abdomen	intracranial, osseous	yes	yes	yes
4	F	517	1247	IV	abdomen	lymphatic	yes	no	yes
5	M	486	alive	IV	adrenal gland (right)	lymphatic	no	no	yes
6	F	1060	alive	IV	adrenal gland (left)	retroperitoneal lymphatic, osteomedullary	no	yes	yes
7	M	1125	1369	IV	adrenal gland	retrobulbary, orbital, intracranial, osseous	yes	no	yes
8	M	123	146	IV	adrenal glands (left and right)	liver, skin, muscular, osseous, renal	yes	no	no

*tumor stage according to INSS classification
F, female. M, male. HSCT, hematopoietic stem cell transplantation.

Table S2. Guidelines for quantification of MRD.

Parameters	ALL EuroMRD guidelines[1]	Neuroblastoma adapted MRD guidelines
Slope	≥ -3.1 to -3.9	≥ -3.1 to -4.4
Regression	≥ 0.98	≥ 0.99
CT of 10^{-1} dilution	not specified	< 29
Distance between each 10-fold dilution	2.6 to 4.0 CT	not specified
Distance between each 2-fold dilution	0.5 to 1.5 CT	not specified
DNA amount used for standard dilution	500 ng	500 ng
Serial dilution range	10^{-1}, 10^{-2}, 10^{-3}, $10^{-3,.3}$, 10^{-4}, $10^{-4.3}$, 10^{-5}	10^{-1}, 10^{-2}, 10^{-3}, $10^{-3.3}$, 10^{-4}, $10^{-4.3}$, 10^{-5}, 10^{-6}
Delta CT of replicates	≤ 1.5	≤ 1.5
Quantitative range / limit of quantification (LoQ)	CT values ≥ 3.0 lower than the lowest Ct value of the background (mutation-negative DNA control)	CT values ≥ 2.0 lower than the lowest C_t value of the background (mutation-negative DNA control)
Sensitivity / limit of detection (LoD)	CT values ≥ 1.0 lower than the lowest CT value of the background (mutation-negative DNA control)	CT values ≥ 1.0 lower than the lowest CT value of the background (mutation-negative DNA control)
PCR input / template	500ng DNA (equivalent to 75.000 cells)	500ng DNA (equivalent to 75.000 cells)
Normalization (reference gene)	beta-globin	beta-globin

[1] van der Velden et al., 2007 (27)
ALL, acute lymphoblastic leukemia. MRD, minimal residual disease. CT, threshold cycle.

Table S3. Genomic breakpoints of neuroblastoma cell line TR-14 and neuroblastoma patient cohort.

Cell line / patient (P)	Name of genomic breakpoint	Breakpoint rearrangement partner 1			Breakpoint rearrangement partner 2		
		Genomic position	Gene	Gene region	Genomic position	Gene	Gene region
TR-14	bkp 1 *MYCN*	chr2:15,952,491	intergenic	upstream *MYCN*	chr2:2,295,975	*MYT1L*	intron 1
	bkp 2 *MYCN*	chr2:15,966,854	intergenic	upstream *MYCN*	chr2:16,400,242	intergenic	downstream *GACAT3*
	bkp 3 *MYCN*	chr2:2,295,975	*MYT1L*	intron 1	chr2:15,952,488	intergenic	upstream *MYCN*
	bkp 4 *MYCN*	chr2:2,406,951	intergenic	downstream *MYT1L*	chr2:2,406,728	intergenic	downstream *MYT1L*
P1	bkp 1 *MYCN*	chr2:6,472,899	intergenic	downstream *SILC1*	chr2:16,077,648	*MYCNOS*	intron 3
	bkp 2 *MYCN*	chr2:15,485,527	*NBAS*	intron 35	chr2:15,485,644	intergenic	intron 35
	bkp 3 *MYCN*	chr2:15,089,801	intergenic	upstream *NBAS*	chr2:15,089,918	intergenic	upstream *NBAS*
	bkp 4 *MYCN*	chr2:16,885,922	intergenic	downstream *CYRIA*	chr2:16,896,040	intergenic	downstream *CYRIA*
P2	bkp 1 *MYCN*	chr2:15,953,878	intergenic	upstream *MYCN*	chr2:16,084,307	*MYCN*	intron 1
	bkp 2 *MYCN*	chr2:16,006,920	intergenic	upstream *MYCN*	chr2:16,007,205	intergenic	upstream *MYCN*
	bkp 3 *MYCN*	chr2:16,047,202	intergenic	upstream *MYCN*	chr2:16,047,488	intergenic	upstream *MYCN*
	bkp 4 *ACE2*	chrX:15,581,593	*ACE2*	intron 19	chrX:15,583,145	*ACE2*	intron 18
	bkp 5 *LOX*	chr5:121,059,231	intergenic	upstream *LOX*	chr5:121,060,010	intergenic	upstream *LOX*
P3	bkp1 *GPNH*	chr14:67,170,333	*GPHN*	intron 2	chr14:67,171,709	*GPHN*	intron 2
	bkp 2 *KIDINS220*	chr2:8,247,304	intergenic	upstream *KIDINS220*	chr2:8,250,390	intergenic	upstream *KIDINS220*
	bkp 3 *ACVR2A*	chr2:147,941,346	intergenic	upstream *ACVR2A*	chr2:147,946,805	intergenic	upstream *ACVR2A*
P4	bkp 1 *GPHN*	chr14:67,170,333	*GPHN*	intron 2	chr14:67,171,708	intron 2	*GPHN*
	bkp 2 *MYCN*	Chr2:15,735,596	*DDX1*	intron 2	chr2:15,736,742	*DDX1*	intron 1
P5	bkp 1 *MYCN*	chr2:13,490,249	intergenic	upstream *DDX1*	chr:15,815,066	intergenic	downstream *DDX1*
	bkp 2 *MYCN*	chr2:15,519,534	*NBAS*	intron 30	chr15,519,574	*NBAS*	intron 30
	bkp 3 *MYCN*	chr2:15,814,998	intergenic	downstream *DDX1*	chr15,815,134	intergenic	downstream *DDX1*
	bkp 4 *TERT*	chr5:1,256,596	intronic	intron 12	chr5:1,256,701	intronic	intron 12
P6	bkp 1 *MYCN*	chr2:15,714,587	intergenic	upstream *DDX1*	chr2:15,715,733	intergenic	downstream *NBAS*
	bkp 2 *TTC6*	chr14:38,133,147	*TTC6*	intron 3	chr14:38,137,807	*TTC6*	intron 3
P7	bkp 1 *MYCN*	chr2:15,387,809	*NBAS*	intron 44	chr2:15,387,945	*NBAS*	intron 44
P8	bkp 1 *MYCN*	chr2:16,365,867	intergenic	downstream *MYCN*	chr2:16,453,683	intergenic	downstream *MYCN*
	bkp 2 *NF1*	chr17:29,557,816	*NF1*	intron 24	chr22:16,311,557	intergenic	downstream *POTEH*

P, patient. Bkp, breakpoint. Chr, chromosome.

Table S4. SNVs of neuroblastoma patient cohort.

Patient (P)	Name of SNV	Genomic position	Type of mutation	cDNA change	Protein change	Sequencing coverage tumor	Allelic fraction tumor
P3	SNV *ALK*	chr2:29,432,664	missense	c.3824G>A	p.R1275Q	362x	55%
P7	SNV *BRCA1*	chr17:41,244,246	missense	c.3302G>A	p.S1101N	513x	27%

P, patient. SNV, single nucleotide variation. Chr, chromosome. cDNA, complementary DNA.

Table S5. Sensitivity and quantitative range of MP-PCR assays.

Cell line / patient (P)	Name of genomic MRD taget*	Assay sensitivity	Quantitative range EuroMRD[1]	Quantitative range NB adapted
TR-14	bkp 1 *MYCN*	E-5	E-4.3	E-5
	bkp 2 *MYCN*	E-5	E-4.3	E-5
	bkp 3 *MYCN*	E-5	E-4.3	E-5
	bkp 4 *MYCN*	E-6	E-4.3	E-5
P1	bkp 1 *MYCN*	E-3.3	E-2	E-3
	bkp 2 *MYCN*	E-6	E-3	E-6
	bkp 3 *MYCN*	E-6	E-3	E-5
	bkp 4 *MYCN*	E-6	E-3	E-5
	bkp5 *LOX*	E-3.3	E-2	E-3
P2	bkp 1 *MYCN*	E-3.3	E-3.3	E-3.3
	bkp 2 *MYCN*	E-4.3	E-3.3	E-4
	bkp 3 *MYCN*	E-5	E-3	E-4.3
	bkp 4 *ACE2*	E-4.3	E-3	E-4.3
P3	bkp1 *GPNH*	E-2	E-2	E-2
	bkp 2 *KIDINS220*	E-4	E-2	E-4
	bkp 3 *ACVR2A*	E-4.3	E-2	E-3.3
	SNV *ALK*	E-4	E-3	E-3.3
P4	bkp 1 *GPHN*	E-4	E-2	E-3.3
	bkp 2 *MYCN*	E-4.3	E-2	E-3.3
P5	bkp 1 *MYCN*	E-5	E-3	E-4.3
	bkp 2 *MYCN*	E-6	E-2	E-4.3
	bkp 3 *MYCN*	E-5	E-3	E-4.3
	bkp 4 *TERT*	E-5	E-3	E-4.3
P6	bkp 1 *MYCN*	E-5	E-3.3	E-4.3
	bkp 2 *TTC6*	E-4.3	E-3.3	E-3.3
P7	bkp 1 *MYCN*	E-4	E-2	E-2
	SNV *BRCA1*	E-4.3	n.q.	E-3.3
P8	bkp 1 *MYCN*	E-4	E-3	E-3.3
	bkp 2 *NF1*	E-4	E-3	E-3.3

[1] van der Velden et al., 2007 (27)
* The color coding represents the fluorescence color channels in which the respective genetic alterations were detected (*green* – FAM, *orange* – Atto-Rho-101, *red* – Atto-647-N, *crimson* – Cy5.5).
P, patient. MRD, minimal residual disease. NB, neuroblastoma. Bkp, breakpoint. SNV, single nucleotide variation. E, Euler´s number. N.q., not quantitative.

Table S6. Sensitivity and quantitative range of MP-PCR assays at individual time points.

P1

	day 0			day 52			day 164			day 226			day 256			day 310			day 614		
	MRD	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**
bkp 1 *MYCN*	2,56E-02			2,22E-04			negative			negative			negative			negative			negative		
bkp 2 *MYCN*	4,21E-02	DD	< 1%	8,29E-04	NDD	< 1%	negative	NDD	0%	negative	NDD	0%	negative	NDD	0%	negative	NDD	0%	negative	NDD	0%
bkp 3 *MYCN*	1,74E-02			6,17E-04			negative			negative			negative			negative			negative		
bkp 4 *MYCN*	1,32E-02			4,96E-04			negative			negative			negative			negative			negative		

P2

	day 0			day 29			day 41			day 121			day 142			day 223		
	MRD	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**
bkp 1 *MYCN*	negative			negative			negative			negative			negative			negative		
bkp 2 *MYCN*	negative			negative			negative			negative			negative			negative		
bkp 3 *MYCN*	negative	DD	< 1%	negative	NDD	0%	negative	NDD	0%	negative	NDD	0%	negative	NDD	0%	negative	NDD	n.a.
bkp 4 *ACE2*	negative			negative			negative			negative			negative			negative		
bkp 5 *LOX*	negative			negative			negative			negative			negative			negative		

P3

	day 0			day 324			day 733			day 850			day 1050			day 1138		
	MRD	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**
bkp 1 *GPHN*	1,15E-01			1,00E+00			9,59E-01			5,16E-01			4,11E-01			5,66E-01		
bkp 2 *KIDINS220*	1,01E-01	DD	0%	1,00E+00	NDD	0%	9,48E-01	NDD	n.a.	6,88E-01	NDD	0%	5,43E-01	NDD	n.a.	7,54E-01	NDD	0%
bkp 3 *ACVR2A*	1,23E-01			1,00E+00			9,49E-01			1,00E+00			7,19E-01			1,00E+00		
SNV *ALK*	9,55E-01			1,00E+00			8,26E-01			1,00E+00			1,00E+00			1,00E+00		

P4

	day 0			day 228		
	MRD	**bm**	**GD2**	**MRD**	**bm**	**GD2**
bkp 1 *GPHN*	7,04E-01	DD	31-100%	4,41E-01	NDD	< 1%
bkp 2 *MYN*	5,33E-01			1,98E-02		

P5

	day 0			day 39			day 60		
	MRD	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**
bkp 1 *MYCN*	3,59E-04			negative			negative		
bkp 2 *MYCN*	negative	NDD	< 1%	negative	NDD	< 1%	negative	NDD	0%
bkp 3 *MYCN*	3,61E-04			negative			negative		
bkp 4 *TERT*	negative			negative			negative		

P6

	day 0			day 185			day 241			day 276			day 553			day 646		
	MRD	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**
bkp 1 *MYCN*	3,56E-01	NDD	0%	negative	NDD	0%	negative	NDD	0%	negative	NDD	< 1%	negative	NDD	0%	negative	NDD	0%
bkp 2 *TTC6*	3,56E-01			4,92E-03			5,60E-03			2,11E-02			2,93E-03			1,35E-02		

P7

	day 0			day 25			day 49			day 77			day 193		
	MRD	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**	**MRD**	**bm**	**GD2**
bkp 1 *MYCN*	6,85E-01	DD	31-100%	1,25E-01	DD	< 1%	3,27E-01	NDD	n.a.	1,00E+00	NDD	< 1%	1,00E+00	NDD	0%
SNV *BRCA1*	1,00E+00			1,80E-01			1,47E-01			1,68E-01			2,59E-01		

P8

	day 0			day 23		
	MRD	**bm**	**GD2**	**MRD**	**bm**	**GD2**
bkp 1 *MYCN*	7,42E-01	DD	31-100%	1,91E-03	NDD	< 1%
bkp 2 *NF1*	8,79E-01			9,25E-01		

P, patient. MRD, minimal residual disease. Bm, bone marrow. GD2, disialoganglioside. Bkp, breakpoint. SNV, single nucleotide variation. DD, detectable disease. NDD, no detectable disease. E, Euler´s number.

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(27) Kipf E, Schlenker F, Borst N, Fillies M, Kirschner-Schwabe R, Zengerle R, et al. Advanced Minimal Residual Disease Monitoring for Acute Lymphoblastic Leukemia with Multiplex Mediator Probe PCR. *J Mol Diagn*. 2022;24(1):57–68.

(28) van der Velden VHJ, Cazzaniga G, Schrauder A, Hancock J, Bader P, Panzer-Grumayer ER, et al. Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. *Leukemia*. 2007;21(4):604–11.

FIGURES

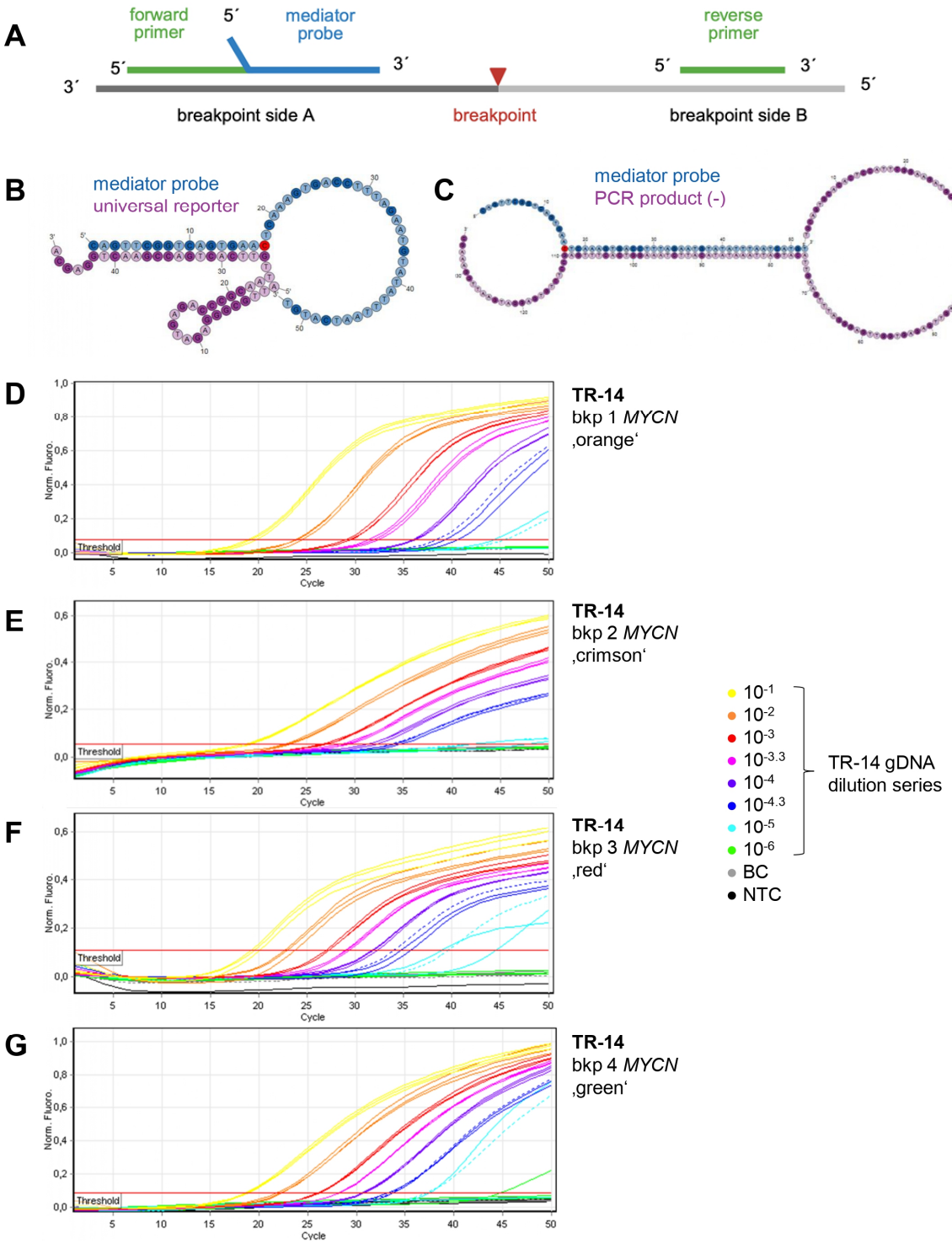

Fig. S1. TR-14 cell line as model for multiplex PCR assay development. A, positioning of primers and mediator probe on TR-14 DNA sequence flanking *MYCN* amplicon breakpoint. **B,** sequence-specific binding between mediator probe and universal reporter. **C,** sequence-specific binding between mediator probe and PCR product. B+C taken from AssayManager software (GNWI mbH, Germany, 27). **D,** dilution series of TR-14 gDNA for assay quantification of *MYCN* breakpoint 1 (fluorophore Atto-Rho-101, color channel *orange*). **E,** dilution series of TR-14 gDNA for assay quantification of *MYCN* breakpoint 2 (fluorophore Cy5.5, color channel *crimson*). **F,** dilution series of TR-14 gDNA for assay quantification of *MYCN* breakpoint 3 (fluorphore Atto-647-N, color channel *red*). **G,** dilution series of TR-14 gDNA for assay quantification of *MYCN* breakpoint 4 (fluorophore FAM, color channel *green*). D-G from Rotor-Gene Q software by Qiagen. Bkp, breakpoint. BC, buffy coat. NTC, no template control. gDNA, genomic DNA.

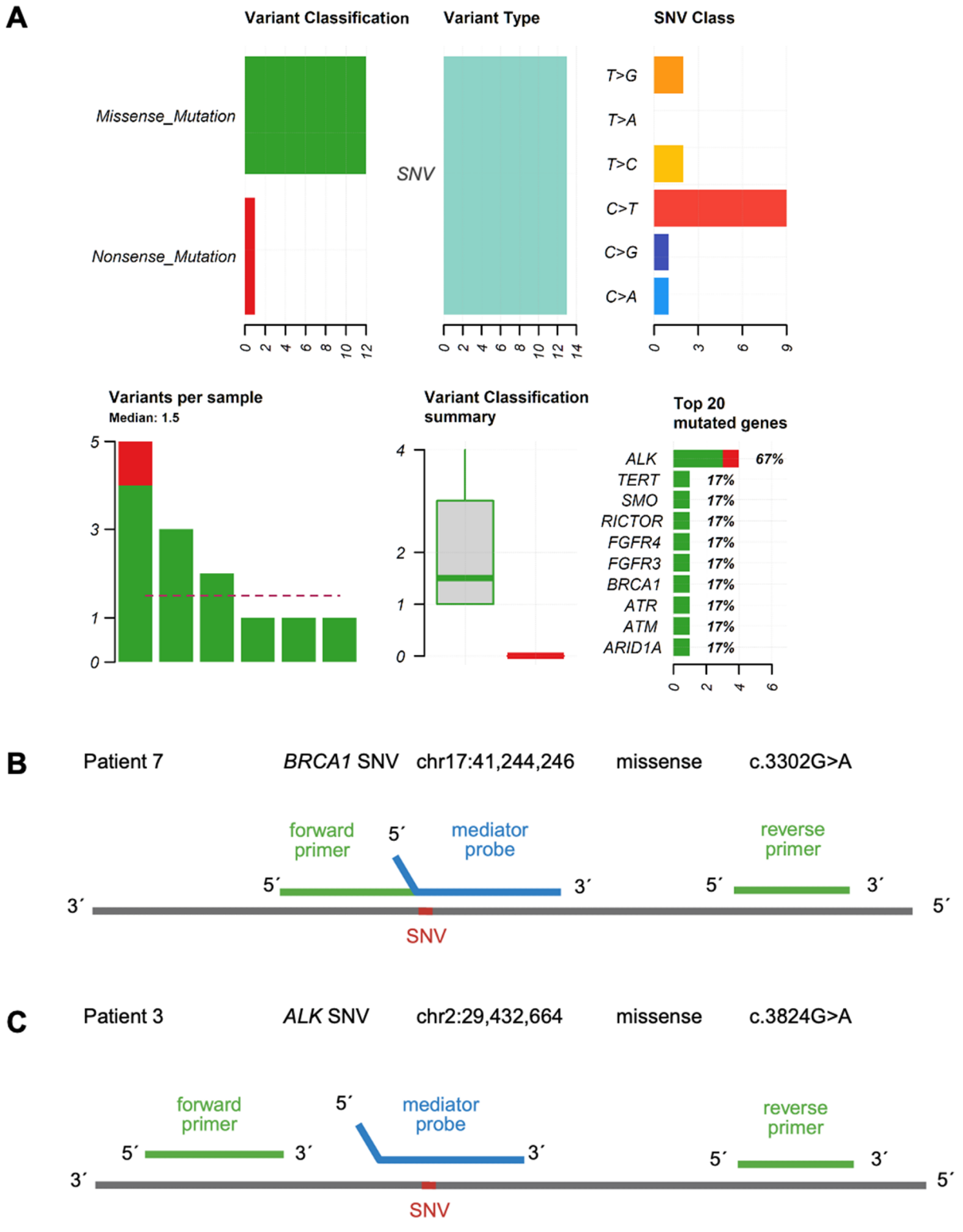

Fig. S2. Detection of SNVs in MP-PCR assays. A, landscape of SNVs in neuroblastoma tumor samples (Szymansky *et al*., 2021; 18). **B,** highly sensitive detection of SNVs applying MP-PCR chemistry using SNV approach (i) demonstrated at SNV c.3302G>A in *BRCA1* of patient P7. The 5´ end of the mediator probe is located on the SNV, with the forward primer directly adjacent to it in upstream position. **C,** highly sensitive detection of SNVs applying MP-PCR chemistry using SNV approach (ii) shown at SNV c.3824G>2 in *ALK* of patient P3. The mediator probe is spanning over the SNV with the forward primer not directly adjacent. P, patient. SNV, single nucleotide variation.

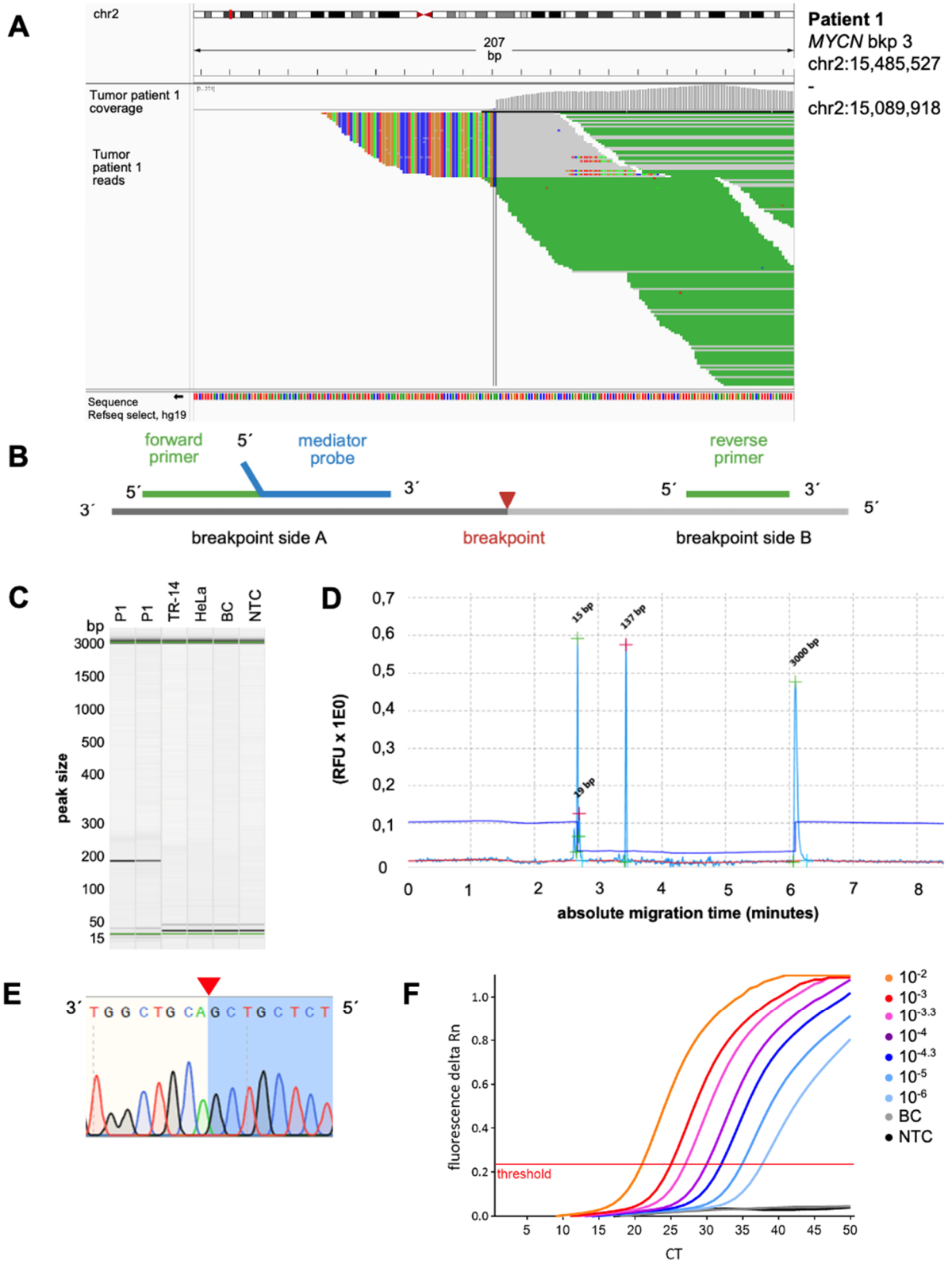

Fig. S3. Assay establishment for highly sensitive detection of genomic breakpoints using MP-PCR chemistry demonstrated at *MYCN* breakpoint 3 of patient P1. A, targeted panel sequencing of tumor tissue (Szymansky et al., 2021; 18). **B,** MP-PCR primer and mediator probe design with primers flanking the *MYCN* breakpoint. **C,** + **D,** QIAxel automated capillary electrophoresis for validation of the MP-PCR assays initially tested with conventional PCR. **E,** sanger sequencing to confirm PCR product sequence. **F,** dilution series of tumor gDNA using Rotorgene Q device for assessment of sensitivity and quantitative range. P, patient. Bkp, breakpoint. Bp, base pairs. BC, buffy coat. NTC, non template control. RFU, relative fluorescence units. CT, cycle threshold.

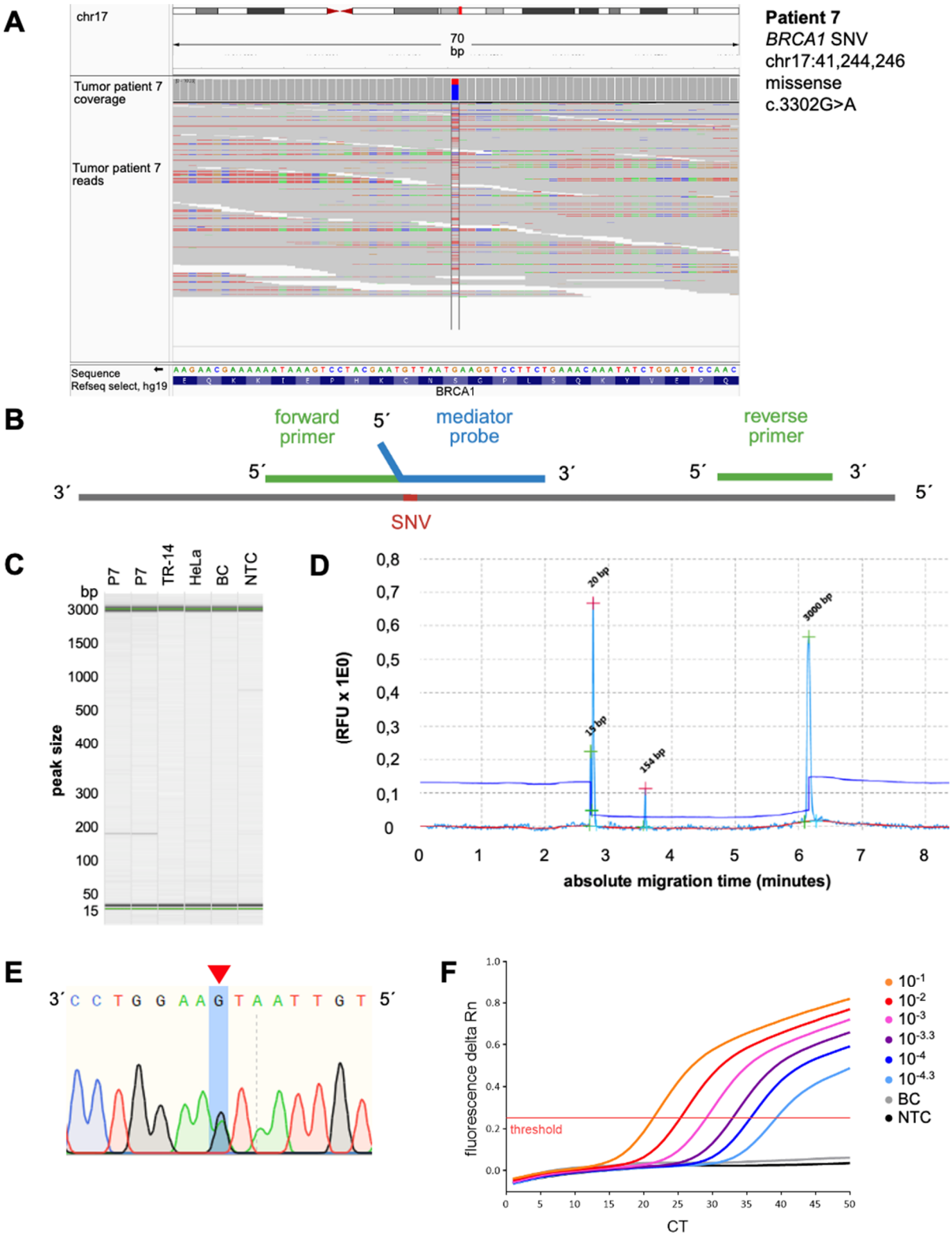

Fig. S4. Assay establishment for highly sensitive detection of SNV c.3302G>A in *BRCA1* of patient P7 using approach (i) for SNV detection. A, targeted panel sequencing of tumor tissue (Szymansky et al., 2021; 16). **B,** MP-PCR primer and mediator probe design with the 5´ terminal end of the probe located at the SNV and the forward primer directly adjacent. **C,** + **D,** QIAxel automated capillary electrophoresis for validation of the assays previosuly tested with conventional PCR. **E,** Sanger sequencing to confirm PCR product sequences. **F,** dilution series of tumor gDNA using Rotorgene Q device for assessment of sensitivity and quantitative range. P, patient. SNV, single nucleotide variation. Bp, base pairs. BC, buffy coat. NTC, non template control. RFU = relative fluorescence units. CT, cycle threshold.

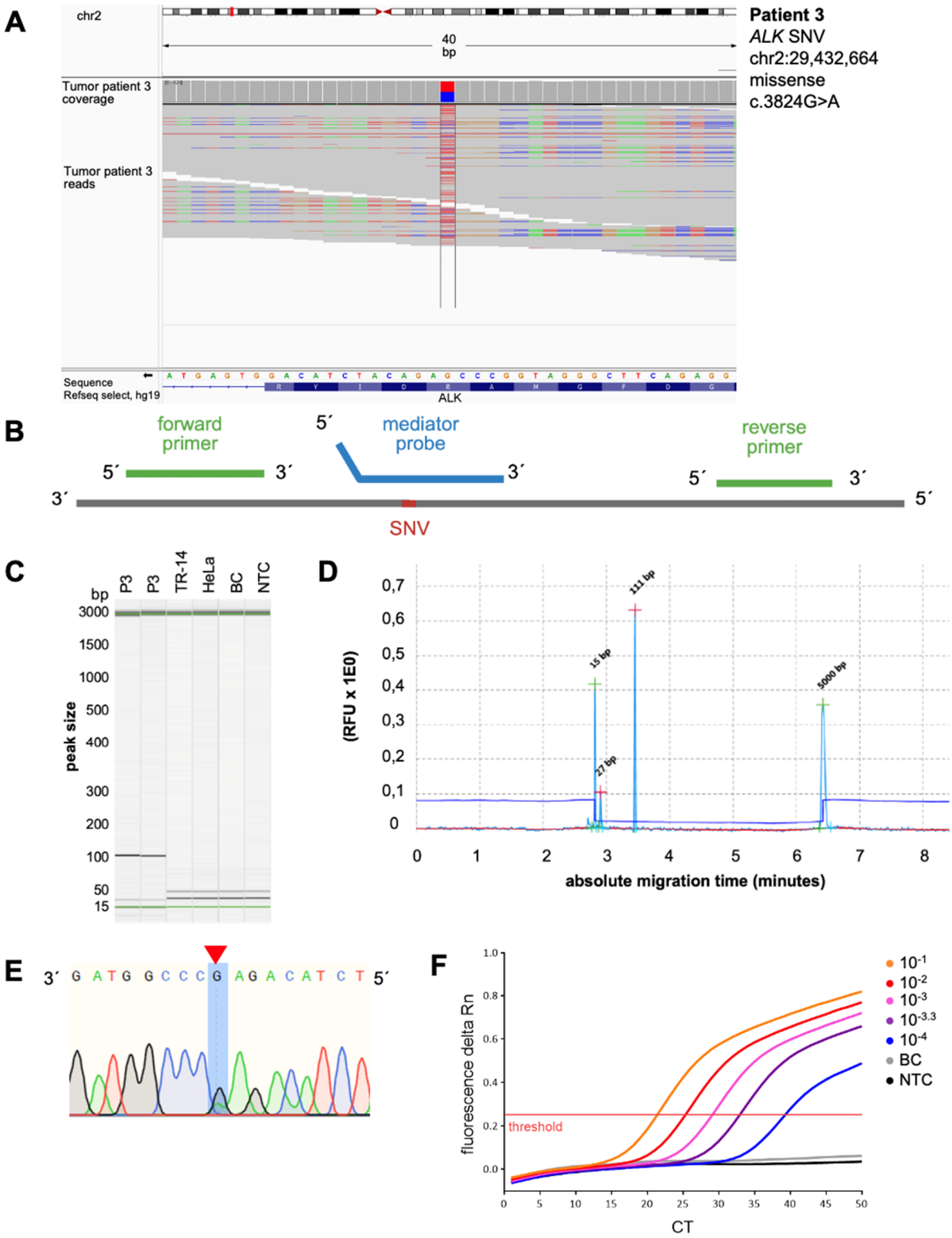

Fig. S5. Assay establishment for highly sensitive detection of SNV c.3824G>2 in *ALK* of patient P3 using approach (ii) for SNV detection. A, targeted panel sequencing of tumor tissue (Szymansky et al., 2021; 18). **B,** MP-PCR primer and mediator probe design with the probe located at the SNV and the forward primer not directly adjacent. **C,** + **D,** QIAxel automated capillary electrophoresis for validation of the assays previously tested with conventional PCR. **E,** Sanger Sequencing to confirm PCR product sequences. **F,** dilution series of tumor gDNA using Rotorgene Q device for assessment of sensitivity and quantitative range. P, patient. SNV, single nucleotide variation. Bp, base pairs. BC, buffy coat. NTC, non template control. RFU, relative fluorescence units. CT, cycle threshold.