Supporting information

Establishment of Primary Reference Measurement Procedures and Reference Materials for *EGFR* variant detection in non-small cell lung cancer

S1 Supporting Methods

S1.1 ctDNA RM preparation

Conventional PCR was conducted in a volume of 100 μL, which comprised 50 μL of 2× PCR Mix (CWBIO, Beijing, China), 5 μL of 5 μM primer mixture (Thermo Scientific, Beijing, China), 35 μL of ddH2O, and 10 μL of template DNA. A conventional PCR machine (Veriti, Applied Biosystems, CA, USA) was used. The thermal cycle consisted of a 10-min denaturation period at 95°C followed by 40 cycles of a three-step thermal profile of 15 s at 95°C, 30 s at 56°C, and 60 s at 72°C and one final extension step at 72°C for 10 min. Each PCR amplicon was gravimetrically mixed with fragmented WT gDNA to prepare the candidate ctDNA RM. The mixture was diluted in yeast total RNA (Sigma, USA) at a concentration of 400 ng/μL. Then the solution was stirred on a magnetic stir plate for 1 h, after which it was pipetted into sterile 0.5-mL screw cap vials at approximately 50 μL/vial and stored at −70°C.

All PCR products were assessed for size and purity using the 2100 Bioanalyzer (Agilent 2100, Agilent, USA) with the Agilent DNA 1000 Kit and purified with a commercial DNA clean-up kit (CWBIO, Beijing, China). To verify each target mutant, the purified PCR product was sequenced using a Sanger sequencer (ABI 3310, Thermo Fisher, USA). The concentrations of PCR products were estimated by UV absorbance (NanoDrop 2000, Thermo, USA), then the copy number concentration was based on the molecular weight of the fragment and the Avogadro constant.

S1.2 dPCR mixture preparation and standard thermal cycling protocol

The dPCR analysis was performed on a QX200 system (BioRad Laboratories, Inc., CA, USA). The reaction mixture, in a total volume of 20 μL, consisted of 10 μL of 2× ddPCR Super Mix for Probe (BioRad Laboratories, Inc.), 3.6 μL of 5 μM primer mixture, 0.5 μL of 10 μM VIC-labeled probe, 0.5 μL of 5 μM FAM-labeled probe (Thermo Scientific, USA), 1.4 μL of $TE_{0.1}$, and 4 μL of template DNA with a concentration of 10 ng/μL. A conventional PCR machine (Veriti) was used for the thermocycling. Thermal cycling consisted of a 10 min denaturation period at 95°C, followed by 40 cycles of a two-step thermal profile of 15 s at 95°C and 60 s at 58°C for combined annealing–extension and one final denaturation step at 98°C for 10 min.

Results were analyzed with the QuantaSoft v.1.7.4 software (BioRad Laboratories, Inc.)[1].

Supporting Equations

Uncertainty evaluation equations for the gravimetrical method.

$$C_{MU} = \frac{C_{PCR} \times \frac{M_{PCR}}{\rho_{PCR}}}{\frac{M_{PCR}}{\rho_{PCR}} + \frac{M_{gWT}}{\rho_{gWT}}} \quad \text{(S1)}$$

$$C_{WT} = \frac{C_{gWT} \times \frac{M_{gWT}}{\rho_{gWT}}}{\frac{M_{PCR}}{\rho_{PCR}} + \frac{M_{gWT}}{\rho_{gWT}}}, \quad \text{(S2)}$$

$$FA = \frac{C_{PCR} \times \frac{M_{PCR}}{\rho_{PCR}}}{C_{PCR} \times \frac{M_{PCR}}{\rho_{PCR}} + C_{gWT} \times \frac{M_{gWT}}{\rho_{gWT}}} \times 100\% \quad \text{(S3)}$$

$$\frac{uFA}{FA} = \sqrt{\left(\frac{uC_{PCR}}{C_{PCR}}\right)^2 + \left(\frac{uM_{PCR}}{M_{PCR}}\right)^2 + \left(\frac{u\rho_{PCR}}{\rho_{PCR}}\right)^2 + \left(\frac{u(C_{PCR} \times \frac{M_{PCR}}{\rho_{PCR}} + C_{WT} \times \frac{M_{gWT}}{\rho_{gWT}})}{C_{PCR} \times \frac{M_{PCR}}{\rho_{PCR}} + C_{WT} \times \frac{M_{gWT}}{\rho_{gWT}}}\right)^2} \quad \text{(S4)}$$

$$u\left(C_{PCR} \times \frac{M_{PCR}}{\rho_{PCR}} + C_{gWT} \times \frac{M_{gWT}}{\rho_{gWT}}\right)^2 = u\left(C_{PCR} \times \frac{M_{PCR}}{\rho_{PCR}}\right)^2 + u\left(C_{gWT} \times \frac{M_{gWT}}{\rho_{gWT}}\right)^2 \quad \text{(S5)}$$

$$\left(\frac{u\left(C_{PCR} \times \frac{M_{PCR}}{\rho_{PCR}}\right)}{C_{PCR} \times \frac{M_{PCR}}{\rho_{PCR}}}\right)^2 = \left(\frac{uC_{PCR}}{C_{PCR}}\right)^2 + \left(\frac{uM_{PCR}}{M_{PCR}}\right)^2 + \left(\frac{u\rho_{PCR}}{\rho_{PCR}}\right)^2 \quad \text{(S6)}$$

$$\left(\frac{u\left(C_{gWT} \times \frac{M_{gWT}}{\rho_{gWT}}\right)}{C_{gWT} \times \frac{M_{gWT}}{\rho_{gWT}}}\right)^2 = \left(\frac{uC_{gWT}}{C_{gWT}}\right)^2 + \left(\frac{uM_{gWT}}{M_{gWT}}\right)^2 + \left(\frac{u\rho_{gWT}}{\rho_{gWT}}\right)^2 \quad \text{(S7)}$$

$$\frac{uC_{MU}}{C_{MU}} = \sqrt{\left(\frac{uC_{PCR}}{C_{PCR}}\right)^2 + \left(\frac{uM_{PCR}}{M_{PCR}}\right)^2 + \left(\frac{u\rho_{PCR}}{\rho_{PCR}}\right)^2 + \left(\frac{u(\frac{M_{PCR}}{\rho_{PCR}} + \frac{M_{gWT}}{\rho_{gWT}})}{\frac{M_{PCR}}{\rho_{PCR}} + \frac{M_{gWT}}{\rho_{gWT}}}\right)^2} \quad \text{(S8)}$$

$$u\left(\frac{M_{PCR}}{\rho_{PCR}}+\frac{M_{gWT}}{\rho_{gWT}}\right)^2=u\left(\frac{M_{PCR}}{\rho_{PCR}}\right)^2+u\left(\frac{M_{gWT}}{\rho_{gWT}}\right)^2 \quad \text{(S9)}$$

$$\left(\frac{u(\frac{M_{PCR}}{\rho_{PCR}})}{\frac{M_{PCR}}{\rho_{PCR}}}\right)^2=\left(\frac{uM_{PCR}}{M_{PCR}}\right)^2+\left(\frac{u\rho_{PCR}}{\rho_{PCR}}\right)^2 \quad \text{(S10)}$$

$$\left(\frac{u(\frac{M_{gWT}}{\rho_{gWT}})}{\frac{M_{gWT}}{\rho_{gWT}}}\right)^2=\left(\frac{uM_{gWT}}{M_{gWT}}\right)^2+\left(\frac{u\rho_{gWT}}{\rho_{gWT}}\right)^2 \quad \text{(S11)}$$

$$\frac{uC_{WT}}{C_{WT}}=\sqrt{\left(\frac{uC_{gWT}}{C_{gWT}}\right)^2+\left(\frac{uM_{gWT}}{M_{gWT}}\right)^2+\left(\frac{u\rho_{gWT}}{\rho_{gWT}}\right)^2+\left(\frac{u(\frac{M_{PCR}}{\rho_{PCR}}+\frac{M_{gWT}}{\rho_{gWT}})}{\frac{M_{PCR}}{\rho_{PCR}}+\frac{M_{gWT}}{\rho_{gWT}}}\right)^2} \quad \text{(S12)}$$

Where M_{PCR} is the mass of the PCR solution as weighed on the balance, ρ_{PCR} is the density of the PCR solution (Table S-2), C_{PCR} is the target mutant copy number concentration in each PCR product determined by dPCR, M_{gWT} is the mass of the fragmented gDNA solution as weighed on the balance, C_{gWT} is the WT copy number concentration of the fragmented gDNA, and ρ_{gWT} is the density of the fragmented gDNA solution.

$$C_{Ref}=-\ln\left(1-\frac{N_{MU}+N_{Combine}}{N_0+N_{MU}+N_{Combine}}\right)\times\frac{D}{V_P} \quad \text{(S13)}$$

$$C_{WT}=-\ln\left(1-\frac{N_{Combine}}{N_0+N_{MU}+N_{Combine}}\right)\times\frac{D}{V_P} \quad \text{(S14)}$$

$$C_{MU}=C_{Ref}-C_{WT} \quad \text{(S15)}$$

$$C_{MU}=-\ln\left(1-\frac{N_{MU}}{N_0+N_{MU}+N_{Combine}}\right)\times\frac{D}{V_P} \quad \text{(S16)}$$

Where C_{Ref} is the copy number concentration based on the reference probe and equal to the sum of those of MU and WT; N_0 is the number of partitions in the double negative

cluster, N_{MU} is the number of partitions in the VIC-only positive cluster, $N_{combined}$ is the number of partitions in the FAM and VIC double-positive cluster, V_P is the partition volume, and D is the dilution factor.

Supporting Results

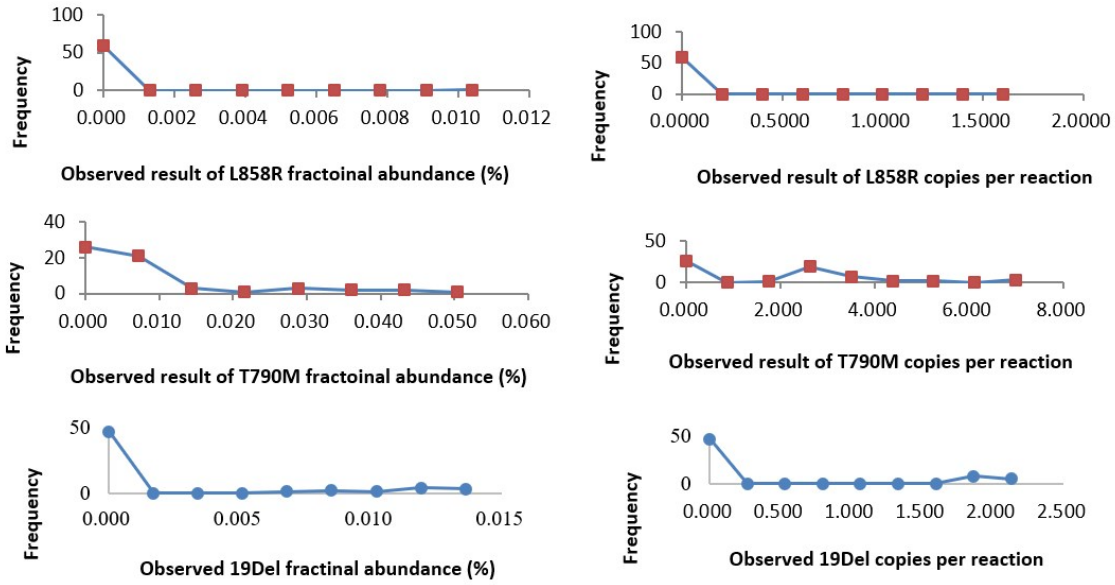


Figure S1. Limit of blank of the L858R, T790M, and 19Del. Recorded distributions of 60 blank mutant measurements for determination of limit of blank of the L858R, T790M, and 19Del mutant copy number per reaction and fractional abundance.

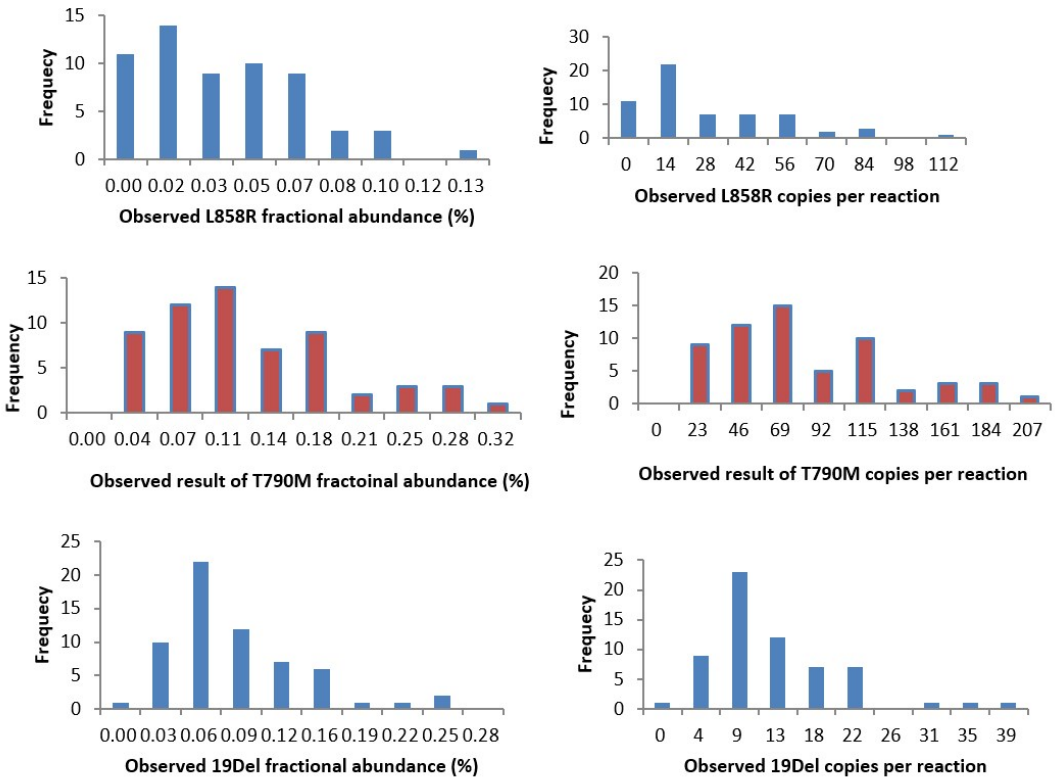


Figure S2. Limit of detection of the L858R, T790M, and 19Del. Recorded distributions of 72 measurements obtained from low concentration samples for determination of limit of detection of the L858R, T790M, and 19Del copies per reaction and fractional abundance.

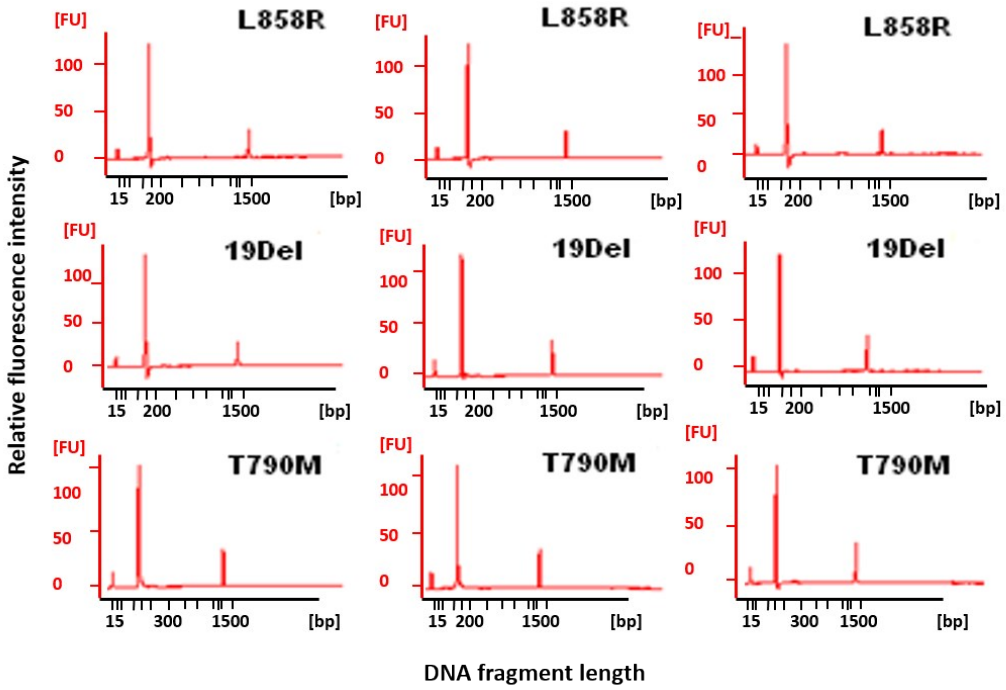
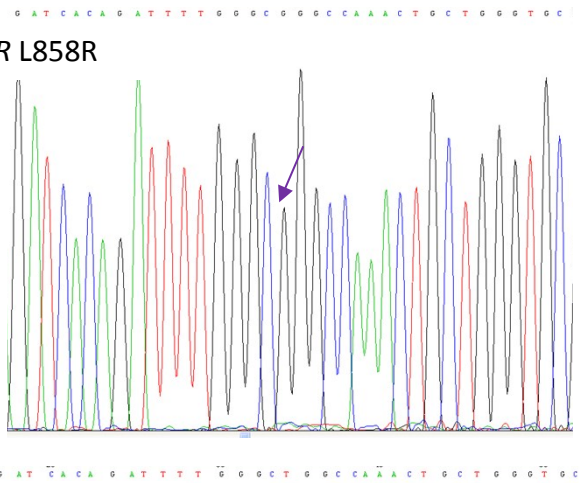
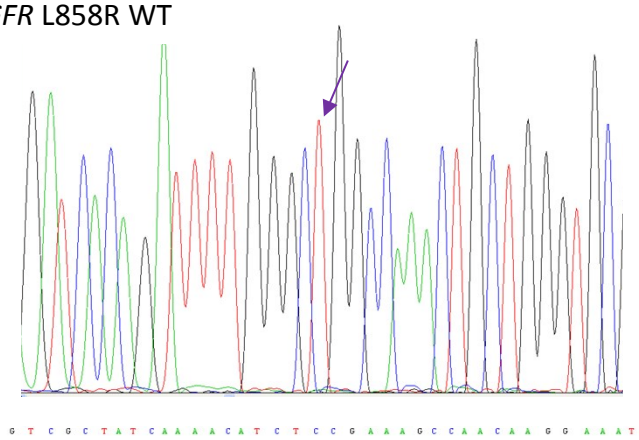


Figure S3. Size of PCR product analyzed by Agilent 2100 fragment analyzer.

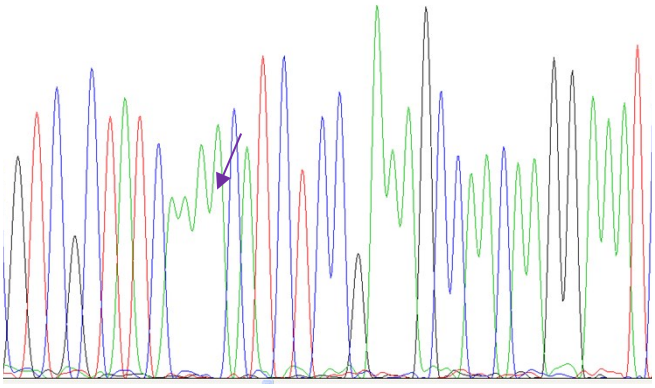
A. *EGFR* L858R



B. *EGFR* L858R WT



C. *EGFR* 19Del



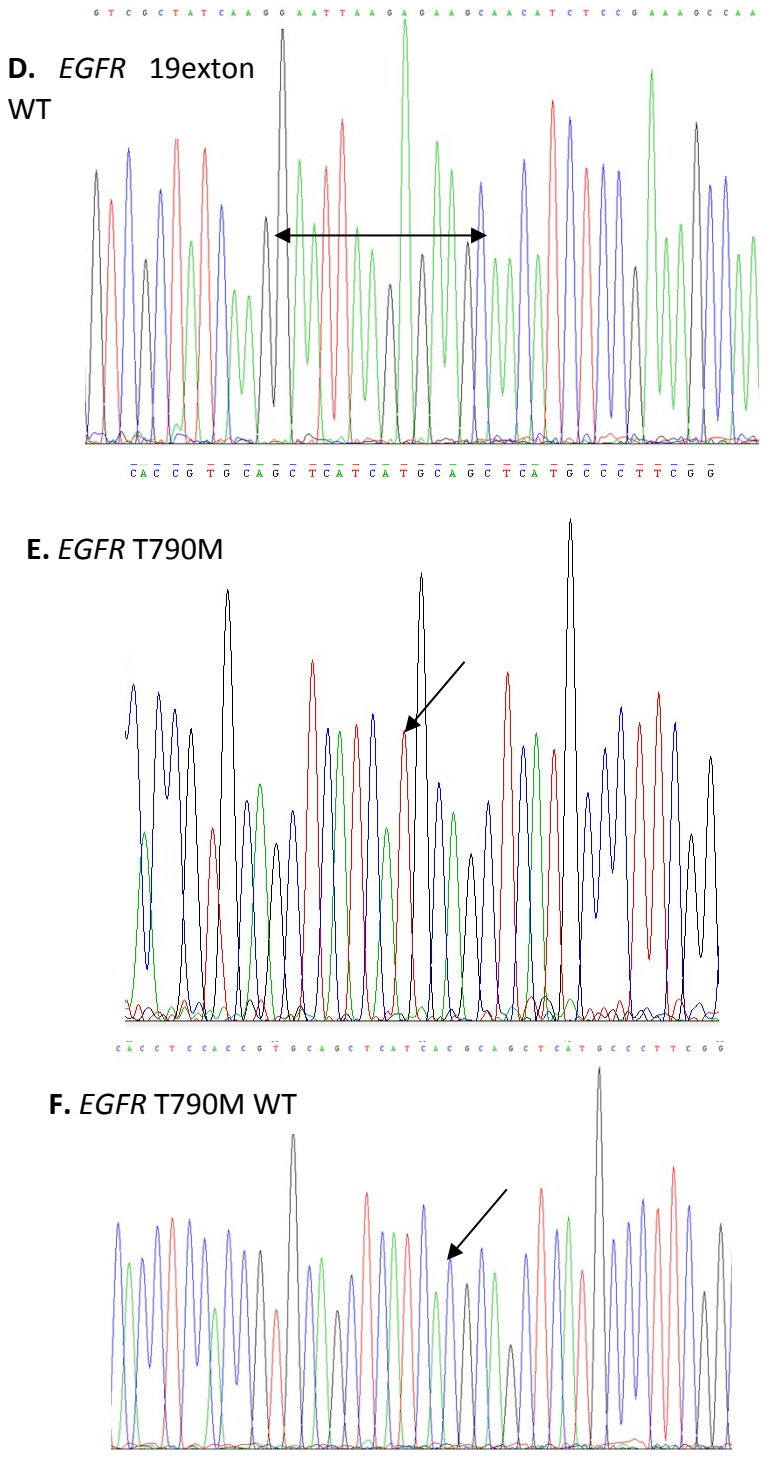


Figure S4. Target sequences identified by Sanger sequencing.

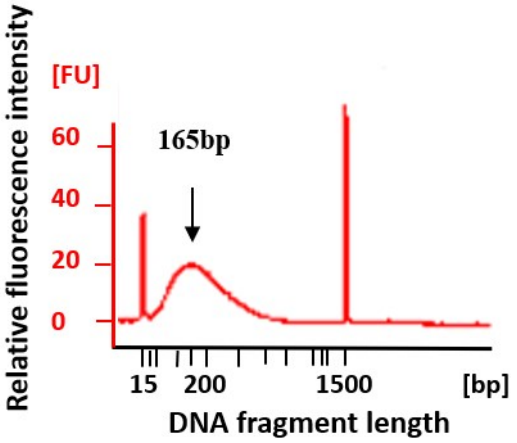


Figure S5. Result of ultrasonic treatment of the genomic DNA.

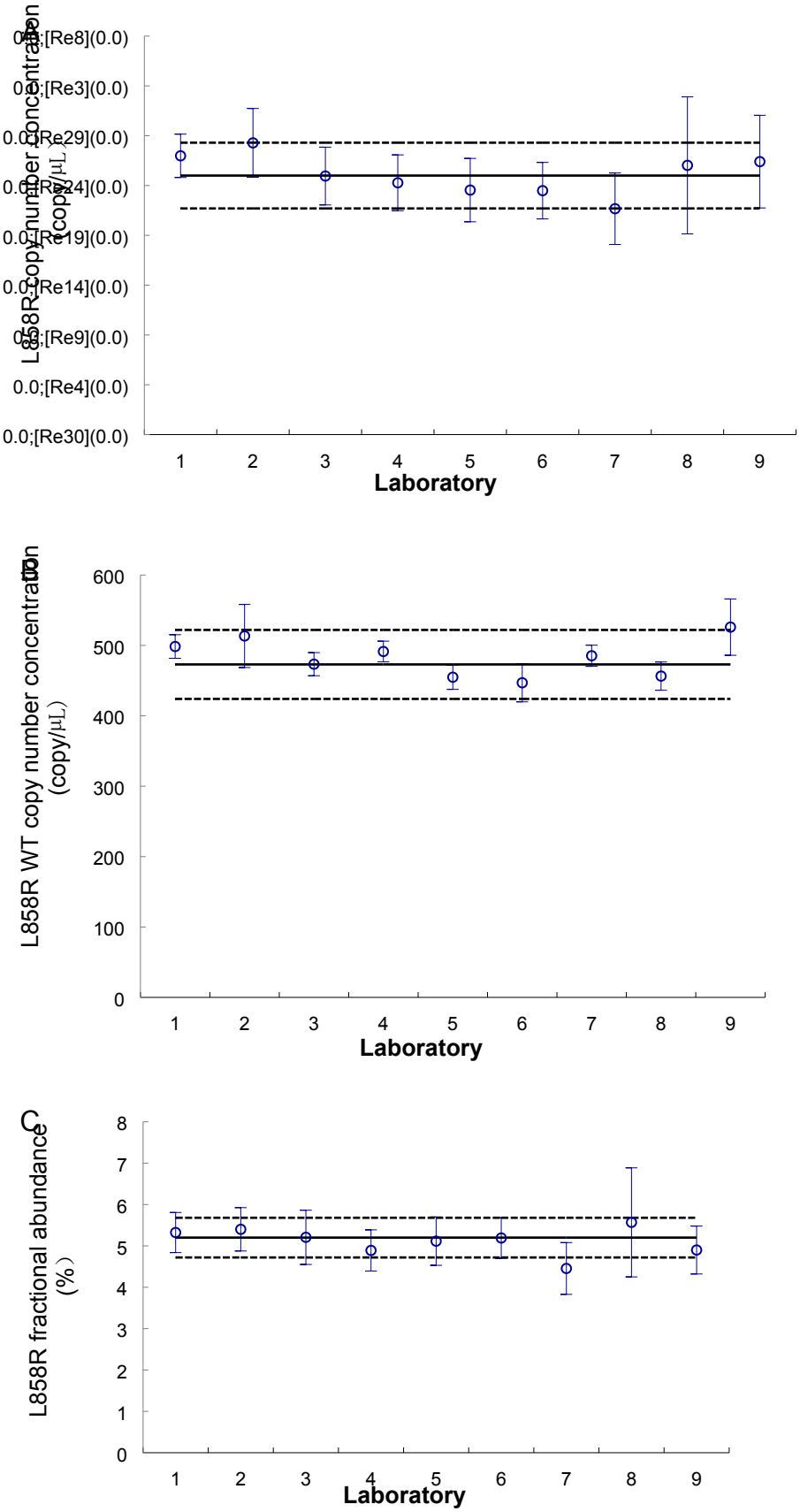


Figure S6. Interlaboratory assessment of candidate reference measurement procedure of *EGFR* L858R.

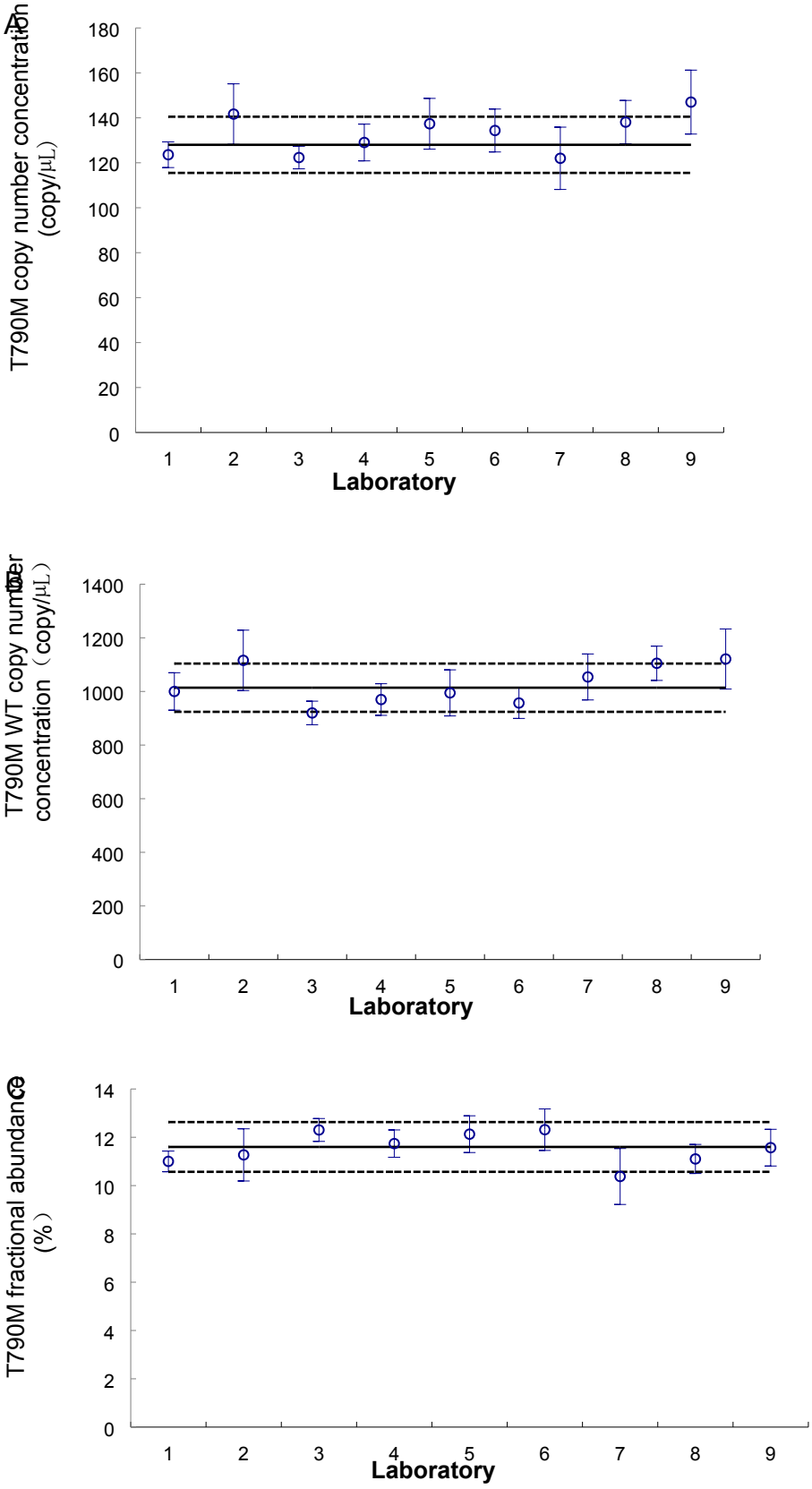


Figure S7. Interlaboratory assessment of candidate reference measurement procedure of *EGFR* T790M.

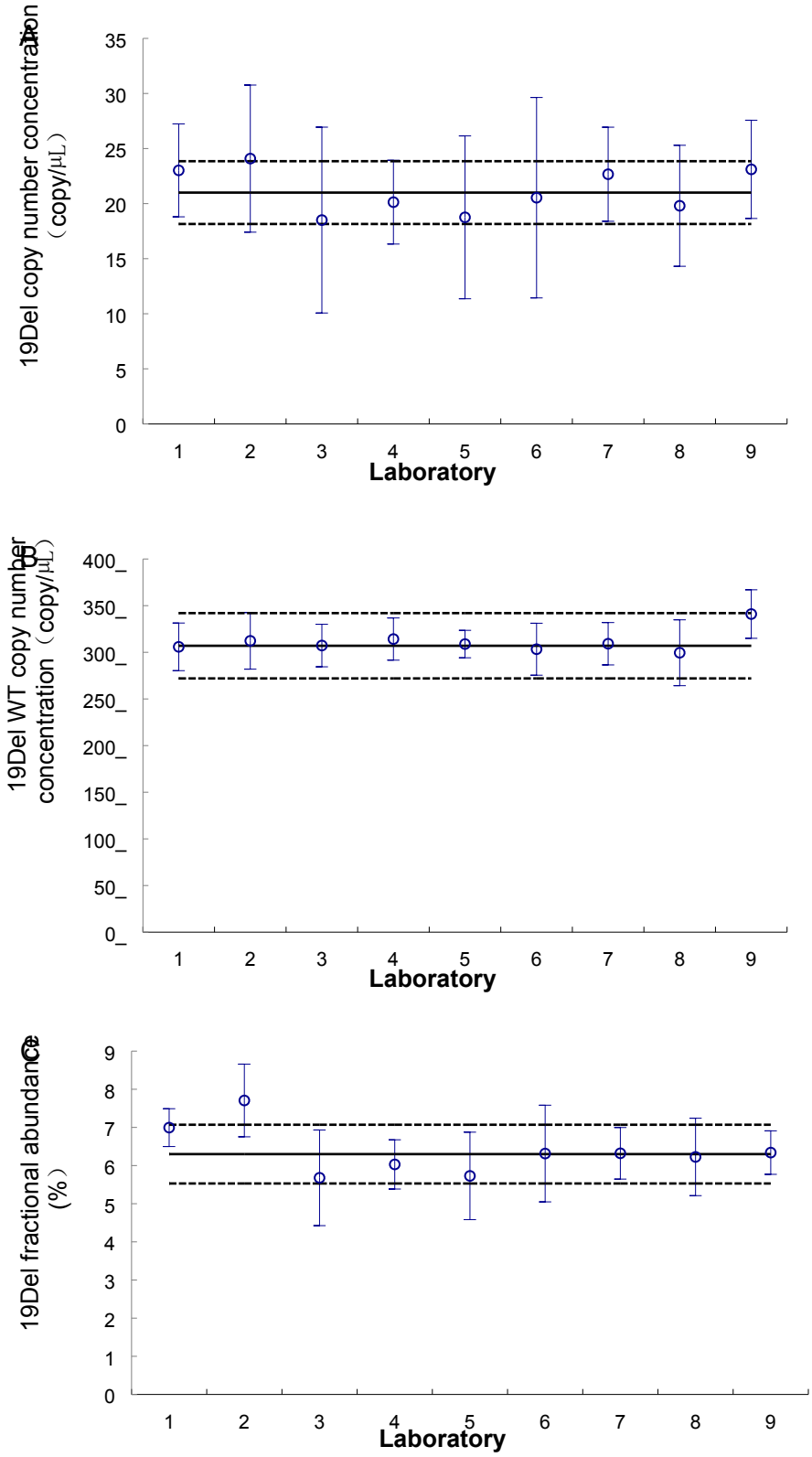


Figure S8. Interlaboratory assessment of candidate reference measurement procedure of *EGFR* 19Del.

Table S1. Primer sequences and sizes of amplicons.

Primer	Sequence 5'-3'	Size of amplicon (bp)
EGFR L858R-F	GGGCATGAACTACTTGGAGGAC	154
EGFR L858R-R	GCCTCCTTCTGCATGGTATTCTTTC	
EGFR 19del-F	TCTTCCTTCTCTCTCTGTCATAGGG	169
EGFR 19del-R	CTGAGGTTCAGAGCCATGGA	
EGFR T790M-F	AGCGTGGACAACCCCCAC	152
EGFR T790M-R2	CACCAGTTGAGCAGGTACTG	

Repeatability and intermediate precision, expressed as the percent coefficient of variation (CV), were evaluated for all assays using mixed template. The mixed template consisted of approximately 60 copies/reaction, which is more akin to the level of ctDNA, and 10^4 copies/reaction, which is close to the optimal concentration. Precision of the L858R assay was 18% and 2% (both repeatability and intermediate precision) for approximately 60 and 10^4 copies/reaction, respectively (Supplementary Table S2). The precision of the 19Del assay was 8–18 % and 2–3% for approximately 60 and 10^4 copies/reaction, respectively (Supplementary Table S3). For the T790M assay, the precision was 10–16 % and 3% for approximately 60 and 10^4 copies/reaction, respectively (Supplementary Table S4). The precision of all three WT assay were about 5–15% and 2–4% for approximately 60 and 10^4 copies/reaction, respectively.

Table S2. Repeatability and intermediate precision of L858R/WT assays.

Replicate	L858R copies/reaction					
	Experiment 1	**Experiment 2**	**Experiment 3**	**Experiment 4**	**Experiment 5**	**Experiment 6**
1	50	42	42	8040	7890	8240
2	70	68	62	8020	8000	8180
3	44	66	66	8120	8240	7940
4	68	56	50	7680	8000	8200
5	60	52	64	7880	8020	8220
Mean	58.4	56.8	56.8	7948	8030	8156
SD	11.26	10.64	10.35	172.97	128.06	122.80
CV (%)	19.28	18.73	18.23	2.18	1.59	1.51
InCV (%)		17.43			1.98	
Replicate	**WT copies/reaction**					
	Experiment 1	**Experiment 2**	**Experiment 3**	**Experiment 4**	**Experiment 5**	**Experiment 6**
1	70	76	68	9580	8890	9780
2	64	82	66	9580	9480	10180
3	70	78	86	9680	9520	9460
4	62	86	80	9220	9400	9640
5	64	86	64	9200	9360	9760
Mean	66	81.6	72.8	9452	9330	9764
SD	3.74	4.56	9.65	224.77	253.97	265.10
CV (%)	5.67	5.59	13.26	2.38	2.72	2.72
InCV (%)		12.19			3.13	

CV, coefficient of variance; SD, standard deviation; InCV, intermediate CV.

Table S3. Repeatability and intermediate precision of 19Del/WT assays.

Replicate	19Del copies/reaction					
	Experiment 1	**Experiment 2**	**Experiment 3**	**Experiment 4**	**Experiment 5**	**Experiment 6**
1	64	80	80	7420	7080	7500
2	58	60	100	7580	6920	7560
3	66	60	80	7460	7120	7560
4	54	80	60	7220	7140	7800
5	62	80	80	7520	7080	7580
Mean	60.8	72	80	7440	7068	7600
SD	4.82	10.95	14.14	137.11	86.72	115.76
CV (%)	7.92	15.21	17.68	1.84	1.23	1.52
InCV (%)		18.08			3.45	
Replicate	**WT copies/reaction**					
	Experiment 1	**Experiment 2**	**Experiment 3**	**Experiment 4**	**Experiment 5**	**Experiment 6**
1	76	62	60	8760	8440	9340
2	60	60	68	9140	8400	9460
3	68	64	74	9040	8500	9280
4	58	72	54	8820	8580	9440
5	66	84	58	8820	8780	9340
Mean	65.6	68.4	62.8	8916	8540	9372
SD	7.13	9.84	8.07	164.56	150.33	75.63
CV (%)	10.86	14.38	12.86	1.85	1.76	0.81
InCV (%)		12.42			4.18	

CV, coefficient of variance; SD, standard deviation; InCV, intermediate CV.

Table S4. Repeatability and intermediate precision of T790M/WT assays.

Replicates	T790M copies/reaction					
	Experiment1	**Experiment2**	**Experiment3**	**Experiment4**	**Experiment5**	**Experiment6**
1	60	52	58	6640	6840	7080
2	68	56	66	6580	7120	6880
3	68	56	68	6600	6560	6740
4	56	60	62	7000	6880	7080
5	45.8	72	52	6380	6960	6920
Mean	59.56	59.2	61.2	6640	6872	6940
SD	9.28	7.69	6.42	224.94	204.74	144.22
CV (%)	15.59	13.00	10.49	3.39	2.98	2.08
InCV (%)		12.26			3.28	
Replicates	**WT copies/reaction**					
	Experiment1	**Experiment2**	**Experiment3**	**Experiment4**	**Experiment5**	**Experiment6**
1	128	120	134	9560	9500	9800
2	138	120	132	9360	9780	9520
3	90	140	130	9360	9220	9640
4	118	122	138	9800	9560	9780
5	118	134	118	9400	9780	9680
Mean	118.4	127.2	130.4	9496	9568	9684
SD	17.91	9.23	7.54	188.89	232.21	113.49
CV (%)	15.13	7.26	5.78	1.99	2.43	1.17
InCV (%)		10.09			1.97	

CV, coefficient of variance; SD, standard deviation; InCV, intermediate CV.

Table S5. Result of mutant and wild–type concentration and mutant fractional abundance in serial dilution determined by gravimetrical value and dPCR for *EGFR* 19Del, L858R, and T790M.

Abundance	Mutant concentration (copy/reaction)				Wild type concentration (copy/reaction)				Mutant fractional abundance (%)			
19Del	Gravimetrical		dPCR*		Gravimetrical		dPCR		Gravimetrical		dPCR*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
25%	5179	183	4991	108	14359	340	14013	724	26.92	0.94	26.12	0.72
5%	927	15	1093	63	14350	245	14149	711	6.14	0.10	7.09	0.40
1%	249	5	223	21	14353	290	14333	173	1.73	0.04	1.52	0.17
0.20%	34	2	26	6	14349	240	14335	307	0.24	0.03	0.18	0.05
0.10%	9	4	14	5	14354	240	14605	300	0.07	0.00	0.09	0.03
0.05%	8	2	8	3	13150	185	12834	285	0.06	0.03	0.07	0.03
0.02%	3	1	4	3	13131	160	12679	273	0.016	0.004	0.05	0.02
0.01%	2	2	7	10	14307	239	14316	169	0.010	0.002	0.05	0.02
L858R	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
25%	5695	144	5825	125	21986	373	21938	377	21.26	0.52	20.98	0.38
5%	1613	14	1685	62	21917	373	21383	531	6.83	0.06	7.31	0.35
1%	167	7	223	51	21982	374	21948	332	1.07	0.03	1.16	0.13
0.20%	34	2	39	7	22000	374	21772	533	0.21	0.01	0.24	0.03
0.10%	15	1	11	5	22023	251	22150	308	0.07	0.00	0.05	0.02
0.05%	8	2	9	4	21928	373	22286	364	0.05	0.01	0.04	0.02
0.02%	3	1	5	3	22035	251	21753	385	0.015	0.003	0.02	0.01
0.01%	2	1	2	1	21978	374	22211	328	0.009	0.003	0.01	0.00
T790M	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
25%	5335	99	5379	98	17970	325	17600	455	22.89	0.42	23.42	0.42
5%	653	18	690	25	17945	325	18025	305	3.51	0.10	3.69	0.15
1%	124	6	140	16	17920	325	18180	395	0.69	0.03	0.76	0.10
0.20%	23	4	35	8	15930	185	16005	300	0.16	0.05	0.24	0.05
0.10%	10	4	20	7	15935	185	15825	275	0.07	0.02	0.13	0.04
0.05%	7	1	12	5	15950	185	16160	400	0.05	0.01	0.07	0.03
0.02%	2	1	11	6	15950	185	16235	300	0.014	0.003	0.07	0.04
0.01%	2	1	10	7	15950	185	16175	325	0.010	0.001	0.06	0.04

*Result of *EGFR* 19Del calculated using method (a) based on subtracting the wild-type copy number concentration from the reference copy number concentration when mutant fractional abundance was >0.24 % and calculated using method (b) based on the number of VIC-only positive droplet when mutant fractional abundance was ⩽ 0.24 %.

Table S6. Comparison of *EGFR* 19Del mutant copy number concentration (copy/reaction) between gravimetrical value and dPCR result calculated by using two different ways.

Sample	MU[a] (copy/reaction)	MU[b] (copy/reaction)	MU[c] (%)	MU[d](%)	*P*[1]	*P*[2]
S1	5179±183	4991±108	2607±79	4724±378	5.4E-15	0.097
S2	927±15	1093±63	588±33	1039±78	2.8E-10	0.172
S3	249±5	223±21	121±10	222±30	7.8E-08	0.923
S4	34±3	40±16	26±6	25±7	0.051	0.051
S5	9±4	14±15	14±5	13±5	0.928	0.931
S6	8±2	14±8	8±3	8±5	0.044	0.044
S7	3±1	7±3	4±3	4±2	0.017	0.017
S8	2±1	13±10	7±10	7±3	0.105	0.072

[a] *EGFR* 19Del mutant copy number concentration prepared by the gravimetric method;

[b] *EGFR* 19Del mutant copy number concentration calculated using method (a): which is based on subtracting the wild-type copy number concentration from the reference copy number concentration;

[c] *EGFR* 19Del mutant copy number concentration calculated using method (b), which is based on the total accepted partitions and single-positive partitions in VIC-only cluster with automatic threshold setting;

[d] *EGFR* 19Del mutant copy number concentration calculated using method (b), which is based on the total accepted partitions and single-positive partitions in VIC-only cluster with manual threshold setting;

[1] The *P* value of *T* test between columns b and c;

[2] The *P* value of *T* test between columns b and d;

* Data are present as mean± standard deviation, *n*=7.

Table S7. Upper 15 blank values (%) for determining the LoB.

Rank	L858R FA (%)	L858R MU (copy/reaction)	T790M FA (%)	T790M MU (copy/reaction)	19Del FA (%)	19Del MU (copy/reaction)
60	0.01039	1.6	0.0575	7.0	0.0139	2.13
59	0	0	0.0474	7.0	0.0137	1.95
58	0	0	0.0403	6.6	0.0127	1.91
57	0	0	0.0367	4.6	0.0123	1.91
56	0	0	0.0342	4.4	0.0120	1.90
55	0	0	0.0311	4.0	0.0117	1.85
54	0	0	0.0268	3.8	0.0111	1.83
53	0	0	0.0253	3.0	0.0109	1.80
52	0	0	0.0222	2.8	0.0104	1.79
51	0	0	0.0146	2.8	0.0102	1.70
50	0	0	0.0125	2.8	0.0082	1.65
49	0	0	0.0093	2.8	0.0073	1.63
48	0	0	0.0077	2.8	0.0066	1.61
47	0	0	0.0066	2.8	0	0
46	0	0	0.0575	2.8	0	0

Table S8. Copy number concentration determined by fragment analyzer/UV and dPCR.

Target	Concentration[1] (ng/μL)	Concentration[2] (copies/μL)	Concentration[3] (copies/μL)	Concentration[4] (copies/μL)
PCR-L858R	15.13±0.50[&]	(8.96±0.30) ×10^{10}	(8.86±0.31) ×10^{10}	8071±291
PCR-T790M	15.50±0.56[&]	(9.30±0.33) ×10^{10}	(9.18±0.31) ×10^{10}	11960±419
PCR-19Del	16.38±0.56[&]	(8.84±0.30) ×10^{10}	(8.75±0.34) ×10^{10}	7768±305
L858R WT	33±0.56[*]	10045±170	9945±278	5096±158
T790M WT	33±0.56[*]	10045±170	9847±266	4085±123
19extron WT	33±0.56[*]	10045±170	9915±248	3318±56

WT, Wild type

[1] mean ± standard deviation (*n*=5) of PCR product or unsonicated wild type genomic DNA measured by fragment analyzer or UV absorbance;

[2] mean ± standard deviation (*n*=5) calculated from the result determined by fragment analyzer or UV absorbance;

[3] mean ± standard deviation (*n*=5) of PCR product or unsonicated wild type genomic DNA determined by dPCR;

[4] copy number concentration ± standard uncertainty of the diluted PCR product or sonicated wild type genomic DNA used in preparation of the reference material; for the detailed uncertainty evaluation please see Supplementary Table S9;

[&] measured by fragment analyzer (Agilent 2100);

[*] measured by UV absorbance (NanoDrop 2000).

Table S9. The relative standard uncertainty of the copy number concentration of PCR product and sonicated wild-type genomic DNA (WT-gDNA).

	Diluted PCR product				Sonicated WT-gDNA			
Target	***V*p**	**Dilution factor**	**Precision**	u_c	**Dilution factor**	***V*p**	**Precision**	u_c
L858R	0.8	0.012	3.5	3.6	0.012	0.8	3.0	3.1
19Del	0.8	0.012	3.4	3.5	0.012	0.8	2.9	3.0
T790M	0.8	0.012	3.8	3.9	0.012	0.8	1.5	1.7

*V*p, droplet volume; $u_{c,}$combined uncertainty.

Table S10. Preparation of *EGFR* L858R/19Del/T790M ctDNA reference material and assignment of property value by gravimetrically mixing PCR product and wild-type genomic DNA.

Genotype	***EGFR* L858R**	***EGFR* 19Del**	***EGFR* T790M**
M_{gWT} (mg)	2001.73	2002.3	5000.53
M_{PCR} (mg)	68.22	60.19	205.31
C_{PCR} (copy/μL)	8071	7768	11960
C_{gWT} (copy/μL)	5096	3318	4085
Dilution factor*	10.00	9.96	4.00
C_{MU} (copy/μL)	27	23	119
C_{WT} (copy/μL)	492	323	981
FA (%)	5.23	6.63	10.82

* Diluted by RNA solution

Table S11. Standard uncertainty evaluation for each parameter related to gravimetrical method.

Parameter	L858R	19del	T790M
$C_{PCR} \times \frac{M_{PCR}}{\rho_{PCR}}$	545770	457642	2403440
$C_{gWT} \times \frac{M_{gWT}}{\rho_{gWT}}$	9896019	6445122	19816807
$\frac{u\rho_{PCR}}{\rho_{PCR}}$	0.0146	0.0229	0.0230
$\frac{u\rho_{gWT}}{\rho_{gWT}}$	0.0063	0.0063	0.0063
$\frac{uC_{PCR}}{C_{PCR}}$	0.0360	0.0350	0.0393
$\frac{uC_{gWT}}{C_{gWT}}$	0.0310	0.0300	0.0170
$\frac{uM_{PCR}}{M_{PCR}}$	0.0028	0.0032	0.0042
$\frac{uM_{gWT}}{M_{gWT}}$	0.0019	0.0021	0.0028
$\frac{u\left(C_{PCR} \times \frac{M_{PCR}}{\rho_{PCR}}\right)}{C_{PCR} \times \frac{M_{PCR}}{\rho_{PCR}}}$	0.0389	0.0420	0.0458
$\frac{u\left(C_{gWT} \times \frac{M_{gWT}}{\rho_{gWT}}\right)}{C_{gWT} \times \frac{M_{gWT}}{\rho_{gWT}}}$	0.0317	0.0307	0.0183
$u\left(C_{PCR} \times \frac{M_{PCR}}{\rho_{PCR}}\right)$	21257	19200	109976

$u\left(C_{gWT} \times \frac{M_{gWT}}{\rho_{gWT}}\right)$	313612	198164	363548
$u\left(C_{PCR} \times \frac{M_{PCR}}{\rho_{PCR}} + C_{gWT} \times \frac{M_{gWT}}{\rho_{gWT}}\right)$	314331	199092	379819
$\frac{u(C_{PCR} \times \frac{M_{PCR}}{\rho_{PCR}} + C_{WT} \times \frac{M_{gWT}}{\rho_{gWT}})}{C_{PCR} \times \frac{M_{PCR}}{\rho_{PCR}} + C_{WT} \times \frac{M_{gWT}}{\rho_{gWT}}}$	0.0301	0.0288	0.0171
$\frac{uFA}{FA}$ (%)	**4.92**	**5.09**	**4.89**
$\frac{u(\frac{M_{PCR}}{\rho_{PCR}})}{\frac{M_{PCR}}{\rho_{PCR}}}$	0.0149	0.0232	0.0234
$\frac{u(\frac{M_{gWT}}{\rho_{gWT}})}{\frac{M_{gWT}}{\rho_{gWT}}}$	0.0066	0.0066	0.0069
$u\left(\frac{M_{PCR}}{\rho_{PCR}}\right)$	1.0053	1.3642	4.6984
$u\left(\frac{M_{gWT}}{\rho_{gWT}}\right)$	12.7784	12.8995	33.4446
$u\left(\frac{M_{PCR}}{\rho_{PCR}} + \frac{M_{gWT}}{\rho_{gWT}}\right)$	12.8178	12.9715	33.7730
$\frac{u(\frac{M_{PCR}}{\rho_{PCR}} + \frac{M_{gWT}}{\rho_{gWT}})}{\frac{M_{PCR}}{\rho_{PCR}} + \frac{M_{gWT}}{\rho_{gWT}}}$	0.0064	0.0065	0.0067
$\frac{uC_{MU}}{C_{MU}}$ (%)	**3.95**	**4.25**	**4.62**
$\frac{uC_{WT}}{C_{WT}}$ (%)	**3.23**	**3.14**	**1.95**

Table S12. Uncertainty evaluation of dPCR method.

Property	Parameter	L858R	19Del	T790M
WT	X_{dPCR}	473	307	1014
	$u_{a,rel}$	2.1	1.0	2.5
	$u_{df,rel}$	0.012	0.012	0.012
	$u_{vp,rel}$	0.8	0.8	0.8
	$u_{dPCR,rel}$	2.2	1.3	2.7
	u^* (cp/μL)	10	4	27
MU	X_{dPCR}	26	21	128
	$u_{a,rel}$	2.5	3.6	2.7
	$u_{df,rel}$	0.012	0.012	0.012
	$u_{vp,rel}$	0.8	0.8	0.8
	$u_{dPCR,rel}$	2.7	3.7	2.8
	u^* (cp/μL)	0.7	0.8	3.6
FA	X_{dPCR}	5.2	6.3	11.6
	$u_{a,rel}$	1.6	4.0	2.9
	$u_{df,rel}$	-	-	-
	$u_{vp,rel}$	0.8	0.8	0.8
	$u_{dPCR,rel}$	1.8	4.1	2.0
	u^* (cp/μL)	0.09	0.26	0.24

X_{dPCR} is the value measured by dPCR, $u_{a,rel}$ is the relative uncertainty of precision of dPCR, $u_{df,rel}$ is the relative uncertainty of dilution factor, $u_{vp,rel}$ is the relative uncertainty of droplet volume measurement, $u_{dPCR,rel}$ is the combined relative standard uncertainty, and u^* combined absolute standard uncertainty.

Table S13. *En* value and absolute expanded uncertainty of gravimetrical method and dPCR (*k*=2).

Genotype Mutant abundance	*EGFR* L858R	*EGFR* 19Del	*EGFR* T790M
U_{FA}[1] (%)	0.51	0.68	1.06
U_{CMU} [1] (copy/μL)	2	2	11
U_{CWT}[1] (copy/μL)	32	20	38
U_{FA} [2](%)	0.19	0.51	0.47
U_{CMU}[2] (copy/μL)	1.4	1.5	7.3
U_{CWT}[2] (copy/μL)	21	8	54
En_{FA}[3]	0.02	0.37	0.70
En_{CMU}[3]	0.41	0.83	0.69
En_{CWT}[3]	0.51	0.75	0.51

[1] Absolute expanded uncertainty of measurement result determined by gravimetrical method using the equation $U_{gravi} = X_{gravi} \times U_{gravi,rel}$;

[2] Absolute expanded uncertainty of measurement result determined by dPCR using the equation $U_{dPCR} = X_{dPCR} \times U_{dPCR,rel}$;

[3] Calculated according to the equation $En = \frac{(X_{dPCR} - X_{gravi})}{\sqrt{U_{gravi}^2 + U_{dPCR}^2}}$, where X_{gravi} is the value determined by gravimetrical method, X_{dPCR} is the value measured by dPCR, U_{gravi} is the absolute expanded uncertainty of gravimetrical method, U_{dPCR} is the absolute expanded uncertainty of dPCR.

Table S14. Homogeneity assessment of L858R RM.

Vial	MU (copy/µL)			WT (copy/µL)			FA (%)		
1	23	20	28	475	491	495	4.57	3.97	5.28
2	31	26	24	458	471	454	6.30	5.23	5.08
3	30	30	29	450	515	495	6.25	5.58	5.57
4	25	23	20	499	495	471	4.73	4.65	5.01
5	23	25	27	515	491	499	4.22	4.80	5.17
6	30	27	26	523	527	484	5.49	4.83	4.56
7	19	20	29	487	503	507	3.69	3.88	5.37
8	24	32	30	475	495	503	4.80	6.08	5.63
9	26	25	22	495	503	511	4.01	4.69	5.04
10	32	26	29	487	515	475	6.18	4.87	5.72
11	32	26	32	488	544	544	5.23	4.56	5.63
Q_1		237			8004			7.3	
V_1		10			10			10	
S_1^2		24			800			0.73	
Q_2		235			8186			7.6	
V_2		22			22			22	
S_2^2		11			372			0.3	
F		2.2			2.1			2.1	
$F_{0.05(10,22)}$		2.3			2.3			2.3	
$u_{bb\,rel}$		3.9%			1.2%			3.7%	

Table S15. Homogeneity assessment of 19Del RM.

Vial	MU (copy/μL)			WT (copy/μL)			FA (%)		
1	24	28	20	361	365	369	6.82	7.37	5.81
2	24	24	28	353	361	373	6.82	7.37	5.81
3	28	28	28	299	341	346	7.78	7.00	7.22
4	28	24	22	333	446	345	7.61	6.25	6.14
5	20	20	28	361	365	329	5.62	5.81	7.87
6	20	28	28	325	345	341	5.68	7.45	7.45
7	23	27	28	337	353	365	6.44	7.86	7.45
8	28	24	22	357	349	337	7.69	7.06	6.11
9	28	26	28	357	349	337	7.61	7.47	7.69
10	26	24	28	357	369	385	7.59	6.32	7.87
11	24	22	24	344	361	345	6.38	6.89	6.38
Q_1		77			2049			4.2	
V_1		10			10			10	
S_1^2		7.7			205			0.42	
Q_2		193			5144			13.3	
V_2		22			22			22	
S_2^2		8.7			234			0.60	
F		0.88			0.88			0.70	
$F_{0.05(10,22)}$		2.3			2.3			2.3	
$u_{bb\,rel}$		3.7%			1.4%			3.5%	

Table S16. Homogeneity assessment of T790M RM.

Vial	MU (copy/µL)			WT (copy/µL)			FA (%)		
1	130	125	137	1035	954	908	11.13	11.59	13.11
2	103	94	152	1070	1076	1101	8.74	8.07	12.14
3	136	132	152	949	974	989	12.54	11.97	13.33
4	116	148	135	969	944	994	10.71	13.56	11.95
5	135	125	121	994	979	1055	11.95	11.35	10.27
6	135	145	129	1005	974	979	11.85	12.96	11.64
7	137	152	127	974	999	999	12.33	13.20	11.27
8	139	142	129	964	1015	964	12.60	12.28	11.80
9	171	137	145	1005	989	1121	14.58	12.16	11.46
10	165	150	131	1080	989	1012	13.28	13.18	11.46
11	178	165	138	1101	998	996	13.93	14.21	12.17
Q_1		4379			40405			29	
V_1		10			10			10	
S_1^2		438			4040			2.9	
Q_2		5452			38890			31	
V_2		22			22			22	
S_2^2		248			1767			1.4	
F		1.76			2.28			2.03	
$F_{0.05(10,22)}$		2.3			2.3			2.3	
u_{bbrel}		3.6%			1.3%			3.1%	

Table S17. Stability assessment of the reference materials (RMs).

Property	Parameter	L858R RM	19Del RM	T790M RM
MU	$s(b1)$	0.07	0.06	0.24
	t (month)	17	17	12
	u_s (cp/μL)	1.1	1.1	2.8
	$u_{s,rel}$ (%)	**4.2**	**4.3**	**1.7**
WT	$s(b1)$	1.32	1.09	2.76
	t (month)	17	17	12
	u_s (cp/μL)	22	19	33
	$u_{s,rel}$ (%)	**4.6**	**5.4**	**3.3**
FA	$s(b1)$	0.01	0.01	0.03
	t (month)	17	17	12
	u_s (cp/μL)	0.11	0.18	0.36
	$u_{s,rel}$ (%)	**2.1**	**2.8**	**2.5**

Table S18. Factors contributing to the relative standard uncertainty for ctDNA reference material.

Property	uncertainty	*EGFR* L858R	*EGFR* 19Del	*EGFR* T790M
MU	u_{char}[a] (%)	2.7	3.7	2.8
	u_{bb}[b] (%)	3.9	3.7	3.6
	u_S[c] (%)	4.2	4.3	1.7
	u_c[d] (%)	6.3	6.8	4.9
	U (k=2)(%)	**13**	**14**	**9.7**
WT	u_{char} (%)	2.2	1.3	2.7
	u_{bb} (%)	1.2	1.4	1.3
	u_S (%)	4.6	5.4	**3.3**
	u_c (%)	5.2	5.7	4.5
	U (k=2) (%)	**11**	**12**	**8.9**
MU%	u_{char} (%)	1.8	4.1	2.0
	u_{bb} (%)	3.7	3.5	3.1
	u_S (%)	2.1	2.8	2.5
	u_c (%)	4.6	6.1	4.5
	U (k=2) (%)	**9.2**	**12**	**8.9**

[a]method characterization; [b]homogeneity; [c]stability; [d]relative combined standard uncertainty; [e]relative expanded uncertainty.

Table S19. Interlaboratory result of MU, WT, and FA for each RM.

	Interlaboratory result of L858R RM								
Lab	**MU**	**SD**	**Ratio***	**WT**	**SD**	**Ratio**	**FA**	**SD**	**Ratio**
1	28.0	2.2	1.08	498.5	16.7	1.05	5.32	0.48	1.02
2	29.3	3.5	1.13	513.4	44.8	1.09	5.40	0.52	1.04
3	26.0	2.9	1.00	473.4	16.4	1.00	5.21	0.66	1.00
4	25.3	2.8	0.97	491.3	14.7	1.04	4.89	0.50	0.94
5	24.5	3.2	0.94	454.8	17.3	0.96	5.11	0.58	0.98
6	24.5	2.8	0.94	446.9	27.1	0.94	5.19	0.49	1.00
7	22.7	3.6	0.87	485.3	15.0	1.03	4.45	0.63	0.86
8	27.0	6.9	1.04	456.4	20.1	0.96	5.57	1.32	1.07
9	27.4	4.7	1.05	526.0	40.0	1.11	4.90	0.58	0.94
	Interlaboratory result of T790M RM								
Lab	**MU**	**SD**	**Ratio**	**WT**	**SD**	**Ratio**	**FA**	**SD**	**Ratio**
1	123.6	5.7	0.97	999.9	34.9	0.99	11.00	0.43	0.95
2	141.7	13.5	1.11	1116.0	37.6	1.10	11.27	1.08	0.97
3	122.3	5.0	0.96	919.8	22.0	0.91	12.30	0.47	1.06
4	129.0	8.2	1.01	969.8	29.5	0.96	11.74	0.57	1.01
5	137.4	11.3	1.07	994.7	43.0	0.98	12.13	0.76	1.05
6	134.4	9.5	1.05	956.9	28.8	0.94	12.31	0.86	1.06
7	122.0	13.9	0.95	1054.0	42.9	1.04	10.38	1.16	0.89
8	138.1	9.7	1.08	1105.1	32.0	1.09	11.10	0.60	0.96
9	147.0	14.2	1.15	1121.0	56.0	1.11	11.57	0.76	1.00
	Interlaboratory result of 19Del RM								
Lab	**MU**	**SD**	**Ratio**	**WT**	**SD**	**Ratio**	**FA**	**SD**	**Ratio**
1	23.0	2.1	1.10	305.8	12.8	1.00	6.99	0.50	1.11
2	24.1	3.3	1.15	312.3	15.2	1.02	7.71	0.95	1.22
3	18.5	4.2	0.88	307.3	11.4	1.00	5.68	1.25	0.90
4	20.1	1.9	0.96	314.2	11.3	1.02	6.03	0.65	0.96
5	18.8	3.7	0.89	308.8	7.4	1.01	5.73	1.15	0.91
6	20.5	4.5	0.98	303.3	13.9	0.99	6.32	1.27	1.00
7	22.7	2.1	1.08	309.2	11.3	1.01	6.32	0.68	1.00
8	19.8	2.7	0.94	299.5	17.6	0.98	6.23	1.01	0.99
9	23.1	2.2	1.10	341.0	13.0	1.11	6.34	0.57	1.01

*The difference between interlaboratory result and property value.

References

[1] L. Dong, Y. Meng, J. Wang, Y. Liu, Analytical and Bioanalytical Chemistry, Online 10.1007/s00216-013-7546-1 (2014) 1701-1712.