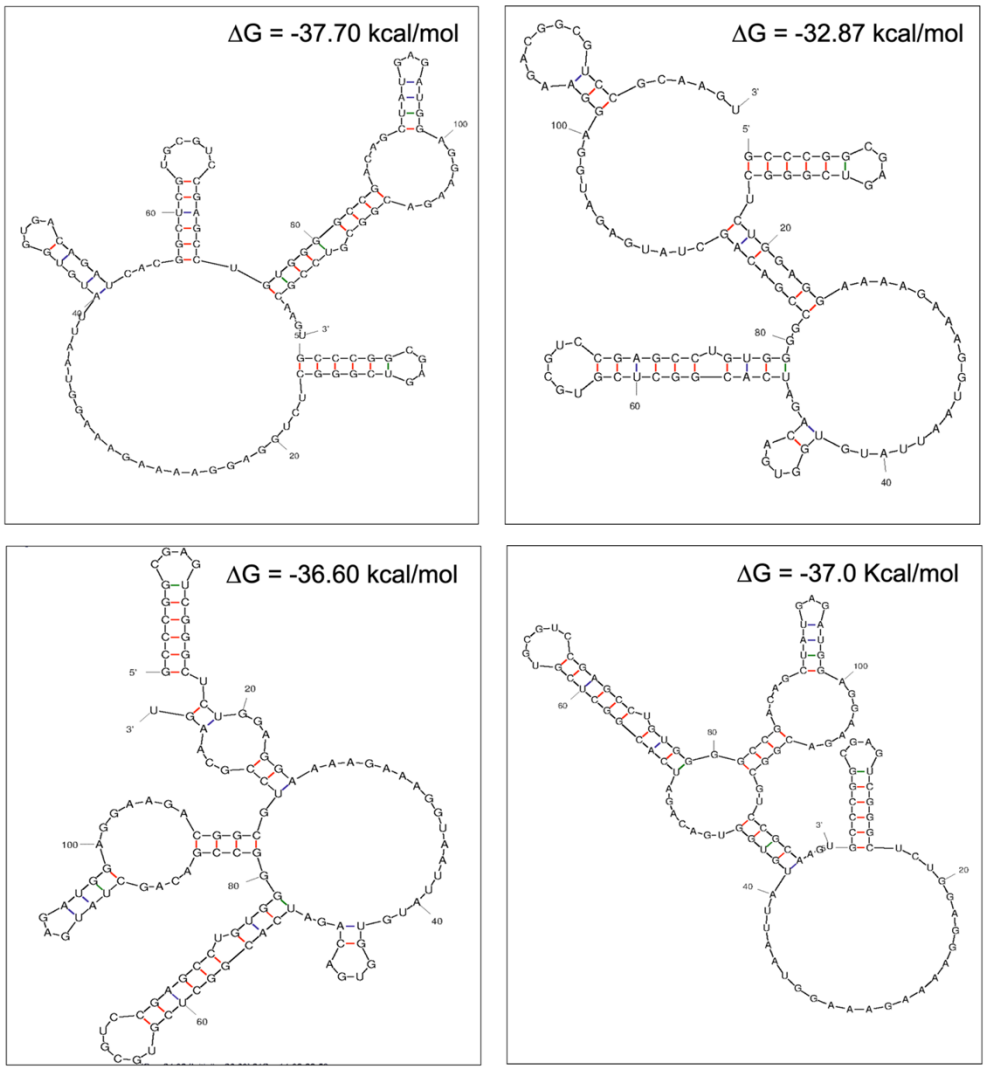
Supplementary Figure 1

EGFRvIII target amplicon

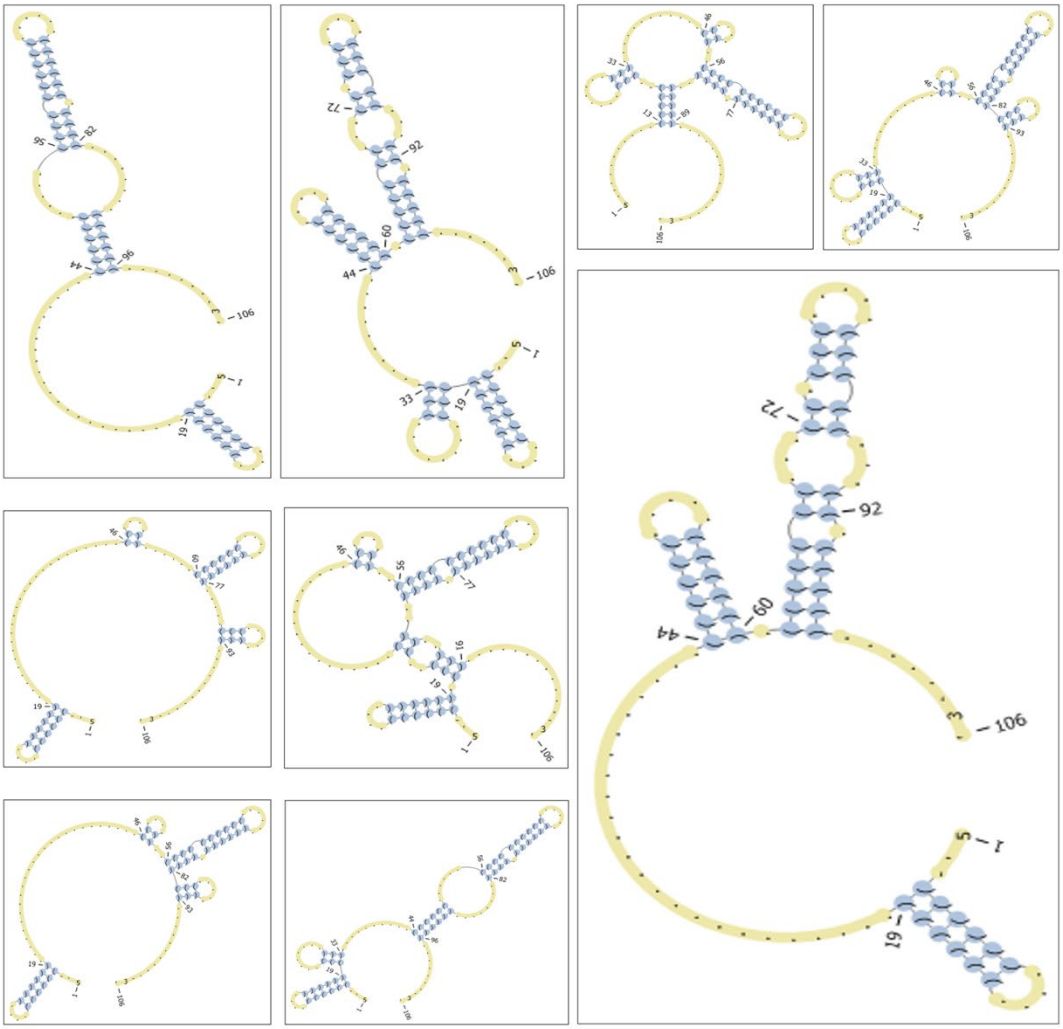
a.



Supplementary Figure 1 (continued)

EGFRvIII target amplicon

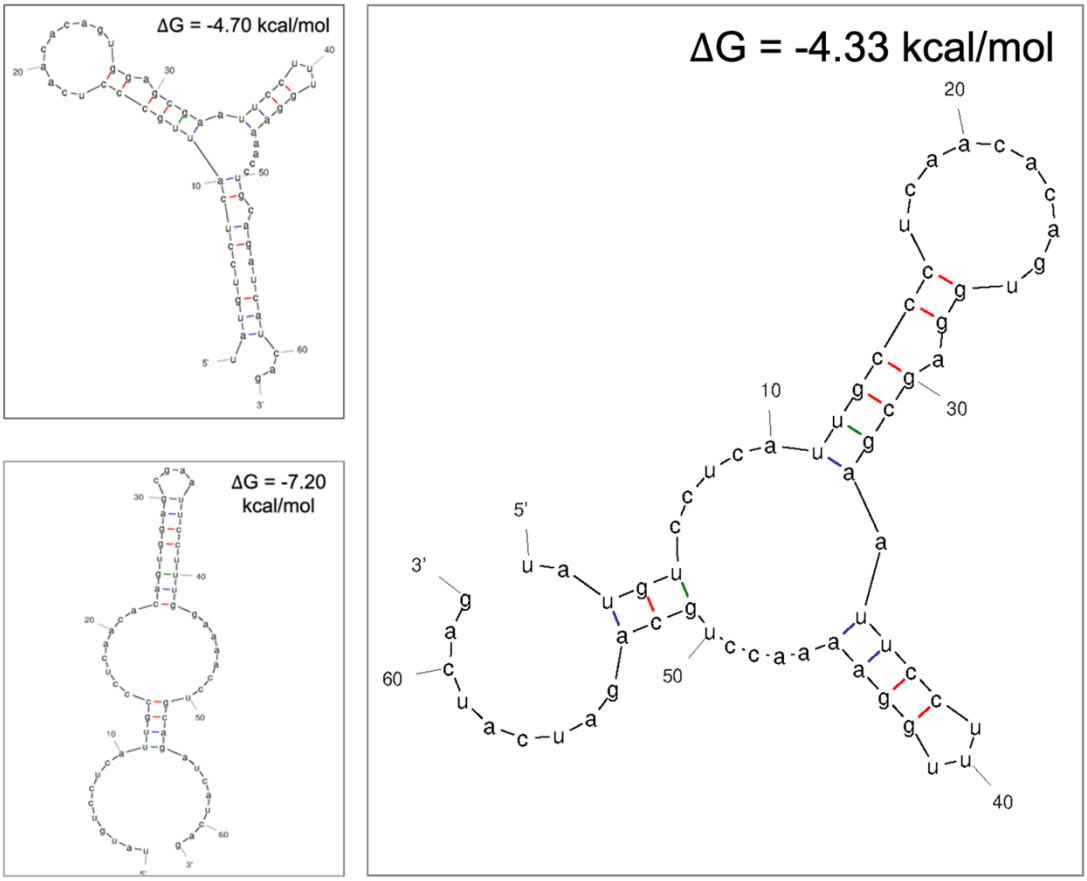
b.



Supplementary Figure 1 (continued)

EGFRwt target amplicon

c.



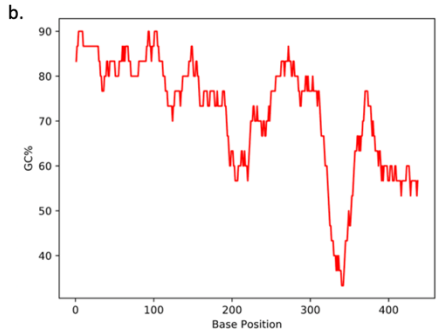
Supplementary Figure 1: Secondary Structure prediction in target amplicon of EGFRvIII and EGFR wt using Mfold algorithm. **(a)** Four distinct secondary structures in target amplicon (96 bp) of EGFRvIII with minimum free energy (△G) in the range of (-32.87 to -37.70) kcal/mol. **(b)** Depiction of nine potential pseudoknots in target amplicon of EGFRvIII as predicted by algorithm. **(c)** Three possible secondary structures predicted in EGFR wt target amplicon (62 bp) with △G in the range of (-4.33 to -7.20) kcal/mol.

Supplementary Figure 2

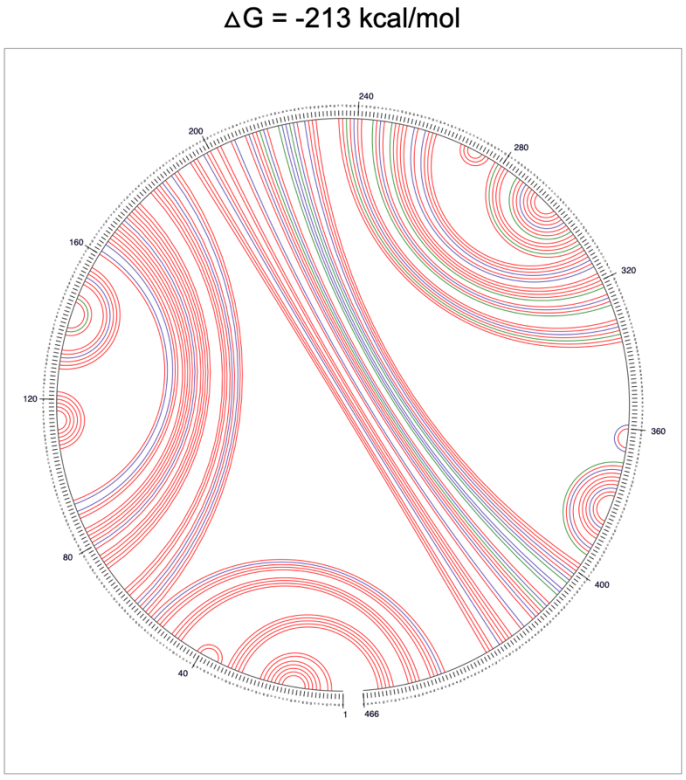
a.

AGACGTCCGGGCAGCCCCCGGCGCAGCGCGGCCGCAGCAGCCTCCGCCCCCCGCACGGTGTGAGCGC
CCGACGCGGCCGAGGCGGCCGGAGTCCCGAGCTAGCCCCGGCGGCCGCCGCCGCCCAGACCGGACGA
CAGGCCACCTCGTCGGCGTCCGCCCCGAGTCCCCGCCTCGCCGCCAACGCCACAACCACCGCGCACGGCC
CCCTGACTCCGTCCAGTATTGATCGGGAGAGCCGGAGCGAGCTCTTCGGGGAGCAGCGATGCGACCCTC
CGGGACGGCCGGGGCAGCGCTCCTGGCGCTGCTGGCTGCGCTCTGCCCGGCGAGTCGGGCTCTGGAG
GAAAAGAAAG//////////GTAATTATGTGGTGACAGATCACGGCTCGTGCGTCCGAGCCTGTGGGGCCG
ACAGCTATGAGATGGAGGAAGACGGGCGTCCGCAAGTGTAAGAAGTGCGAAGGGCCTTGCCGCAAAG

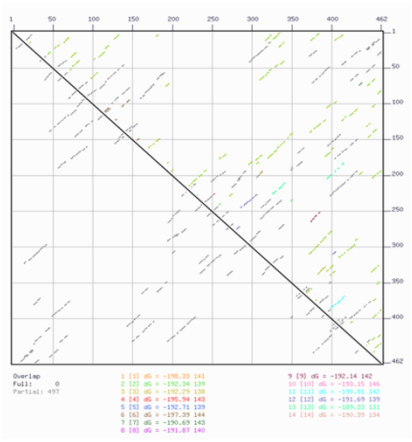
b.



c.

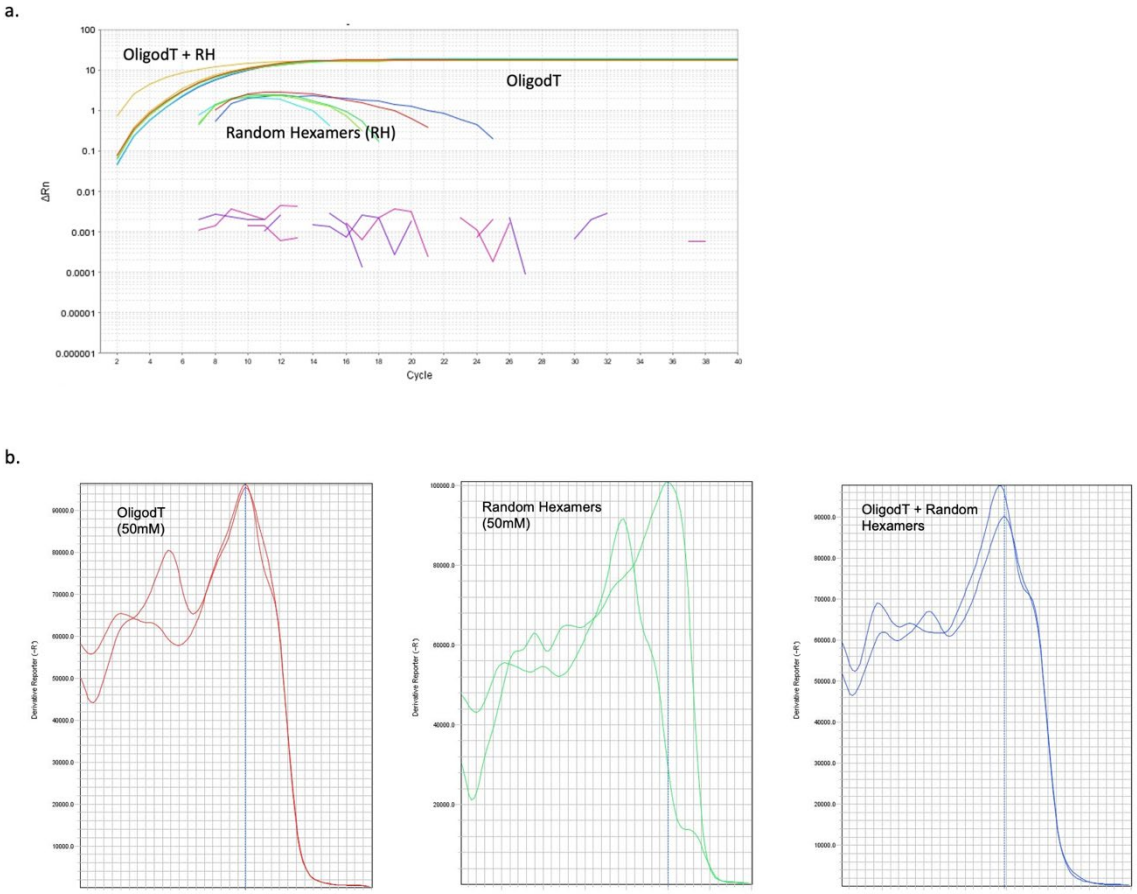


d.



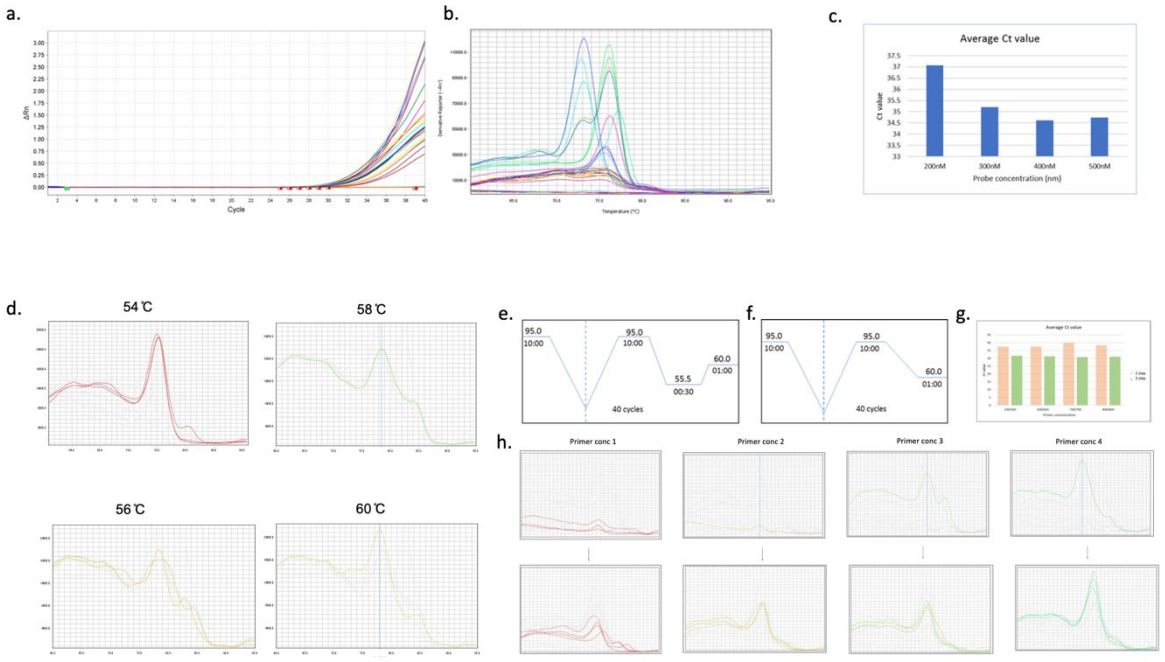
Supplementary Figure 2: Analysis of nucleotide sequence (467 bp) unique to EGFRvIII at exon1:exon8 junction site (a) Nucleotide sequence of exons 1:8 fusion where EGFRvIII occurs, highlighting GC, CG and G repeats. **(b)** Graphical representation of the GC content (percentage) in the nucleotide sequence of exon1:exon8 with the calculation based on window size of 30 bases. (**c)** Circle graph obtained from secondary structure prediction analysis illustrating the individual base pair interactions in 20 predicted foldings. Each color represents a distinct base pair interaction. The ΔG indicating the thermal stability of the predicted foldings is -213 kcal/mol. **(d)** Energy dot plot of the computed foldings, with different dots representing the superposition and relative thermal stability of all possible foldings.

Supplementary Figure 3



Supplementary Figure 3: Summary of optimization measures. **(a)** Amplification plot demonstrating the effect of using oligodT and Random hexamers in different combinations during reverse transcription on resulting Ct value in qPCR**. (b)** Melt curve plots illustrating the maximum peak and Tm value of the qPCR product in the three different reverse transcription conditions.

Supplementary Figure 4



Supplementary Figure 4: Summary of qPCR optimization measures. Amplification plot (a) and melt curve plot (b) obtained by testing different concentrations of forward and reverse primers. Amplification plot demonstrating the Cycle threshold (Ct) values obtained at different probe concentrations (c). Annealing temperature gradient (54-60)℃ assessment to determine the peak of target product and prevalence of non-specific products as shown on melt curve plots (d). Schematic depicting the three step (e) and two step (f) qPCR cycling conditions. Bar graph (g) demonstrating the lowering of Ct value at four different forward/reverse primer concentrations with the use of three step (green bar) vs two step (orange) cycling conditions. Melt curve plots (h) from two step (top panel) and three step (bottom panel) cycling conditions at four different primer concentrations comparing the maximum peak of the target product.

Supplementary Table 1: Summary of patient Data

Patient Number	Medications	Levetiracetam	Dexamethasone	Clinical Trial Participant	Chemo-RT	Age	Gender	WHO Grade	Race	Ethnicity	Diagnosis	Location	Tumor parameters (mm)	Contrast Enhancement	Duration (symptom onset to surgery)/months	Recurrent	IDH1 status	TERT Status	EGFR Status (snapshot)	EGFR status (qPCR)	EGFRvIII status (qPCR)	MGMT Status	Survivability	rep 1	rep 2	rep 3	rep 4	Average (Copies / 20 µl)	Copies /ml
P1	[illegible]		✓		Bavituximab	49	F	NA	White	Non-Hispanic	GBM	Right Parietal/Frontal	50 x 37 x 50	yes	02/28/2018-08/12/2020	yes	wt	mutant	amplified, vIII	amplified	positive	NA	2/25/2018-present	114	106	122	158	125	500
P2	[illegible]	✓	✓		Bevacizumab	76	M	IV	White	Non-Hispanic	GBM	Left Temporal	3.2 x 3 x 3	yes	02/19/2018-10/10/2018	yes	wt	mutant	amplified, vIII	amplified	positive	negative	02/2018-06/2019 (deceased)	136	122	106	84	112	448
P3	[illegible]	✓		✓	Bavituximab + Radiation + TMZ	63	F	IV	White	Non-Hispanic	GBM	Septum pellucidum	14 x 14	yes	08/18/2017-08/21/2017	no	wt	mutant	amplified, vIII	amplified	positive	positive	08/18/2017-present	4.2	4.8	3.8	0	3.2	12.8
P4	[illegible]	✓	✓			63	M	IV	White	Non-Hispanic	GBM	Right Frontal	NA	yes	12/13/2019-06/01/2020	yes	wt	NA	amplified, vIII	amplified	positive	positive	03/14/2018-present	12	9.6	1.4	0	7.7	30.8
P5	[illegible]	✓	✓		antineoplastic RT + chemo (doxorubicin, bleomycin, vinblastine sulfate, dacarbazine)	40	M	IV	White	Non-Hispanic	GBM	Right Fronto-parietal	25 x 14 x 10	no	05/21/2018-03/26/2019	yes	wt	mutant	amplified, vIII	amplified	positive	positive	2015-7/1/2019(deceased)	100	96	148	180	131	524
P6	[illegible]		✓		Bavituximab + Radiation	56	M	IV	White	Non-Hispanic	GBM	Right Temporal	5.6 x 4.5 x 5.6	yes	05/01/2019-12/12/2019	yes	wt	mutant	amplified, vIII	amplified	positive	negative	2019-03/4/2020(deceased)	0	0	0	0	0	0
P7	[illegible]	✓	✓	✓	Bavituximab + Radiation + TMZ	64	M	IV	White	Non-Hispanic	HGG	Left Temporal	1.8 x 1.8 x 2.2	yes	04/25/2018-06/10/2019	yes	wt	mutant	amplified, vIII	amplified	positive	negative	3/2018-07/29/2020(deceased)	0	0	0	0	0	0
Wt1	[illegible]		✓	✓	antineoplastic RT + abemaciclib (INSIGht)	41	F	IV	White	Non-Hispanic	GBM	Left Temporal	5.6 x 4.2 x 4.9	yes	06/28/2018-02/18/2020	NA	wt	NA	wild-type	wild-type	negative	negative	06/12/2018-present	0	0	0	0	0	0
H1	N/A	N/A	N/A	N/A	N/A	53	M	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0
H2	N/A	N/A	N/A	N/A	N/A	47	M	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0
H3	N/A	N/A	N/A	N/A	N/A	50	M	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0
P8	[illegible]	✓	✓		RT + Chemotherapy	62	M	IV	White	Non-Hispanic	GBM	Left Parietal	35 x 35 x 31	yes	08/2020-08/11/2020	no	wt	NA	amplified	amplified	positive	positive	08/2020-present	1.2	3.6	1.2	0	1.5	6
P9	[illegible]	✓	✓			68	F	IV	White	Non-Hispanic	GBM	Left Frontal	42 x 42 x 52	yes	10/24/2018-11/12/2018	yes	wt	NA	NA	amplified	positive	positive	10/24/2018-present	0	0	0	0	0	0
P10	[illegible]	✓	✓		Chemotherapy (etoposide, Cisplatin)	56	M	IV	White	Non-Hispanic	GBM	Left Temporal	20 x 29 x 29	yes	2/18/2020-10/20/2020	yes	wt	mutant	amplified, vIII	amplified	positive	negative	7/31/2021(deceased)	1	3.6	2	5	2.9	11.6
P11	[illegible]			✓	RT + TMZ + bevacizumab	62	F	IV	White	Non-Hispanic	GBM	Right Frontoparietal	NA	yes	02/25/2019-04/06/2020	NA	wt	mutant	amplified, vIII	amplified	positive	NA	2019-present	2.2	4.6	4	2	3.2	12.8
P12	[illegible]	✓	✓		RT + Chemotherapy	73	M	IV	White	Non-Hispanic	GBM	Right Frontal	39 x 36 x 46	yes	07/2020-07/21/2020	no	wt	mutant	amplified	amplified	positive	positive	07/21/2020-present	0	0	0	0	0	0
P13	[illegible]	✓	✓			76	M	IV	White	Non-Hispanic	GBM	Right Temporal	56 x 43	yes	10/3/2019-10/07/2019	no	wt	NA	amplified	amplified	positive	negative	10/3/2019-12/29/2019	0	0	0	0	0	0
P14	[illegible]	✓	✓			82	M	IV	White	Non-Hispanic	GBM	Right Frontal	50 x 34 x 47	yes	03/08/2020-03/11/2020	no	wt	mutant	amplified	amplified	positive	positive	03/08/2020-4/9/2021	3740	3400	3560	3380	3520	14080
P15	[illegible]				RT	60	F	IV	White	Non-Hispanic	GBM	Right Temporal	33 x 25 x 29	yes	(History of seizures since 2014, weakness starting 01/2021)-01/28/2021	NA	wt	NA	amplified	amplified	positive	positive	01/2021-present	4603	5187	4663	4801	4813.5	19254
P16	[illegible]	✓	✓		RT	51	M	III	White	Non-Hispanic	Anplastic Astrocytoma	Right Temporal	NA	no	05/29/2018-03/13/2019	yes	wt	mutant	amplified	amplified	positive	negative	05/11/2018-11/9/2019	90	56	50	58	63.5	254
P17	[illegible]					67	M	IV	White	Non-Hispanic	GBM	Left Temporal	53 x 41 x 53	yes (peripheral)	6/11/2018-6/15/2018	NA	wt	NA	amplified	amplified	positive	positive	6/11/2018-present	78	34	32	11.4	38.85	155.4
Wt2	[illegible]	✓	✓			79	M	IV	White	Non-Hispanic	GBM	Right Temporal	76 x 55 x 49	yes	6/2019-9/24/2019	no	NA	NA	amplified	amplified	negative	positive	6/2019-01/12/2020 (deceased)	0	0	0	0	0	0
Wt3	[illegible]	✓	✓		Chemotherapy (TMZ)	64	M	IV	White	Non-Hispanic	GBM	Right Frontal	43 x 43 x 44	yes	11/19/2019-11/25/2019	no	wt	mutant	amplified	amplified	negative	positive	11/19/2019-present	0	0	0	0	0	0
H4	N/A	N/A	N/A	N/A	N/A	23	M	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0
H5	N/A	N/A	N/A	N/A	N/A	31	F	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0
H6	N/A	N/A	N/A	N/A	N/A	29	M	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0
H7	N/A	N/A	N/A	N/A	N/A	24	F	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0
P18	[illegible]	✓			RT	68	M	IV	White	Non-Hispanic	GBM	Right Temporal	NA	yes	12/2020-04/01/2021	no	wt	NA	NA	amplified	positive	negative	12/2020-present	9.4	2.2	0	4.4	4	16
P19	[illegible]					66	M	IV	White	Non-Hispanic	GBM	Corpus callosum	48 x 48 x 42	yes	01/14/2021-01/20/2021	no	wt	NA	NA	amplified	positive	negative	01/14/2021-3/14/2021	0	0	0	0	0	0
P20	[illegible]				RT + Chemotherapy	45	M	IV	White	Non-Hispanic	GBM	Right Parieto Occipital	49 x 32 x 37	yes	03/13/2021-03/18/2021	no	wt	NA	NA	amplified	positive	positive	03/13/2021-present	26	14	30	10	20	80
P21	[illegible]	✓			RT	74	M	IV	White	Non-Hispanic	GBM	Corpus callosum	NA	yes	04/18/2021-04/22/2021	no	wt	NA	NA	amplified	positive	positive	4/18/2021-7/1/2021	2.4	1.2	1.2	4.6	2.35	9.4
P22	[illegible]				Chemotherapy (TMZ)	58	M	III	White	Non-Hispanic	Anaplastic Oligodendro glioma	Left Frontal	60 x 49 x 56	yes	late 2020-02/17/2021	no	wt	NA	NA	amplified	positive	NA	late 2020-present	78	88	76	110	88	352
P23	[illegible]				RT	61	F	IV	White	Non-Hispanic	GBM	Right Temporal	33 x 25 x 29	yes	01/25/2021-01/28/2021	no	wt	NA	amplified	amplified	positive	positive	01/25/2021-present	0	0	0	0	0	0
P24	[illegible]	✓			RT	65	F	IV	White	Non-Hispanic	GBM	Left Frontal	40 x 30 x 34	yes (peripheral)	08/11/2020-02/19/2021	yes	wt	NA	NA	amplified	positive	negative	08/08/2020-present	0	0	0	0	0	0
P25	[illegible]	✓			RT + Chemotherapy	59	F	IV	White	Non-Hispanic	GBM	Right Parietal	49 x 54 x 56	yes	early 2021(before 03/2021)-03/31/2021	no	wt	NA	NA	amplified	positive	negative	early 2021-present	1030	1200	1150	1320	1175	4700
P26	[illegible]					78	F	IV	White	Non-Hispanic	GBM	Right Frontal	44 x 38 x 42	yes	02/26/2021-03/01/2021	no	wt	NA	NA	amplified	positive	positive	02/26-6/24/2021	78	54	86	80	74.5	298
P27	[illegible]	✓	✓		RT + Chemotherapy	32	M	III	White	Non-Hispanic	Anaplastic Astrocytoma	Left Frontal	41 x 51 x 47	no	04/05/2021-10/26/2021	yes	wt	NA	NA	amplified	positive	positive	01/20/2021-present	4.2	7.8	10	6.4	7.1	28.4
P28	[illegible]	✓			RT + Chemotherapy (Tamodar)	65	F	IV	White	Non-Hispanic	GBM	Left Frontal	32 x 28 x 34	yes	07/16/2020-07/21/2020	no	wt	mutant	amplified	amplified	positive	positive	07/16/2020-present	32	54	46	26	39.5	158
P29	[illegible]		✓		RT	60	F	IV		Non-Hispanic	GBM	Left Frontal	56 x 50 x 50	yes (peripheral)	07/24/2020 (also kidney/adrenal issues in 2018)-08/04/2020	no	wt	NA	NA	amplified	positive	negative	07/24/2020-present	26	26	4.6	17	18.4	73.6
P30	[illegible]					55	M	IV	White		GBM	Right Parietal	61 x 39 x 45	yes	02/2021-04/09/2021	no	wt	NA	NA	amplified	positive	positive	NA	3.6	3.6	0	0	1.8	7.2
Wt4	[illegible]	✓			RT	79	M	IV	White	Non-Hispanic	GBM	Right Temporal	76 x 55 x 49	yes	09/2019-09/24/2019	no	wt	NA	amplified	amplified	negative	positive	9/1/2019-01/12/2020 (deceased)	0	0	0	0	0	0
Wt5	[illegible]				Chemotherapy (TMZ)	85	M	IV	White	Non-Hispanic	GBM	Left Frontal	33 x 34 x 36	yes	07/2020-08/13/2020	no	wt	NA	NA	amplified	negative	positive	07/2020-2/1/2021 (deceased)	0	0	0	0	0	0
Wt6	[illegible]	✓	✓		Chemotherapy (Temodar)	64	M	IV	White	Non-Hispanic	GBM	Right Frontal	43 x 43 x 44	yes	11/2019-11/25/2019	no	wt	mutant	amplified	amplified	negative	positive	11/2019-present	0	0	0	0	0	1
Wt7	[illegible]	✓	✓			37	M	IV	White	Non-Hispanic	GBM	Left Frontal	6 x 37 x 51	yes (peripheral)	08/25/2020-08/27/2020	no	wt	mutant	amplified	amplified	negative	negative	08/25/2020-present	0	0	0	0	0	0
Wt8	[illegible]				RT + Chemotherapy	71	F	IV	White	Non-Hispanic	GBM	Left Parietal	22 x 22 x 24	yes (peripheral)	10/03/2020-10/06/2020	no	wt	mutant	wild-type	wild-type	negative	negative	10/03/2020-present	0	0	0	0	0	0
Wt9	[illegible]					32	M	NA	White	Non-Hispanic	Recurrent Glioneuronal tumor	Right Frontal	NA	yes (peripheral)	12/2018-12/27/2019	yes	NA	NA	NA	amplified	negative	NA	12/2018-present	2.4	1.2	0	0	0.9	3.6
Wt10	[illegible]	✓		✓	RT + Chemotherapy	54	M	IV	White	Non-Hispanic	GBM	Right Temporal	54 x 44 x 45	yes (peripheral)	11/17/2020-10/04/2021	yes	wt	mutant	amplified	amplified	negative	negative	11/04/2020-present	0	0	0	0	0	0
H8	N/A	N/A	N/A	N/A	N/A	24	M	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0
H9	N/A	N/A	N/A	N/A	N/A	76	F	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0
H10	N/A	N/A	N/A	N/A	N/A	85	M	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0
H11	N/A	N/A	N/A	N/A	N/A	32	F	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0
H12	N/A	N/A	N/A	N/A	N/A	56	F	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0
H13	N/A	N/A	N/A	N/A	N/A	67	F	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0
H14	N/A	N/A	N/A	N/A	N/A	64	M	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0	0	0
P10 longitudinal																								1.2	5.2	7	4.2	4.4	17.6
P11 longitudinal																								0	0	0	0	0	0
P16 longitudinal																								6320	5940	6260	6180	6175	24,700
P20 longitudinal																								1.2	1.2	1	0	0.85	3.4

RT = Radiotherapy
TMZ = Temozolamide
NA = Not available
HGG = High grade glioma
GBM = Glioblastoma

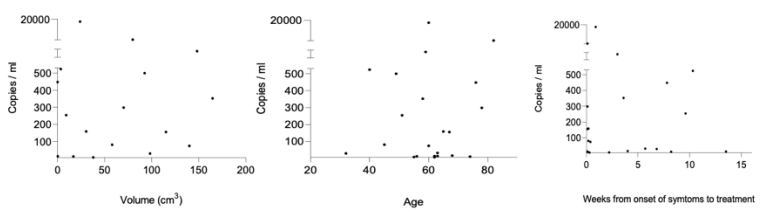
Duration
Primary — From first symptom to first surgery
Recurrent — First surgery to second surgery

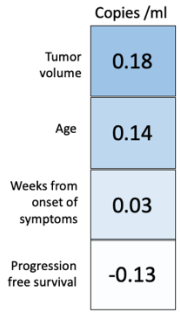
Supplementary Table 2. qPCR EGFR amplification cut-off data for Cohort 3

Patient ID	Diagnosis	IDH status	Tumor Grade	Recurrence	Ct rep 1	Ct rep 2	Avg_GAPDH	Ct rep 1	Ct rep 2	Avg_EGFRwt	Norm_GAPDH	Norm_EGFRwt
BC-18012	GBM	wt	IV	No	21.15153694	21.25002289	21.20077991	26.86749268	26.16753006	26.51751137	0	26.51751137
BC-18018	Residual HGG	mut	IV	Yes	24.56344795	23.9705658	24.26700687	30.31254959	30.03812218	30.17533588	3.066226959	27.10910892
MGH-18128	Diffuse Astrocytoma	mut	II	No	24.33731461	24.45201492	24.39466476	29.96534157	29.49748421	29.73141289	3.19388485	26.53752804
MGH-18122	Diffuse Astrocytoma	mut	II	No	24.84611053	24.88852692	24.86731873	28.82067299	28.48605347	28.65336323	3.666538811	24.98682442
MGH-19132	Anaplastic Oligodendroglioma	mut	III	No	24.17763901	24.11063004	24.14413452	29.74443245	29.32966042	29.53704643	2.943354607	26.59369183
MGH-19019	Diffuse Astrocytoma	mut	II	No	22.96496964	22.50131226	22.73314095	28.81457329	28.75448227	28.78452778	1.532361031	27.25216675
Average of Normalized_EGFRwt of all samples (n=6): 26.4994719 (defined as cut-off)												

Supplementary Figure 5

(a)





(b)

Patient ID	T1	T2	Longitudinal sample
Wt2	0	0	N/A
Wt5	0	0	N/A
P20	5.2	3.1	8.6
Wt8	0	0	N/A
P16	4.7	3.3	3.5
P10	6.9	4.2	9.1
Wt3	0	0	N/A

Supplementary Figure 5: Reproducibility and intratumoral heterogeneity

(a) Linear regression (top) and correlation matrix (bottom) between copies/ml and clinical parameters. (b) Detection of EGFRvIII mutant levels in plasma derived EV RNA, in a group of EGFRvIII patients (P10, P16, P20) and controls (Wt3, Wt5, Wt8) at two different time points, T1 and T2. The copies of EGFRvIII are normalized to GAPDH run in the same well. Third column represents EGFRvIII copies (normalized to GAPDH) in longitudinal samples.

Supplementary Table 3

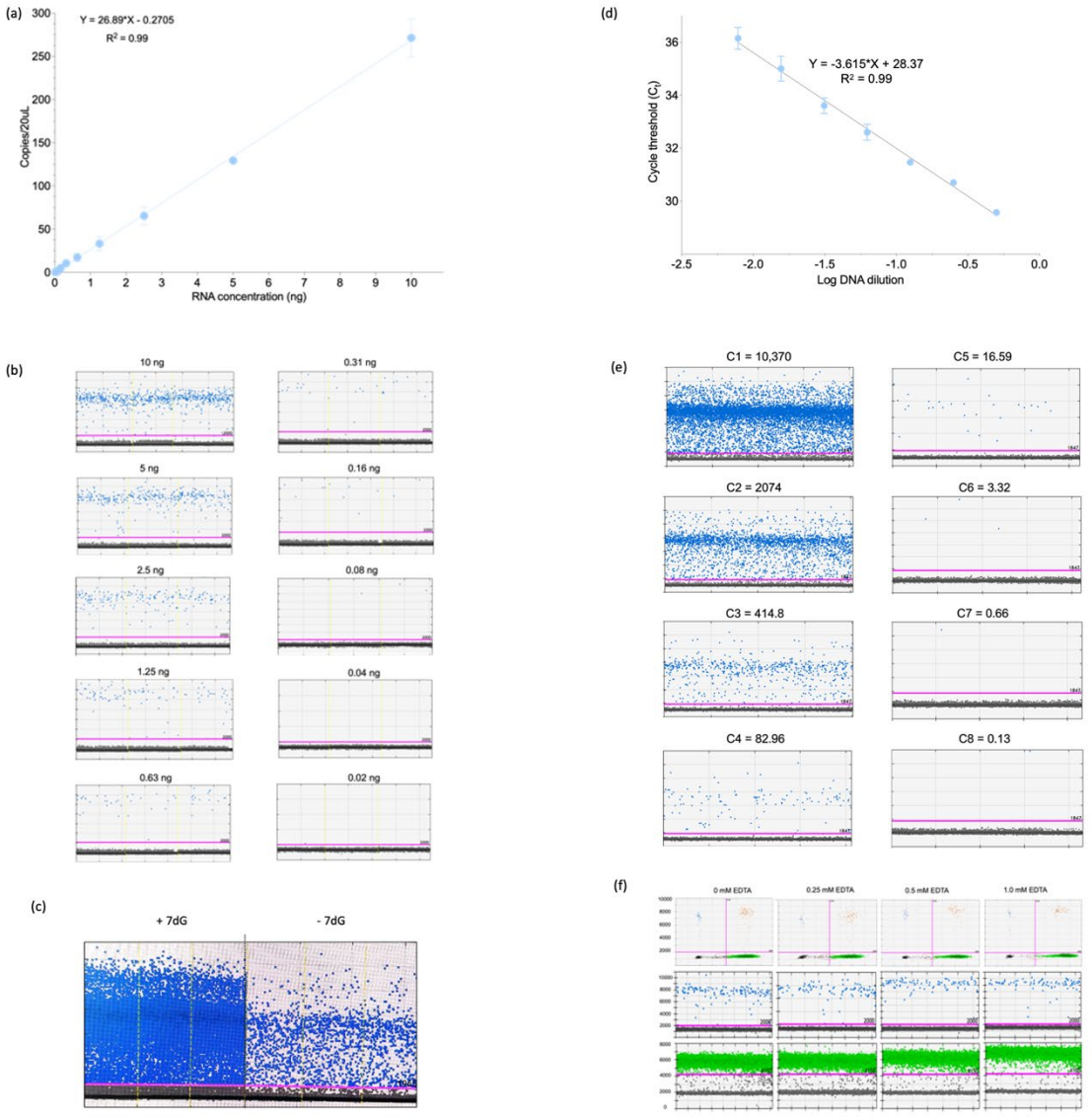
Cohort 2

Study ID	Ct rep 1	Ct rep 2	Avg_GAPDH	Ct rep 1	Ct rep 2	Avg_EGFRwt	Ct rep 1	Ct rep 2	Avg_EGFRvIII	Norm_GAPDH	Norm_EGFRwt	Norm_EGFRvIII
MGH-19012	18.3007984	18.2145844	18.25769138	25.49560928	25.08634567	25.29097748	28.79283333	28.88248825	28.83766079	0.807244301	24.48373318	28.03041649
MGH-19006	18.243618	17.7154045	17.97951126	22.68240547	22.69255638	22.68748093	35.62703	36.73383	36.18042946	0.529064178	22.15841675	35.65136528
BC-17014	17.4554996	18.2439938	17.8497467	16.46021843	16.56148529	16.51085186	35.34437561	32.78369904	34.06403732	0.399299622	16.11155224	33.6647377
MGH-18056	19.1898201	19.122864	19.1563421	18.9460113	19.0589161	19.0024637	21.43121719	21.75722504	21.59422112	1.705895017	17.29656868	19.8883261
MGH-19108	18.0961285	18.2877941	18.19196129	16.64451599	16.93544579	16.78998089	30.032938	30.70248604	30.36771202	0.741514206	16.04846668	29.62619781
MGH-21017	17.6818218	17.6644592	17.67314053	16.83738327	16.50945473	16.673419	30.32335281	27.02560425	28.67447853	0.222693443	16.45072556	28.45178509
MGH-20035	18.2074375	17.3450603	17.77624893	20.22495842	19.74502563	19.98499203	23.72048	23.76638	23.74342918	0.325801849	19.65919018	23.41762733
MGH-20084	18.5624428	17.7538853	18.15816402	24.99819183	25.07386589	25.03602886	34.96829987	35.30978775	35.13904381	0.707716942	24.32831192	34.43132687
MGH-18134	18.2077579	18.4575672	18.33266258	19.78232384	20.00311279	19.89271832	31.90046883	29.71730042	30.80888462	0.8822155	19.01050282	29.92666912
BC-17017	18.1269112	17.8028183	17.96486473	19.69542694	19.06052589	19.37797642	25.68898392	25.82143402	25.75520897	0.514417648	18.86355877	25.24079132
MGH-18126	16.874136	19.7245007	18.29931831	22.41389847	21.30988884	21.86189365	24.10434151	23.56221771	23.83327961	0.848871231	21.01302242	22.98440838
MGH-18067	18.128294	17.8565865	17.99244022	17.4209919	17.51852226	17.46975708	35.49660492	32.58495712	34.04078102	0.541993141	16.92776394	33.49878788
MGH-20101	17.5925808	17.4765072	17.53454399	21.76619911	21.11041237	21.43330574	25.35233498	25.66563225	25.50898361	0.084096909	21.34920883	25.4248867
MGH-20062	17.5004539	17.4350948	17.46777439	23.95347595	23.94470978	23.94909286	31.50045967	31.62404251	31.56225109	0.017327309	23.93176556	31.54492378
MGH-19149	17.4770203	17.4238739	17.45044708	25.36227226	24.76921654	25.0657444	40	40	40	0	25.0657444	40
BC-18032	17.5512638	17.6874275	17.61934566	22.28536415	22.23291588	22.25914001	22.32438278	22.00032616	22.16235447	0.168898582	22.09024143	21.99345589
MGH-19093	17.8883724	18.3876057	18.13798904	30.82641	30.81092	30.818665	40	40	40	0.687541962	30.13112304	39.31245804
MGH-19149	17.9953671	17.7853165	17.89034176	22.44848061	21.95455933	22.20151997	40	40	40	0.439894676	21.76162529	39.56010532
BC-18037	18.0925293	17.6941471	17.8933382	21.95669365	22.41148758	22.18409061	27.92067528	27.77781868	27.84924698	0.442891121	21.74119949	27.40635586
MGH-18061	17.743124	17.552309	17.64771652	16.4209919	16.64805603	16.53452396	31.90290642	33.7375412	32.82022381	0.19726944	16.33725452	32.62295437

Cohort 3

Patient ID	Ct rep 1	Ct rep 2	Avg_gapdh	Ct rep 1	Ct rep 2	Avg_egfr	Ct rep 1	Ct rep 2	Avg_EGFRvIII	Norm_gapdh	Norm_egfr	Norm_EGfrvIII
MGH-20104	27.50389862	27.72080231	27.61235046	30.18013763	30.34205055	30.26109409	40	40	40	7.6563797	22.60471439	32.3436203
MGH-20192	23.25525856	24.09257507	23.67391682	25.15888405	24.94959831	25.05424118	36.36100388	37.01246262	36.68673325	3.717946053	21.33629513	32.96878719
MGH-20112	25.95660973	25.65935898	25.80798435	27.65223694	27.9166832	27.78446007	40	40	40	5.852013588	21.93244648	34.14798641
MGH-21067	24.6169529	25.60253334	25.10974312	28.36639977	28.60464287	28.48552132	36.59282303	37.4393959	37.01610947	5.153772354	23.33174896	31.86233711
MGH-20152	24.40704918	24.97827721	24.69266319	20.07348633	19.44060898	19.75704765	34.93694305	34.45499039	34.69596672	4.736692429	15.02035522	29.95927429
MGH-21029	19.74980736	20.16213417	19.95597076	21.94208145	21.76089287	21.85148716	31.34794807	32.48620605	31.91707706	0	21.85148716	31.91707706
MGH-21053	27.92509651	27.9609127	27.94300461	26.7177906	26.40461731	26.56120396	33.49728012	34.1177697	33.80751991	7.987033844	18.57417011	25.82048607
MGH-21038	24.78341866	24.4810276	24.63222313	27.94438171	28.32129288	28.1328373	36.57641602	37.40880203	36.99260902	4.676252365	23.45658493	32.31635666
MGH-21042	27.91183662	27.75788689	27.83486176	29.03050804	28.60367393	28.81709099	40	40	40	7.878890991	20.9382	32.12110901
MGH-21017	20.97009277	20.61476135	20.79242706	18.9414463	19.12094116	19.03119373	32.08921432	32.68430328	32.3867588	0.836456299	18.19473743	27.83802223
MGH-21046	22.45641899	22.27855873	22.36748886	28.46891594	28.19516945	28.33204269	34.95549393	34.93353653	34.94451523	2.411518097	25.9205246	32.53299713
MGH-21056	23.96917152	24.82483292	24.39700222	24.57865143	24.5433445	24.56099796	34.83654022	33.89217377	34.36435699	4.441031456	20.11996651	29.92332554
MGH-20086	24.62406921	24.44667625	24.53537273	22.67568779	22.82577705	22.75073242	34.00020981	33.97887421	33.98954201	4.57940197	18.17133045	29.41014004
MGH-20094	25.42221069	25.41974258	25.42097664	28.03621483	28.40188026	28.21904755	37.81202698	37.87554932	37.84378815	5.465005875	22.75404167	32.37878227
MGH-20100	25.87053871	25.7077198	25.78912926	27.37074471	27.69473839	27.53274155	34.56914902	34.27066803	34.41990852	5.833158493	21.69958305	28.58675003
MGH-20112	29.91491699	29.56949615	29.74220657	31.9421463	32.36932755	32.15573692	40	40	40	9.786235809	22.36950111	30.21376419
MGH-21058	24.41893196	24.28207207	24.35050201	23.77349854	24.12952614	23.95151234	31.32673836	31.91280746	31.61977291	4.39453125	19.55698109	27.22524166

Supplementary Figure 6



Supplementary Figure 6: Assessment of assay efficiency and linearity

EGFRvIII cDNA reverse transcribed from serially diluted tumor tissue RNA was amplified using (a) ddPCR, and (d) qPCR. (a) Linear Regression graph depicting the EGFRvIII copies detected via ddPCR plotted against the RNA input. Regression analysis reported R^2 = 0.99. (b) ddPCR 1D plots demonstrating the mutant signal generated at each RNA concentration. (d) qPCR standard curve demonstrating Cycle threshold (C_t) values plotted against the log RNA input. Linear regression analysis reported R^2 = 0.99. (c) ddPCR 1D plots depicting the mutant signal generated when EGFRvIII synthetic RNA is reverse transcribed into cDNA with (+) and without (-) 7dG followed by ddPCR amplification. (e) ddPCR 1D plots demonstrating the mutant signal generated when cDNA from serially diluted EGFRvIII synthetic RNA was amplified using ddPCR. C number corresponds to the data point and the EGFRvIII copy number input in each RT condition. (f) ddPCR 2D plots

(top row) and 1D plots depicting cluster density, tightness, and separation of mutant events (blue) and GAPDH events (green) at different concentrations of EDTA versus no EDTA addition to ddPCR.