Supplementary data for

Personalized Treatment Selection and Disease Monitoring by Circulating Tumor DNA Profiling in Real-World Cancer Patient Management

Julius Wehrle, Ulrike Philipp, Martina Jolic, Marie Follo, Saskia Hussung, Silvia Waldeck, Max Deuter, Michael Rassner, Jan Braune, Justyna Rawluk, Christine Greil, Cornelius F. Waller, Heiko Becker, Jesús Duque-Afonso, Anna-L. Illert, Ralph M. Fritsch, Frank Meiss, Justus Duyster, Nikolas von Bubnoff#*, Florian Scherer#*

#Equal contribution
*Corresponding authors. E-mails: florian.scherer@uniklinik-freiburg.de; nikolas.vonbubnoff@uksh.de

The file includes:

A

***EGFR* del19**			
Primer			
Gene	Target	Direction	Sequence
EGFR	Ex.19del	forward	5'- CCA GAA GGT GAG AAA GTT A-3'
		reverse	5'- CAG CAA AGC AGA AAC TCA-3'
Probes			
Gene	Target	Mutation	Sequence
EGFR	Ex.19del	Reference LNA	5'- /5HEX/CAA+CAA+GGA+AAT+CCT CGA T /3IABkFQ/ -3'
		Hotspot LNA	5'- /56-FAM/TGC+TTC+TCT+TAA+TTC CTT G /3IABkFQ/ -3'

***EGFR* T790M**			
Primer			
Gene	Target	Direction	Sequence
EGFR	T790M	forward	5'- CAG GAA GCC TAC GTG ATG-3'
		reverse	5'- GTG TTC CCG GAC ATA GTC-3'
Probes			
Gene	Target	Mutation	Sequence
EGFR	T790M	wild type	5'- /5HEX/C ATG+AGC+TGC+GTG+ATG AGC/3IABkFQ/ -3'

		c.2369C>T	5'- /56-FAM/C ATG+AGC+TGC+ATG+ATG AGC/3IABkFQ/ -3'

***EGFR* L858R**			
Primer			
Gene	Target	Direction	Sequence
EGFR	L858R	forward	Bio-Rad Laboratories, Inc. (UniqueAssayID: dHsaMDV2010021)
		reverse	Bio-Rad Laboratories, Inc. (UniqueAssayID: dHsaMDV2010021)
Probes			
Gene	Target	Mutation	Sequence
EGFR	L858R	wild type	Bio-Rad Laboratories, Inc. (UniqueAssayID: dHsaMDV2010021)
		c.2573T>G	Bio-Rad Laboratories, Inc. (UniqueAssayID: dHsaMDV2010021)

***EGFR* C797S**			
Primer			
Gene	Target	Direction	Sequence
EGFR	C797S	forward	5'- CAC CGT GCA GCT CAT CAC -3'
		reverse	5'- CAC ACC AGT TGA GCA GGT A -3'
Probes			
Gene	Target	Mutation	Sequence
EGFR	C797S	wild type	5'- /5HEX/ T TCG+GCT+GCC+TCC+TGG A /3IABkFQ/ -3'
		c.2390G>C	5'- /56-FAM/ T TCG+GCT+CCC+TCC+TGG A /3IABkFQ/ -3'

***EGFR* L861Q**			
Primer			
Gene	Target	Direction	Sequence
EGFR	L861Q	forward	Bio-Rad Laboratories, Inc. (UniqueAssayID: dHsaMDV2010043)
		reverse	Bio-Rad Laboratories, Inc. (UniqueAssayID: dHsaMDV2010043)
Probes			
Gene	Target	Mutation	Sequence
EGFR	L861Q	wild type	Bio-Rad Laboratories, Inc. (UniqueAssayID: dHsaMDV2010043)
		c.2582T>A	Bio-Rad Laboratories, Inc. (UniqueAssayID: dHsaMDV2010043)

***BRAF* V600E**			
Primer			
Gene	Target	Direction	Sequence
BRAF	V600E	forward	5'- GAT CCA GAC AAC TGT TCA -3'
		reverse	5'- ACA CCT CAG ATA TAT TTC TTC A -3'
Probes			
Gene	Target	Mutation	Sequence
BRAF	V600E	wild type	5'- /5HEX/ T CGA+GAT+TTC+ACT+GTA GCT /3IABkFQ/ -3'
		c.1799T>A	5'- /56-FAM/ T CGA+GAT+TTC+TCT+GTA GCT /3IABkFQ/ -3'

***BRAF* V600K**			
Primer			
Gene	Target	Direction	Sequence
BRAF	V600K	forward	5'- CAC CTC AGA TAT ATT TCT TCA TG-3'
		reverse	5'- GAT CCA GAC AAC TGT TCA A-3'

Probes			
Gene	Target	Mutation	Sequence
BRAF	V600K	wild type	5'- /5HEX/ CTA+GCT+ACA+GTG+AAA TCT C /3IABkFQ/ -3'
		c.1798_1799delinsAA	5'- /56-FAM/ TAG+CTA+CAA+AGA+AAT CTC G/3IABkFQ/-3'

***BRAF* V600R**			
Primer			
Gene	Target	Direction	Sequence
BRAF	V600R	forward	5'- ACA CCT CAG ATA TAT TTC TTC A-3'
		reverse	5'- CCA GAC AAC TGT TCA AAC-3'
Probes			
Gene	Target	Mutation	Sequence
BRAF	V600R	wild type	5'- /5HEX/ C GAG+ATT+TCA+CTG+TAG C/3IABkFQ/ -3'
		c.1798_1799delinsAG	5'- /56-FAM/ C GAG+ATT+TCC+TTG+TAG C/3IABkFQ/-3'

***KRAS* G12V**			
Primer			
Gene	Target	Direction	Sequence
KRAS	G12V	forward	5'- GCC TGC TGA AAA TGA CTG-3'
		reverse	5'- GCT GTA TCG TCA AGG CAC-3'
Probes			
Gene	Target	Mutation	Sequence
KRAS	G12V	wild type	5'- /5HEX/ CC+A +C+C+A G+CT C/3IABkFQ/ -3'
		c.35G>A	5'- /56-FAM/ CG+C C+A+A +CAG +CT/3IABkFQ/-3'

***NRAS* Q61R**			
Primer			
Gene	Target	Direction	Sequence
NRAS	Q61R	forward	5'- TCC TCA TGT ATT GGT CTC-3'
		reverse	5'- AGT GGT TAT AGA TGG TGA A-3'
Probes			
Gene	Target	Mutation	Sequence
NRAS	Q61R	wild type	5'- /5HEX/ CT CTT+CT+TG+TC+CAG C/3IABkFQ/ -3'
		c.182A>G	5'- /56-FAM/ CT CTT+CT+CG+TC+CAG C/3IABkFQ/-3'

***KIT* V559A**			
Primer			
Gene	Target	Direction	Sequence
KIT	V559A	forward	5'- CAC AGA AAC CCA TGT ATG-3'
		reverse	5'- GTT GGG TCT ATG TAA ACA TAA-3'
Probes			
Gene	Target	Mutation	Sequence
KIT	V559A	wild type	5'- /5HEX/ CCT CAA+CAA+CCT+TCC+ACT/3IABkFQ/ -3'
		c.1676T>C	5'- /56-FAM/ CCT CAA+CAG+CCT+TCC+ACT/3IABkFQ/-3'

***KIT* D816V**
Primer

Gene	Target	Direction	Sequence
KIT	D816V	forward	5'- CAT GGT CGG ATC ACA AAG-3'
		reverse	5'- CTG TCA AGC AGA GAA TGG-3'
Probes			
Gene	Target	Mutation	Sequence
KIT	D816V	wild type	5'- /5HEX/ TAG CCA+GAG+ACA+TCA+AGA A/3IABkFQ/ -3'
		c.2447A>T	5'- /56-FAM/ TAG CCA+GAG+TCA+TCA+AGA AT/3IABkFQ/-3'

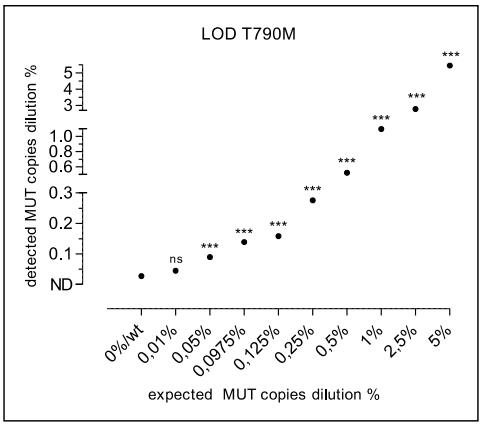
B

Cycling Step	**Temperature, °C**	**Time**	**Number of Cycles**
Enzyme activation	95	10 min	1
Denaturation	95	30 sec	40 (*EGFR*del, *EGFR* T790M, *EGFR* L858R, *EGFR* C797S, *EGFR* L861Q, *BRAF* V600E, *BRAF* V600K, *BRAF* V600R, *KRAS* G12V, *KIT* V559A, *KIT* D816V) 50 (*NRAS* Q61R)
Annealing/ Extension	55.0 (*EGFR*del, *EGFR* L858R, *EGFR* L861Q, *BRAF* V600K, *BRAF* V600R) 56.8 (*NRAS* Q61R) 57 (*BRAF* V600E) 58 (*KIT* D816V) 58.5 (*KIT* V559A) 59.4 (*KRAS* G12V) 63 (*EGFR* T790M, *EGFR* C797S)	60 sec (*EGFR*del, *EGFR* T790M, *EGFR* L858R, *EGFR* C797S, *EGFR* L861Q, *KIT* V559A) 90 sec (*BRAF* V600E, *BRAF* V600K, *BRAF* V600R, *KRAS* G12V, *NRAS* Q61R, *KIT* D816V)	
Enzyme deactivation	98	10 min	1
Hold (optional)	12	Infinite	1

Suppl. Fig. S1. Specifications of digital droplet PCR assay development. Shown are primer/probe sequences (A) and cycling conditions (B) for all ddPCR assays. Primers and probes for the *EGFR* L858R and L861Q assays were obtained from Bio-Rad Laboratories. The *KRAS* G12V assay was developed by Hussung et al., J Mol Diagn., 2020, PMID: 32376474.

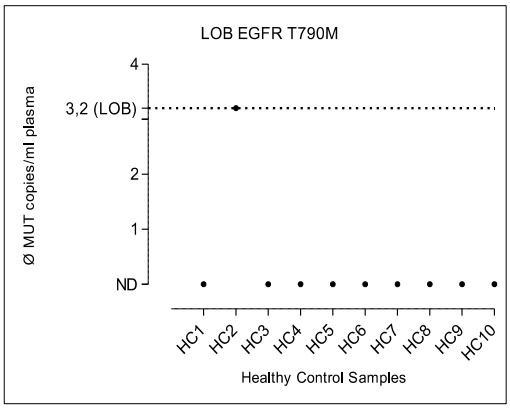
A

Dilution – Expected AF (%)	Ø MUT copies/well	Ø WT copies/well	MUT:WT	SD	Detected AF (%)	Significance
NTC	0	0				
WT	5.05	18710		2	0.027	
5%	1035.5	19015	1:18	28	5.45	***
2,50%	528.5	19015	1:36	24	2.78	***
1%	207.5	18915	1:91	8	1.1	***
0,50%	98.5	18845	1:191	13	0.52	***
0,25%	52	18925	1:364	3	0.27	***
0,125%	29.35	18620	1:634	5	0.16	***
0,0975%	25.2	18210	1:723	3	0.14	***
0,05%	16.75	18798	1:1122	4	0.089	***
0,01%	8.03	18110	1:2255	3	0.044	n.s.



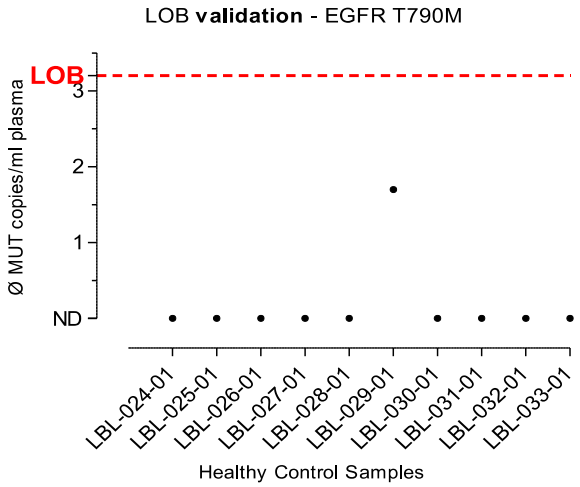
B

Sample	Vol. plasma (mL)	Vol. eluate (µl)	mL plasma / well	Ø WT copies/ well	Ø WT copies /mL plasma	Ø MUT copies /well	Ø MUT copies /mL plasma
NTC				0		0	
WT				850		1	
MIX				2680		607	
HC 1	1.8	67	0.19	220	1170	0	0
HC 2	1.9	65	0.20	262	1280	0.65	3.2
HC 3	1.85	72	0.18	220	1223	0	0
HC 4	1.95	71	0.19	206	1072	0	0
HC 5	2	74	0.19	182	962	0	0
HC 6	1.98	63	0.22	87	395	0	0
HC 7	1.9	65	0.20	156	762	0	0
HC 8	1.95	73	0.19	476	2546	0	0
HC 9	2	65	0.22	153	708	0	0
HC 10	2	66	0.21	113	533	0	0
Mean						0.1	0.3
σ						0.195	0.953
LOB *EGFR* T790M						**0.7**	**3.2**



Suppl. Fig. S2. Digital droplet PCR assay development. Design and development of the *EGFR* T790M digital droplet PCR (ddPCR) assay, representative of all the assays developed in this work. (A) Left: Specifications and results of the dilution series performed to assess limit of detection (LOD), using recombinant mutant DNA fragments in the presence of human genomic DNA. LOD was determined as the ratio of mutant vs. total number of copies from the highest dilution that was significantly above the background, in this case 1:1122 copies/well (= 0.089%). Right: Scatter plot showing the correlation of expected mutant copies (in %) with detected mutant copies (in %) in the dilution series. (B) Determining limit of blank (LOB) by analyzing cell-free DNA (cfDNA) extracted from 10 healthy controls. Left: Table showing plasma/cfDNA input information and test results, with 3.2 mutant copies per mL of plasma being the LOB calculated for the *EGFR* T790M assay (see Materials and Methods). Right: Number of positive mutant copies per mL of plasma in each healthy control sample (HC1 – HC10). Red dashed line: LOB. LODs and LOBs of all assays are depicted in Suppl. Table 2. AF, allele frequency; Vol., volume; MUT, mutant; WT, wildtype; SD (σ), standard deviation; mL, milliliters; µl, microliters; NTC, non-template control; HC, healthy control.

Sample	Vol. plasma (mL)	Vol. eluate (µl)	mL plasma/ well	Ø MUT copies/well	Ø WT copies/well	Ø MUT copies/ mL plasma	Ø WT copies/ mL plasma	AF [%]
LBL-024	1.95	75	0.18	0	127	0	697.8	0
LBL-025	1.7	73	0.16	0	148	0	907.9	0
LBL-026	1.7	74	0.16	0	349	0	2170.3	0
LBL-027	1.65	66	0.18	0	52	0	297.1	0
LBL-028	1.7	64	0.19	0	112	0	602.4	0
LBL-029	1.65	65	0.18	0.3	130	1.7	731.6	0.23
LBL-030	1.6	65	0.17	0	119.5	0	693.5	0
LBL-031	1.55	66	0.16	0	150.5	0	915.5	0
LBL-032	1.75	71	0.17	0	138	0	799.8	0
LBL-033	1.9	67	0.199	0	246.5	0	1241.8	0

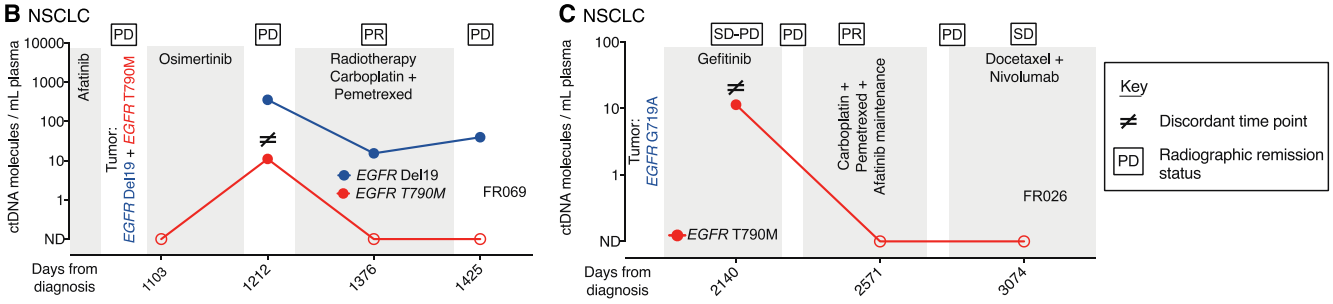


Suppl. Fig. S3. Digital droplet PCR assay validation. Validation of the *EGFR* T790M ddPCR assay, representative of all the assays developed in this work. Specificity was assessed by running the *EGFR* T790M assay on cfDNA obtained from an independent cohort of 10 healthy donors. Top: Table showing plasma/cfDNA input information and test results. Bottom: Number of positive mutant copies per mL of plasma in each healthy control sample (LBL-024 – LBL-033). Dashed red line: LOB defined in Suppl. Figure 1. AF, allele frequency; Vol., volume; MUT, mutant; WT, wildtype; mL, milliliters; LOB, limit of blank.

A

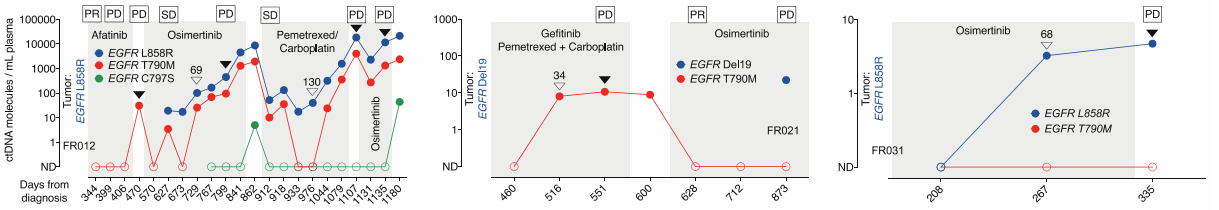
	Tumor positive	Tumor negative	**Total (*n*)**	**Performance**
Plasma positive	14	2*	**16**	**PPV: 87.5% (*100%)**
Plasma negative	2	38	**40**	**NPV: 95%**
Total (*n*)	**16**	**40**	**56**	
Performance	**Sensitivity: 87.5%**	**Specificity: 95% (*100%)**		

*** Tumor results likely false negative**

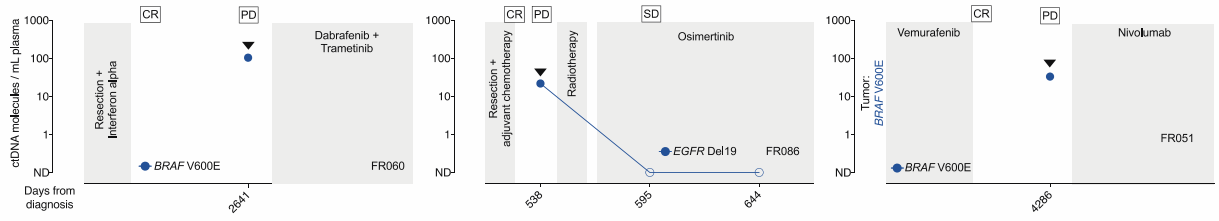


Suppl. Fig. S4. Digital droplet PCR performance. (A) Table showing sensitivity, specificity, positive predictive value, and negative predictive value of all ddPCR assays developed in this work compared to tumor tissue genotyping as a gold standard. Asterisk: 2 plasma samples showed *EGFR* T790M mutations while genotyping from tumor biopsies did not identify these aberrations. (B, C) Shown are the concentrations of ctDNA molecules (*EGFR* Del19 and *EGFR* T790M) per mL of plasma in serial blood samples obtained from the two NSCLC patients with discordant results of matched tumor-plasma samples. Crossed equal signs represent discordant time points when ctDNA was detected in plasma but mutations were not found in tumor biopsies. PPV, positive predictive value; NPV, negative predictive value; PD, progressive disease; PR, partial response; SD, stable disease; mL, milliliters; ND, not detected; NSCLC, non-small cell lung cancer.

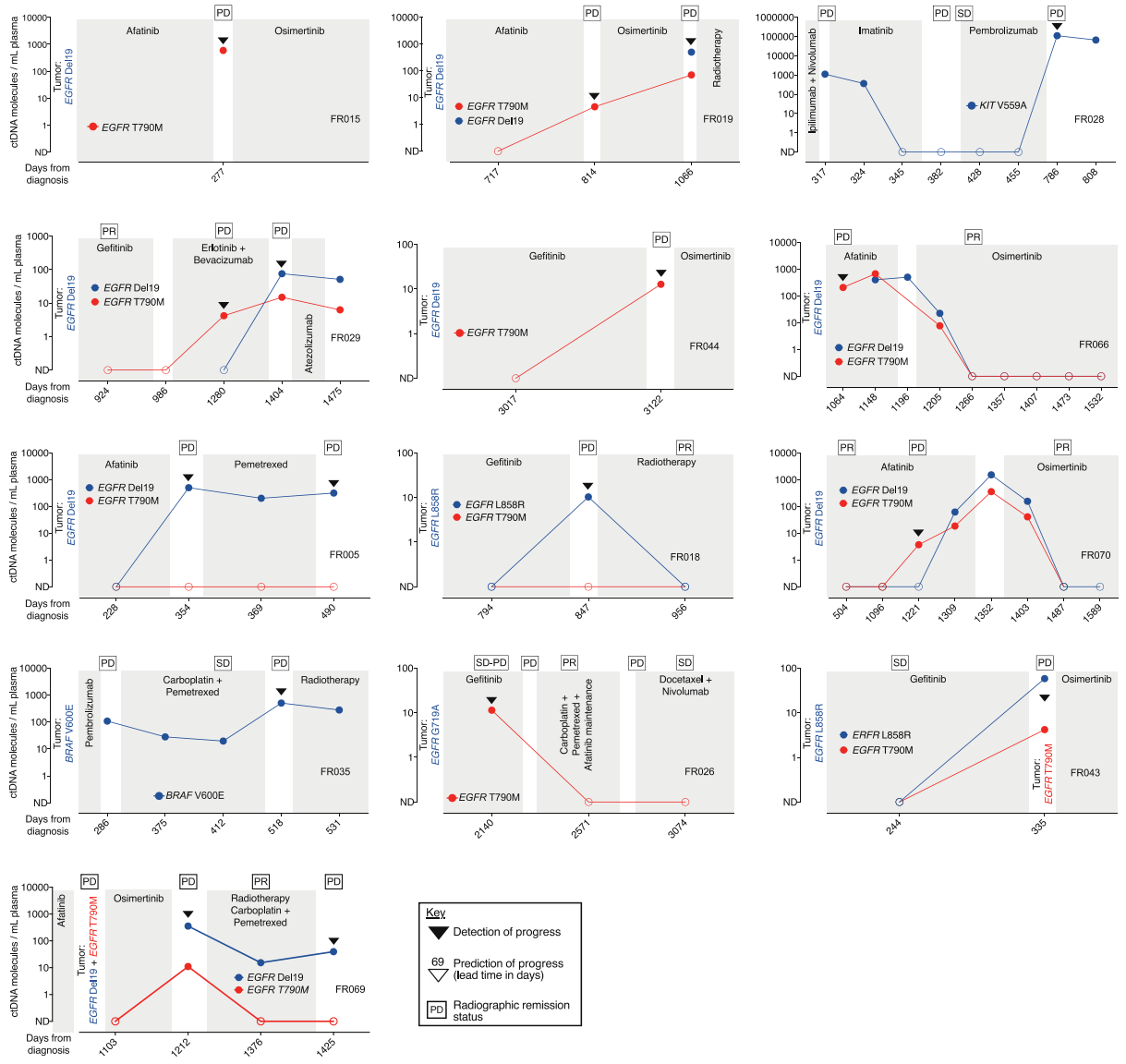
A Prediction of disease progression



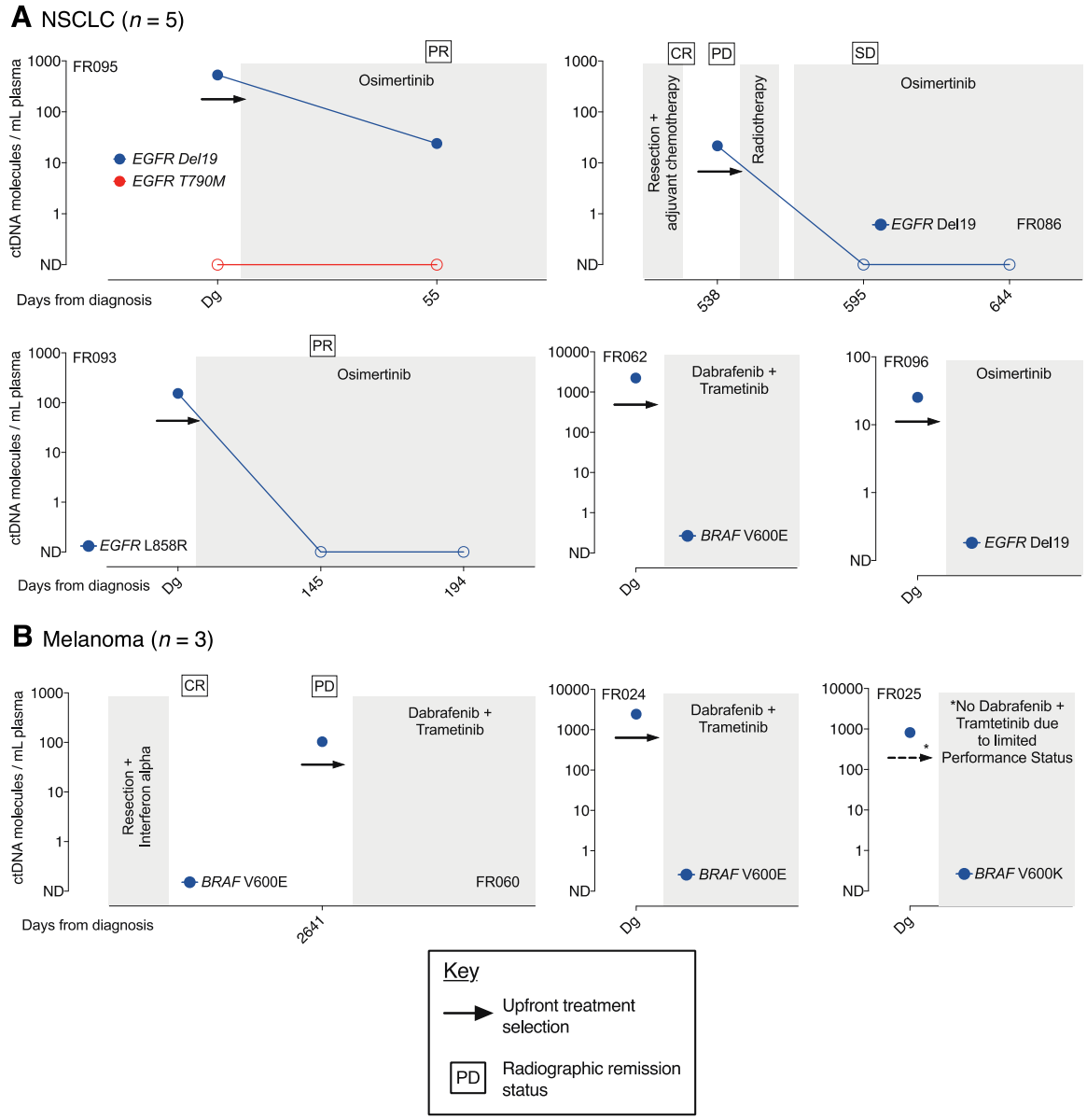
B Detection of disease relapse



C Detection of disease progression



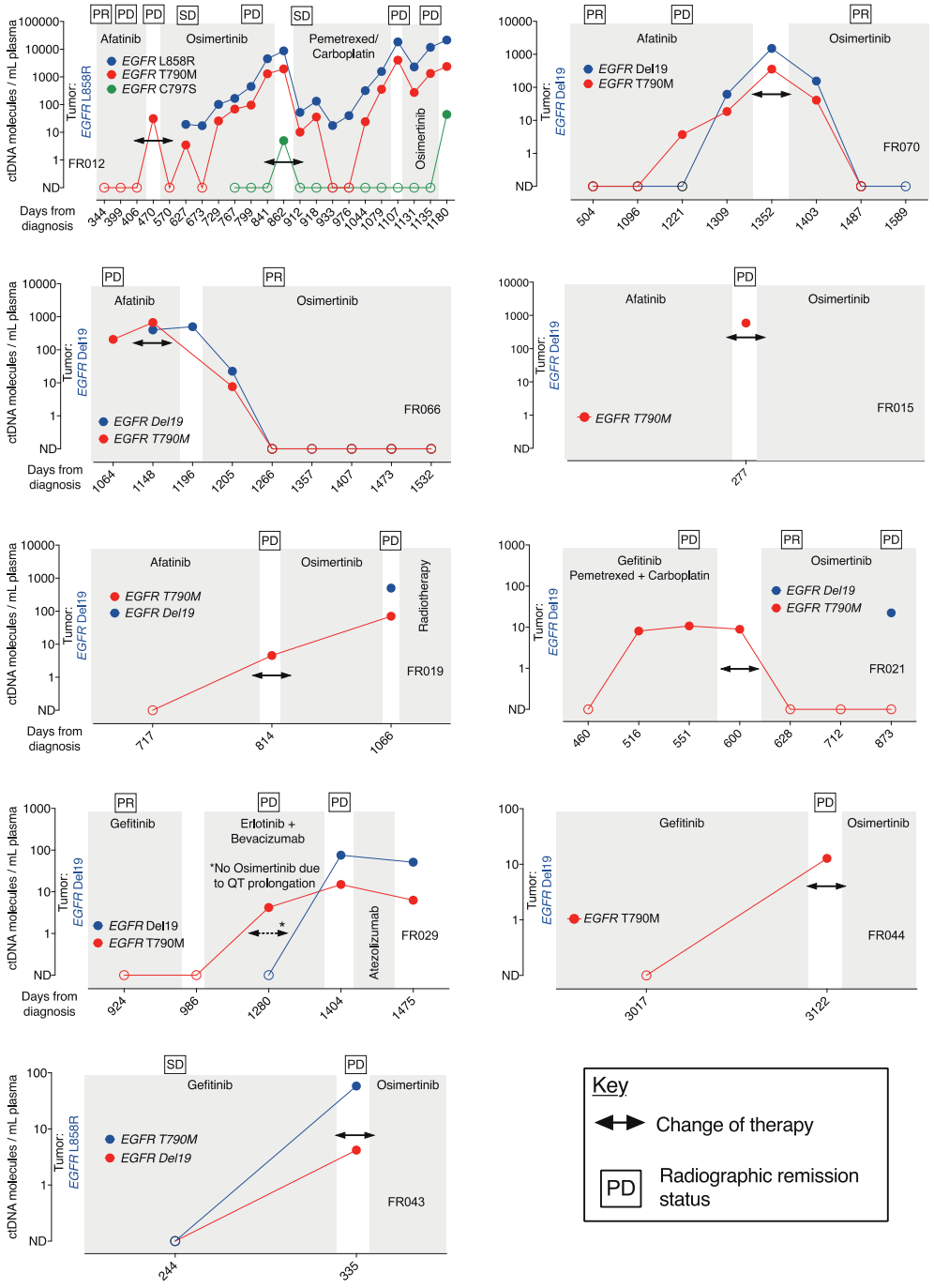
Suppl. Fig. S5. Prediction and detection of disease progression by ctDNA monitoring. Serial monitoring of blood plasma highlighting the value of ctDNA as a biomarker for prediction (A) and detection (B, C) of disease progression or relapse after complete response, either by an increase of ctDNA concentrations over time or the identification of new aberrations reflecting emerging subclones. Triangles highlight plasma sample with increasing ctDNA levels in plasma, either predicting (empty triangles) or detecting radiographic disease progression (black triangles). Numbers above empty triangles represent the time from increase of ctDNA concentrations to radiographic disease progression in days. Tumor genotyping results at diagnosis are depicted on the left side of each graph. Black rectangle, radiographic remission status; PD, progressive disease; PR, partial response; SD, stable disease; CR, complete response; mL, milliliters; ND, not detected.



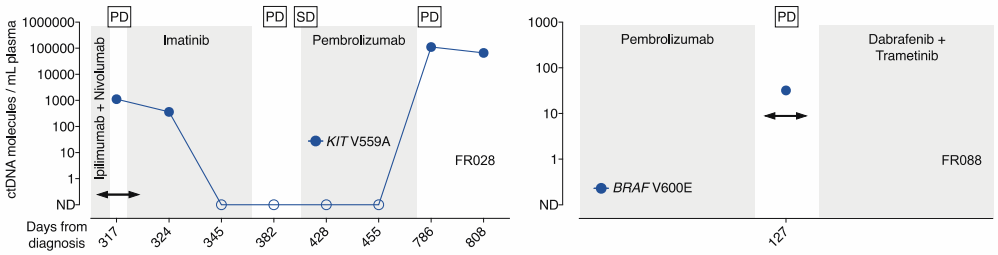
Suppl. Fig. S6. Identification of actionable gene targets by ctDNA profiling. Shown are five NSCLC cases (A) and three melanoma patients (B) in whom detection of tumor

mutations in druggable gene targets led to targeted treatment selection and in whom these mutations were also detected in plasma ctDNA. Black arrows highlight treatment selection based on mutation detection in druggable gene targets. Black rectangle, radiographic remission status; PD, progressive disease; PR, partial response; SD, stable disease; CR, complete response; mL, milliliters; ND, not detected; NSCLC, non-small cell lung cancer.

A NSCLC (*n* = 9)



B Melanoma (*n* = 2)



Suppl. Fig. S7. Detection of resistance mutations by noninvasive ctDNA assessment. This figure demonstrates 9 NSCLC (A) and 2 melanoma patients (B) in whom treatment was adapted based on the detection of novel or resistance mutations and in whom these aberrations were also identified in plasma ctDNA. Black arrows highlight the time point of mutation detection that led to treatment adjustment. Tumor genotyping results at diagnosis are depicted on the left side of each graph. Black rectangle, radiographic remission status; PD, progressive disease; PR, partial response; SD, stable disease; CR, complete response; mL, milliliters; ND, not detected; NSCLC, non-small cell lung cancer.