Supplementary Material

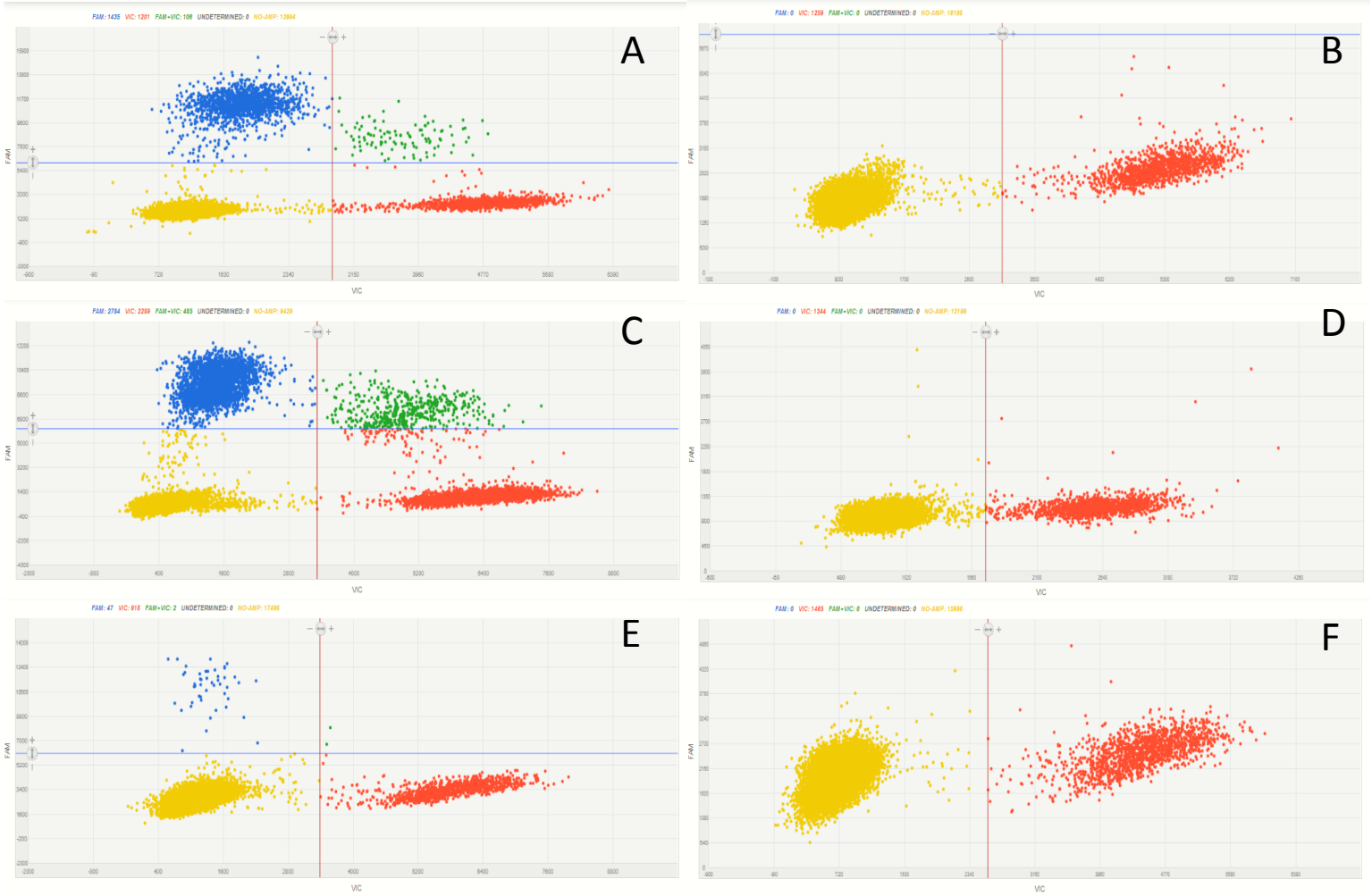

Figure S1: Examples of positive and negative samples for each analyzed mutations. Image A, C and E correspond to positive model cases from mutation H1047R, E545K and H1047L, respectively. Images B, D and F correspond to negative cases for each mutation.

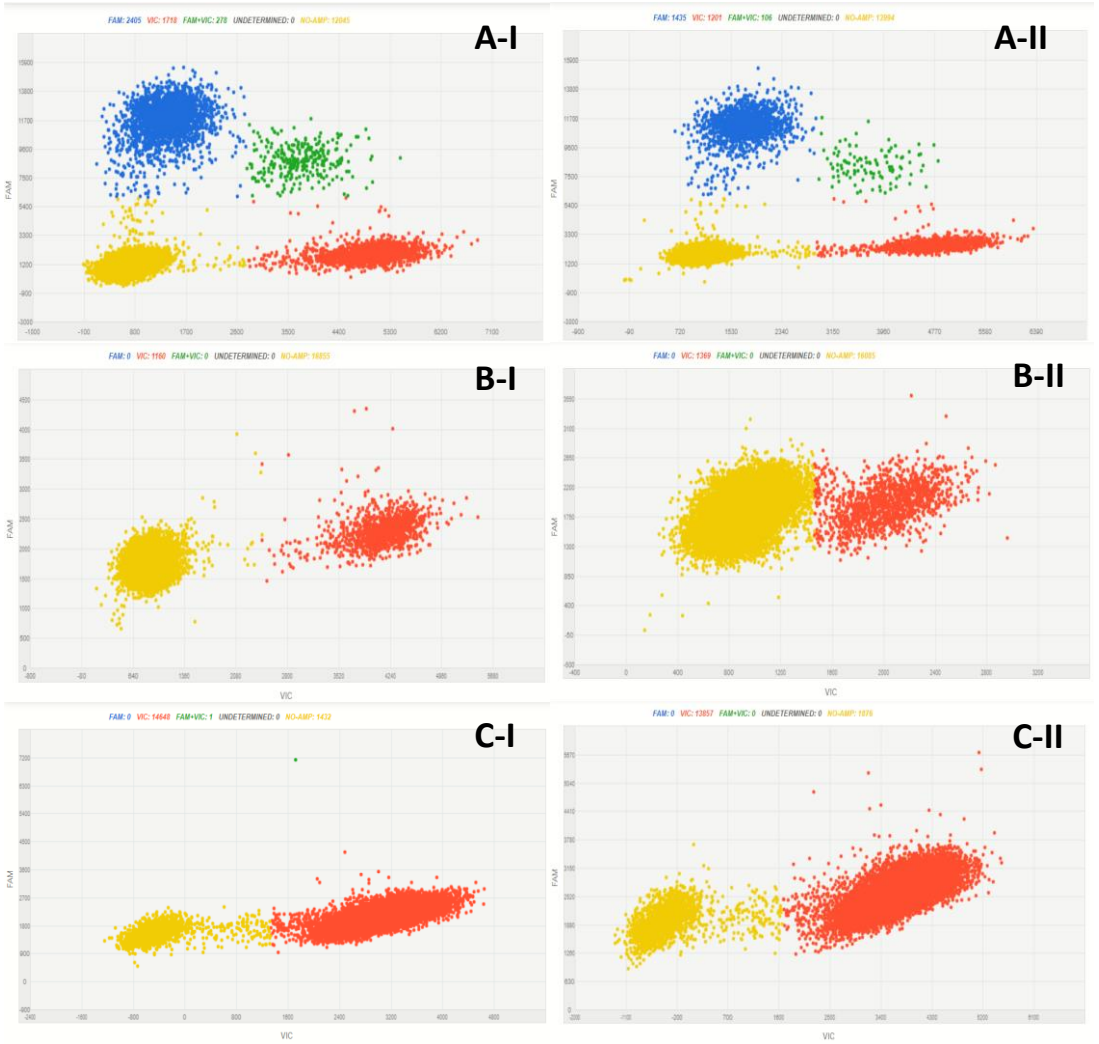

Figure S2: Three examples from the dilution assays´ samples. Figure A I and II correspond to sample I-02 H1047R, B I and II to I-33 H1047R, C I and II to I-68 H1047L.

Table S1. Catalog number of QuantStudio ™ 3D Digital PCR Products

Product	Catalog Identification Code
Essay E545K	Hs000000086_rm
Essay H1047R	Hs000000088_rm
Essay H1407L	Hs000000089_rm
Master Mix v2 (5ml)	A26359
QuantStudio ™ 3D Digital PCR 20K Chip Kit v2	A26316

Table S2. Primers used for conventional PCR

Gene	Exon	Size (bp)	Forward Primer 5′-3′	Reverse Primer 5′-3′
PIK3CA	9	bp	CTGTGAATCCAGAGGGGAAA	CTCCATTTTAGCACTTACCTGTGACT
	20	245 bp	GATGACATTGCATACATTCG	CCTATGCAATCGGTCTTTGC

bp base pairs

Table S3: Cycling conditions PIK3CA primers exon 20

Phase	Temperature	Time	Cycles
Initial Denaturation	95°C	5 minutes	1
Denaturation	95°C	15 seconds	45
Annealing	55°C	30 seconds	
Extension	72°C	20 seconds	
Final Extension	72°C	7 minutes	1
Final stage	10°C	5 minutes	1

Table S4: Cycling conditions PIK3CA primers exon 09

Phase	Temperature	Time	Cycles
Initial Denaturation	95°C	1 minute	1
Denaturation	95°C	15 seconds	40
Annealing	53°C	15 seconds	
Extension	72°C	30 seconds	
Final Extension	72°C	7 minutes	1
Final stage	4°C	5 minutes	1

Table S5: Mean λ, partition volume, total partition number and total volume of part

Template	Mutation Essay	Mean copies per partition (λ) (Median)	Partition volume (nL)	Total partition number (median)	Total volume of partitions measure μL (median)
Tumor DNA	H1047R	0.039	0.755	17084	12.90
	E545K	0.050		17065	12.88
	H1047L	0.045		17080	12.90

Table S6: *PIK3CA* H1047R mutations dilution assay by dPCR.

Sample	Theorical Target/total (Y)	Target/Total (X)	CI Target/Total	Copies/microliter (VIC)	CI Copies/microliter (VIC)	Copies/microliter (FAM)	CI Copies/microliter (FAM)	Chips
d20R-1%	1.00%	0.82%	0.602% -- 1.123%	3692.6	3581.1 -- 3807.6	30.665	22.578 -- 41.649	1
d20R-0.10%	0.10%	0.20%	0.107% -- 0.367%	3831.8	3719.3 -- 3947.7	7.62	4.1 -- 14.161	1
d20R-0.01%	0.01%	0.12%	5.23E-2% -- 0.253%	4438.7	4306.5 -- 4574.9	5.138	2.308 -- 11.437	1

Linear Regression Output	
Observations	3
R square	1.00
Adjusted R square	0.999
Prob > F	0.014

LOD	0.04
lOQ	0.11

	coefficients	standard error	t	p	95% CI	
Variable (x)	1.428	0.032	44.841	0.014	1.024	1.833
Y intercept	-0.173	0.016	-11.022	0.058	-0.372	0.026

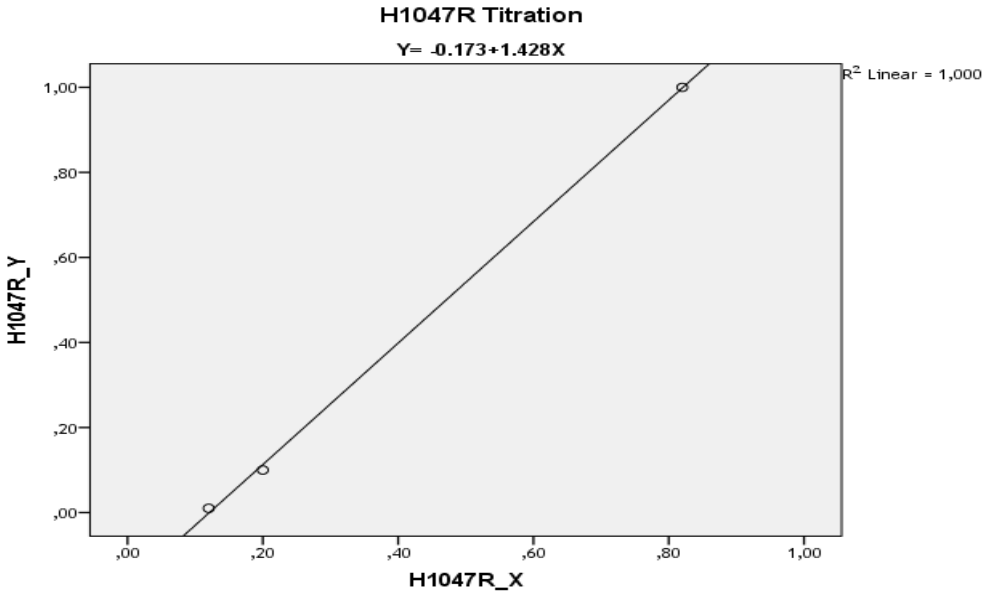

Table S7: *PIK3CA* H1047L mutations dilution assay by dPCR.

Sample	Theorical Target/total (Y)	Target/Total (X)	CI Target/Total	Copies/microliter (VIC)	CI Copies/microliter (VIC)	Copies/microliter (FAM)	CI Copies/microliter (FAM)	Chips
Dl-1	1%	0.88%	0.568% -- 1.371%	1754.3	1680.7 -- 1831.2	15.641	10.09 -- 24.243	1
dl-0.1	0.10%	0.14%	4.77E-2% -- 0.419%	1727	1651.5 -- 1805.9	2.455	0.792 -- 7.612	1
dl-0.01	0.01%	0%	NA	1631.3	1561.9 -- 1703.9	0	NA	1

Linear Regression Output	
Observations	3
R square	0.996
Adjusted R square	0.991
P> F	0.042

LOD	0.11
lOQ	0.34

	coefficients	standard error	t	p	95% CI	
Variable (x)	1.155	0.077	15.064	0.042	0.181	2.130000
Y intercept	-0.023	0.039	-0.577	0.667	-0.524	0.4790000

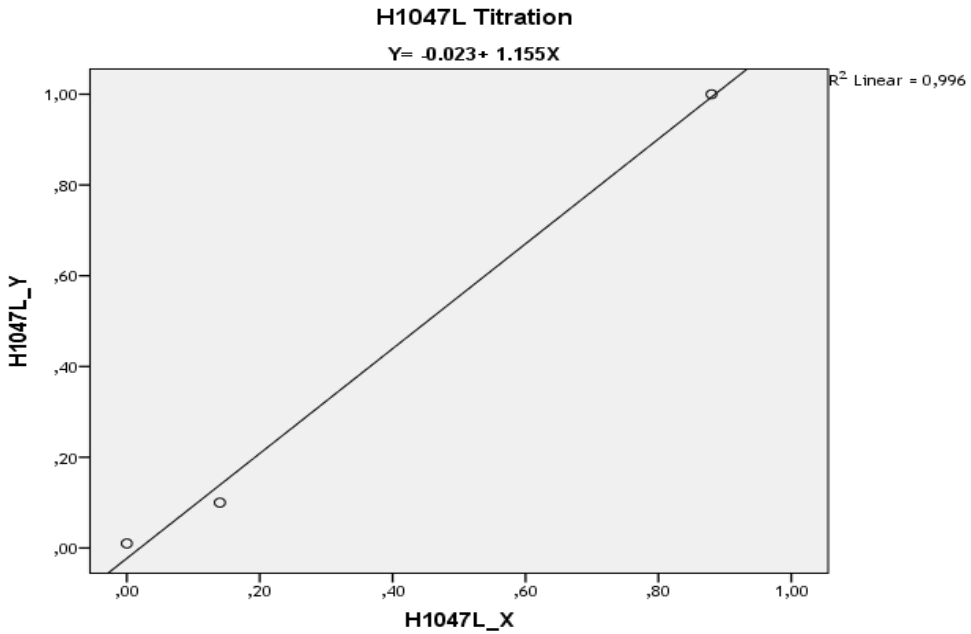

Table S8: *PIK3CA* E545K mutations dilution assay by dPCR.

Sample	Theorical Target/total (Y)	Target/Total (X)	CI Target/Total	Copies/microliter (VIC)	CI Copies/microliter (VIC)	Copies/microliter (FAM)	CI Copies/microliter (FAM)	Chips
d9-1	1.00%	0.92%	0.758% -- 1.118%	9479.5	9268 -- 9695.9	88.261	73.395 -- 106.14	1
d9-0.1	0.10%	0.10%	5.42E-2% -- 0.177%	8792.3	8599.3 -- 8989.5	8.664	4.798 -- 15.646	1
d9-0.01	0.01%	0.03%	1.23E-2% -- 8.37E-2%	9634.7	9425.3 -- 9848.7	3.128	1.174 -- 8.335	1

Linear Regression Output	
Observations	3
R square	1.00
Adjusted R square	1.00
Prob > F	0.00891

LOD	0.02
lOQ	0.06

	coefficients	standard error	t	p	95% CI	
Variable (x)	1.106	0.013	86.891	0.007	0.944	1.268
Y intercept	-0.017	0.007	-2.521	0.240	-0.104	0.069

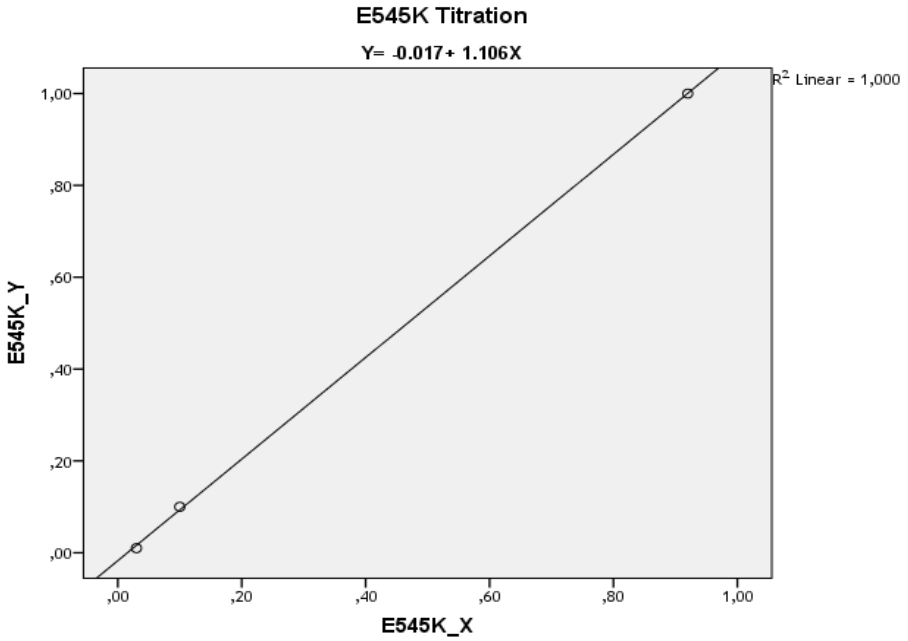

Table S9: *PIK3CA* mutations dilution assay by dPCR.

Sample	Theorical Target/total (Y)	Target/Total (X)	CI Target/Total	Copies/microliter (VIC)	CI Copies/microliter (VIC)	Copies/microliter (FAM)	CI Copies/microliter (FAM)
d20R-1%	1.00%	0.82%	0.602% -- 1.123%	3692.6	3581.1 -- 3807.6	30.665	22.578 -- 41.649
d20R-0.10%	0.10%	0.20%	0.107% -- 0.367%	3831.8	3719.3 -- 3947.7	7.62	4.1 -- 14.161
d20R-0.01%	0.01%	0.12%	5.23E-2% -- 0.253%	4438.7	4306.5 -- 4574.9	5.138	2.308 -- 11.437
d9-1	1.00%	0.92%	0.758% -- 1.118%	9479.5	9268 -- 9695.9	88.261	73.395 -- 106.14
d9-0.1	0.10%	0.14%	5.42E-2% -- 0.177%	8792.3	8599.3 -- 8989.5	8.664	4.798 -- 15.646
d9-0.01	0.01%	0.03%	1.23E-2% -- 8.37E-2%	9634.7	9425.3 -- 9848.7	3.128	1.174 -- 8.335
Dl-1	1.00%	0.88%	0.568% -- 1.371%	1754.3	1680.7 -- 1831.2	15.641	10.09 -- 24.243
dl-0.1	0.10%	0.14%	4.77E-2% -- 0.419%	1727	1651.5 -- 1805.9	2.455	0.792 -- 7.612
dl-0.01	0.01%	0.00%	NA	1631.3	1561.9 -- 1703.9	0	NA

Linear Regression Output	
R square	0.986
Adjusted R square	0.984
P> F	0
observations	9

LOD	0.08
lOQ	0.23

	coefficients	standard error	t	p	95% CI	
Variable (x)	1.208	0.055	22.014	0.000	1.079	1.338
Y intercept	-0.066	0.028	-2.346	0.051	-0.133	0.001

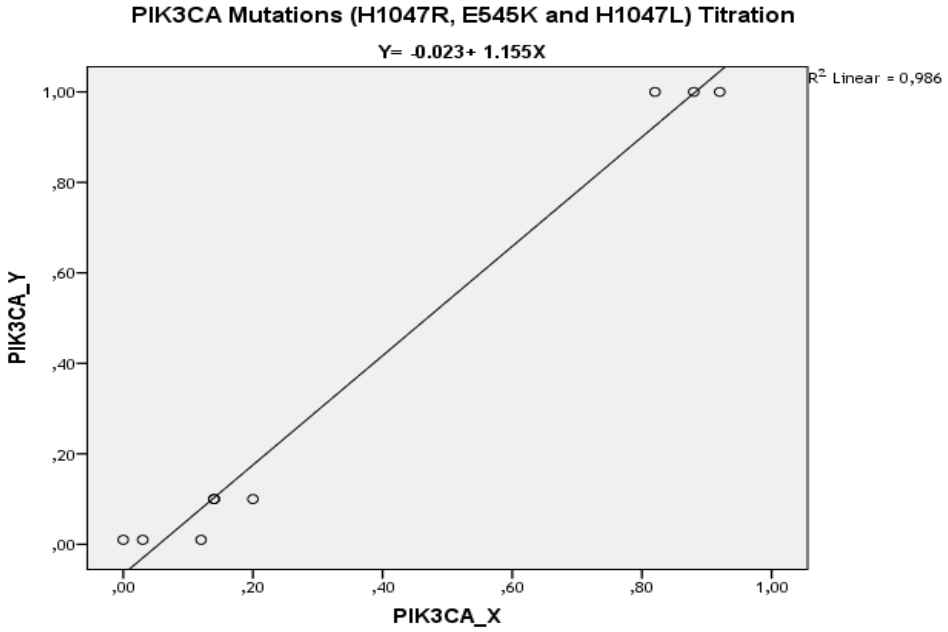

Table S10: Inter-rater assay results from mutant allele frequency.

Patient sample	First Assay	Second Assay
I-02 (H1047R)	57.940%	54.300%
I-02 (H1047L)	0.065%	0.061%
I-24 (E545K)	54.590%	51.290%
I-28 (H1047L)	0.300%	0.000%
I-33 (H1047R)	0.000%	0.000%
I-35 (H1047L)	0.853%	0.807%
I-65 (H1047L)	0.000%	0.034%
I-68 (H1047R)	0.020%	0.000%
I-68 (H1047L)	0.025%	0.000%
I-70 (H1047L)	5.131%	3.402%

Table S11: Digital MIQE checklist for authors, reviewers and editors.

ITEM TO CHECK	IMPORTANCE	CHECKLIST
EXPERIMENTAL DESIGN		
Definition of experimental and control groups	**E**	Materials & Methods
Number within each group	**E**	Materials & Methods
Assay carried out by core lab or investigator's lab?	D	Core Laboratory
Power analysis	D	Not applicable
SAMPLE		
Description	**E**	Materials & Methods
Volume/mass of sample processed	D	Not applicable
Microdissection or macrodissection	**E**	Not applicable
Processing procedure	**E**	Materials & Methods
If frozen - how and how quickly?	**E**	Not applicable
If fixed - with what, how quickly?	**E**	Not available
Sample storage conditions and duration (especially for FFPE samples)	**E**	Materials & Methods
NUCLEIC ACID EXTRACTION		
Nucleic acid quantification	**E**	NanoDropTM Lite Spectrophotometer (ThermoFisher Scientific, Boston, MA, USA).
DNA or RNA quantification	**E**	DNA
Quality/Integrity, method/instrument, e.g. RNA integrity	**E**	Supplementary File 3
Template structural information	**E**	Not applicable
Template modification (digestion, sonication, preamplification etc)	**E**	Not applicable
Template treatment	**E**	Not applicable
Inhibition dilutions or spike	**E**	Not applicable
DNA contamination assessment of RNA samples	**E**	Not applicable
Details of DNase treatment where performed	**E**	Not applicable
Manufacturer of reagents used and catalogue number	D	Materials & Methods/Additional Files
Storage conditions (Nucleic acid): temperature, concentration, duration, buffer)	**E**	Materials & Methods
REVERSE TRANSCRIPTION (if necessary)		
cDNA priming method and concentration	**E**	Not applicable

One or two-step protocol	**E**	Not applicable
Amount of RNA used per reaction	**E**	Not applicable
Detailed reaction components and conditions	**E**	Not applicable
RT efficiency	D	Not applicable
Estimated copies measured with and without addition of RT	D	Not applicable
Manufacturer of reagents and catalogue numbers	D	Not applicable
Reaction volume	D	Not applicable
Storage conditions of cDNA	D	Not applicable
dPCR TARGET INFORMATION		N/A
Sequence accession number	**E**	Included in article
Location of amplicon	D	Not applicable
Amplicon length	**E**	Not applicable
In silico specificity screen (BLAST, etc)	**E**	Not applicable
Pseudogenes, retropseudogenes or other homologs?	D	Not applicable
Sequence alignment	D	Not applicable
Secondary structure analysis of amplicon and GC content	D	Not applicable
Location of each primer by exon or intron (if applicable)	**E**	Not applicable
What splice variants are targeted?	**E**	Not applicable
dPCR OLIGONUCLEOTIDES		
Primer sequences	**E**	Not available
RTPrimerDB Identification Number	D	Not available
Probe sequences	D	Not available
Location and identity of any modifications	**E**	Not available
Manufacturer of oligonucleotides	D	Not available
Purification method	D	Not available
dPCR PROTOCOL		Supplementary Information 2, Table S1
Complete reaction conditions	**E**	Materials & Methods
Reaction volume and amount of cDNA/DNA	**E**	Materials & Methods
Primer, (probe), Mg++ and dNTP concentrations	**E**	Materials & Methods
Polymerase identity and concentration	**E**	Materials & Methods
Buffer/kit identity and manufacturer	**E**	Materials & Methods/ Supplementary File 1
Exact chemical constitution of the buffer	D	Not available

Additives (SYBR Green I, DMSO, etc.)	**E**	Not applicable
Plates/tubes catalogue number and manufacturer	D	Materials & Methods/ Supplementary File 1
Complete thermocycling parameters	**E**	Materials & Methods
Reaction setup (manual/robotic)	D	Manual
Gravimetric or volumetric dilutions (manual/robotic)	D	Volumetric dilutions
Total PCR volume prepared	D	Materials & Methods
Partition number	**E**	Supplementary File 1
Individual partition volume	**E**	Supplementary File 1
Total volume of the partitions measured (effective reaction size)	**E**	Supplementary File 1
Partition volume variance/SD	D	Not available
Comprehensive details and appropriate use of controls	**E**	Materials & Methods
Manufacturer of dPCR instrument	**E**	Materials & Methods
dPCR VALIDATION		
Optimisation data for the assay	D	Materials & Methods (dilution essay)
Specificity (when measuring rare mutations, pathogen sequences etc)	**E**	Results: ROC curve (dPCR vs Sanger sequencing)
Limit of detection of calibration control	D	Materials & Methods, Additional File
If multiplexing, comparison with singleplex assays	**E**	Not applicable
DATA ANALYSIS		
Mean copies per partition (λ or equivalent)	**E**	Supplementary File 1
dPCR analysis program (source, version)	**E**	Available upon request
Outlier identification and disposition	**E**	QuantStudio 3D Analysis SuiteTM Cloud Software
Results of NTCs	**E**	Available upon request
Examples of positive(s) and negative experimental results as supplemental data	**E**	Supplementary File 3
Where appropriate, justification of number and choice of reference genes	**E**	Included in article
Where appropriate, description of normalization method	**E**	Not applicable
Number and concordance of biological replicates	D	Not applicable
Number and stage (RT or qPCR) of technical replicates	**E**	Not applicable
Repeatability (intra-assay variation)	**E**	Materials & Methods, Results and Supplementary File 3
Reproducibility (inter-assay/user/lab etc variation)	D	Not applicable

Experimental variance or CI d	**E**	Materials & Methods
Statistical methods for analysis	**E**	Materials & Methods
Data submission using RDML (Real-time PCR Data Markup Language)	D	Not applicable

Essential information (E); Desirable information (D).