SUPPLEMENTARY MATERIALS

Goldberg et al., Early Assessment of Lung Cancer Immunotherapy Response via Circulating Tumor DNA

TABLE OF CONTENTS:

Supplementary Methods: Detailed Description of Plasma Processing and ctDNA Quantification

Collection and processing of plasma samples:

Blood was collected in 10 mL EDTA-containing Vacutainer tubes (Beckton Dickinson). Tubes were centrifuged within 4 hours of collection at 1000 x g for 10 minutes in a clinical centrifuge with a swinging bucket rotor with slow acceleration and deceleration (brake off). Plasma was transferred to cryovials in 1mL aliquots, being careful to avoid the buffy coat, and was stored at -80^{o}C.

Extraction of DNA from plasma:

Plasma was thawed at room temperature for ~30 minutes and was then centrifuged at 6800 x g for 3 minutes to remove any cryoprecipitate. The supernatant was transferred to a fresh tube for further processing. A QiaAmp MinElute Virus Vacuum Kit (Qiagen) was used for purification of DNA from 1 mL of plasma according to the manufacturer's instructions.

Library preparation for next-generation sequencing:

Libraries for next-generation sequencing were prepared from total plasma-derived cell-free DNA using a highly multiplexed PCR enrichment approach. An enhanced version of the Error-Suppressed Deep Sequencing method previously published by our laboratory was employed.[15] Multiplexed PCR was used to simultaneously amplify multiple genomic regions that are prone to acquiring somatic mutations in a variety of malignancies, including non-small cell lung cancer. The targeted regions are listed in **Supplementary Table S1**. Primers were designed to amplify both wild-type and mutant sequences with similar efficiency. The resulting amplicon library was subjected to massively parallel sequencing enabling identification and quantification of mutant sequences within a background of wild-type sequences. Detection of

very low-abundance mutant DNA molecules was permitted by suppression of errors arising from sequencer misreads and from PCR polymerase misincorporations, as described below.

Plasma-derived DNA was first subjected to 3 cycles of multiplexed PCR to produce copies of template molecules to which degenerate sequence tags were attached. Each reaction consisted of 43 target-specific primer pairs to amplify mutation hotspot and warm-spot regions within 24 genes. The forward primer in each pair was labeled with a 5'-biotin tag to facilitate purification of PCR copies. Reverse primers contained a target-specific sequence, a degenerate sequence (referred to as the molecular lineage tag [MLT]), and a common sequence that served as a primer-binding site for subsequent rounds of PCR. Primer sequences for this first round of PCR are provided in **Supplementary Table S7**. The following reaction components were assembled at room temperature in a total volume of 20 µL: (1) plasma-derived cell-free DNA in 10 µL EB buffer (Qiagen), (2) 1 x Phusion buffer HF (Thermo Fisher), (3) 200 mM of each dNTP, (4) a mixture of 43 forward and 43 reverse primers, 100 nM each, (5) molecular grade water as needed to make the total volume of 20 µL, and (6) Phusion Hot Start DNA polymerase (Thermo Fisher), (0.04 U/ µL). A separate reaction volume was used for each plasma DNA sample. Thermal cycling was carried out using the following protocol: (1) 98^{o}C for 30 seconds, (2) 98^{o}C for 10 seconds, (3) 68^{o}C down to 56^{o}C by 1^{o}C every 10 seconds, (4) 55^{o}C for 2 minutes, (5) 72^{o}C for 20 seconds, and (6) repeat steps 2 to 5 for a total of 3 cycles. EDTA was added to terminate the reaction (10mM final concentration), along with 5 µL of high capacity streptavidin agarose bead slurry (Thermo Fisher) to capture the biotin-labeled products. Tubes containing streptavidin beads were turned end-over-end at room temperature for at least 2 hours to permit biotin-capture.

The products of the first round of PCR were purified by removing the streptavidin beads from the reaction solution and washing twice with 200 µL of 10 mM Tris (pH 7.6) and 50 mM NaCl solution. The first wash was at room temperature, whereas the buffer for the second wash was heated to 60^{o}C for 15 minutes. Captured PCR products were eluted from the streptavidin

beads by heat denaturation and release of the non-biotin-labeled strand. DNA was eluted directly into the PCR cocktail of the next reaction, as indicated below. Beads were heated to 98°C for 20 seconds in the PCR cocktail, and then after brief shaking and centrifugation, the supernatant containing the eluted DNA was transferred to a clean tube.

A second round of limited-cycle PCR was performed to selectively amplify and assign sample-specific barcodes to the products from the first round. The 43 mutation-prone targets were amplified using 43 gene-specific forward primers and a single reverse primer designed to hybridize to the common primer-binding sequence introduced in the first PCR round. Sample-specific barcodes were incorporated into both the forward and reverse primers, so that amplicons would be labeled with dual barcodes. The forward primers used in this PCR round were nested relative those used in the previous round, and also contained a common sequence permitting subsequent amplification using universal primers. Because the forward primers contained multiple sample-specific barcodes in combination with multiple gene-specific priming sequences, a modular oligonucleotide synthesis approach was utilized, as previously described by our lab.[16] The reverse primer was labeled with biotin to facilitate purification of the round 2 PCR products. Forward primer sequences are provided in **Supplementary Table S8**, and the reverse primer sequence is provided below:

Biotin-CAAGCAGAAGACGGCATACGAGAT[10 nt barcode]GTGACTGGAGTTCAGACGTGTGCT

The Round 2 PCR cocktail consisted of the following components: (1) 1 x Phusion buffer HF, (2) 200 mM of each dNTP, (3) a mixture of 43 nested forward primers, 100 nM each, and (4) molecular grade water as needed to achieve a total volume of 30 µL. After elution of the single-stranded product from the first PCR round into the above cocktail, the biotinylated reverse primer was added to achieve a final concentration of 200 nM. Finally, Phusion Hot Start DNA polymerase was added to the cocktail to a final concentration of 0.04 units per µL. Thermal cycling conditions were identical to those used in the first round of PCR, except that the reaction was slowly cooled from 68°C down to 61°C in step (3), and was held at 60°C for 2 minutes in

step (4). A total of 3 PCR cycles were performed. The steps of reaction termination, streptavidin bead binding, washing, and elution were all carried out in the same way as after the first PCR round.

Finally, a third round of PCR was performed to amplify the DNA molecules that were specifically tagged, copied, and purified in the first 2 rounds of PCR. The third PCR round used universal forward and reverse primers, and was carried out for 27 cycles. Since the products of the second round contained dual sample-specific barcodes, they could be pooled prior to amplification in the third PCR round. The final amplification products contained all necessary adapter sequences for paired-end sequencing on the Illumina platform. The forward and reverse primer sequences, respectively, were:

Forward:

AATGATACGGCGACCACCGAGATCTACAC[6 nt barcode]ACACTCTTTCCCTACACGACGCT

Reverse:

CTCGTATGCCGTCTTCTGCTTGA

The round 3 PCR cocktail consisted of: (1) 1 x Phusion buffer HF, (2) 200 mM of each dNTP, (3) forward and reverse universal primers, 500 nM each, and (4) molecular grade water as needed to achieve a total volume of 30 µL per sample. The products of the second PCR round were eluted directly from streptavidin beads into this cocktail by heat denaturation, as was done previously. Phusion Hot Start DNA polymerase was then added to a final concentration of 0.04 units per µL. Thermal cycling parameters were: (1) 98^{o}C for 30 seconds, (2) 98^{o}C for 10 seconds, (3) 62^{o}C for 50 seconds, (4) 72^{o}C for 20 seconds, and (5) repeat steps 2 to 4 for 27 cycles total. EDTA was added to terminate the reaction (10 mM final concentration).

Preparation of PCR products for next-generation sequencing:

The pooled PCR reaction products were purified on a 2% agarose gel. Since the products were not of a homogeneous length, a gel piece was cut that included a diffuse band

centered at ~250 bp with a few millimeters margin above and below to ensure inclusion of any low-intensity products that may have been difficult to visualize. DNA was extracted from the gel slice using a QIAquick Gel Extraction kit (Qiagen), and was eluted into 50 μL of EB buffer.

Next-generation sequencing:

To prepare the gel-extracted DNA for sequencing, its concentration was measured using an Agilent Bioanalyzer and qPCR. The DNA was diluted to the concentration recommended for loading onto an Illumina HiSeq 2500 flow cell. Amplicons from up to 100 individual patient samples (carrying dual sample-specific barcodes) were multiplexed onto a single lane of a flow cell. Sequencing was performed in 75 base-pair paired-end mode on an Illumina HiSeq 2500. The length of the first index read was increased from the standard 7 nucleotides up to 11 nucleotides so that our longer custom barcodes could be appropriately read.

Sequence analysis and error suppression:

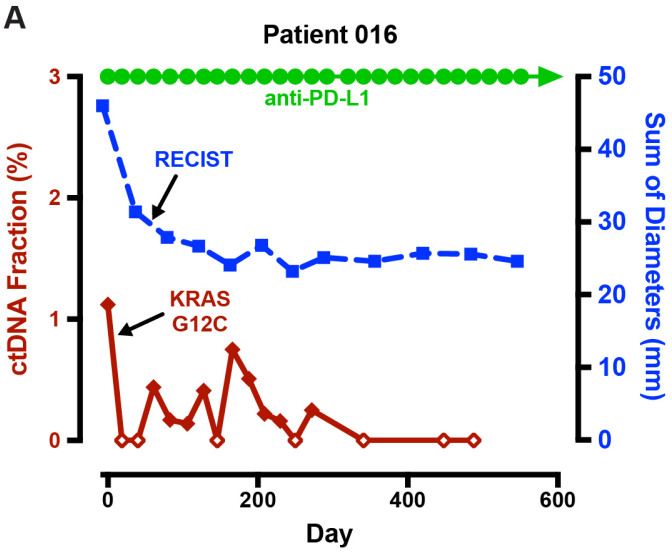
A custom computer code was written to filter, assort, align, and enumerate the millions of paired-end reads generated by the sequencer. First, a read pair was assigned to a sample-specific data bin based on the identity of sample-specific barcodes. Then, the pair was assigned to one of the reference targets based on PCR primer sequences. Next, the longest stretch of perfect sequence agreement between each pair of reads was determined, and this was used to align the reads to the target reference sequence. A read pair was discarded if either member did not pass Illumina filtering; if a nucleotide was reported to be "."; if sample-specific barcodes were inconsistent; or if the region of sequence agreement was less than 36 contiguous nucleotides in length. Variant sequences and wild-type sequences that were confirmed by reads from both strands were identified and counted within each data bin. Finally, true plasma-derived mutations were distinguished from probable PCR artifacts based on analysis of the distribution of molecular lineage tags (MLTs). A variant sequence was more

likely to be derived from a true mutant template molecule if it was associated with a greater number of distinct MLT sequences, each having multiple copies. For pre-treatment plasma samples, a variant was considered to be a candidate for longitudinal tracking if it was supported by at least 4 distinct MLT sequences, each having 2 or more copies. If more than one mutation was found at baseline, the one having the highest allelic fraction was chosen for tracking. Since a specific mutation was being measured in post-treatment samples, a lower detectability threshold could be applied: at least 2 distinct MLT sequences, each having 2 or more copies, were required. The allelic fraction of the mutant sequence was determined by calculating the ratio of mutant to total (mutant plus wild-type) sequences, post-filtration, within a given sample/target data bin.

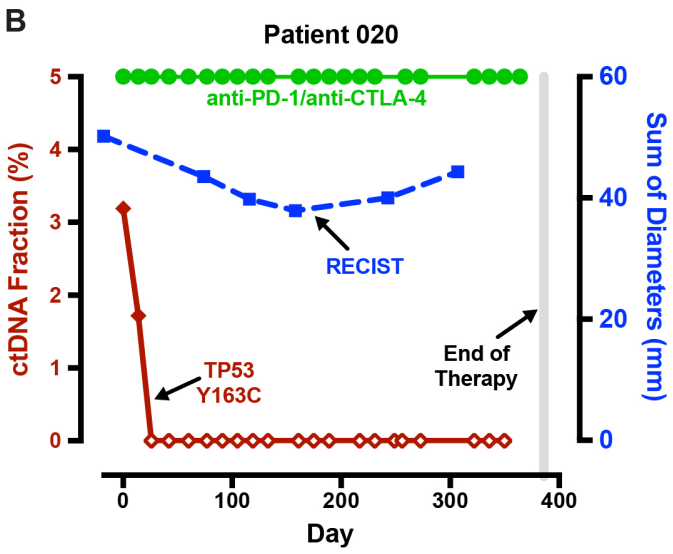
Supplementary Figure S1: Radiographic and ctDNA Measurements for Remaining Patient Cases

A-Z, Levels of mutant circulating tumor DNA (ctDNA), measurements of radiographic tumor burden, and the type and duration of immunotherapy received are plotted for the remaining 26 patients in the study that were not shown in Figure 1. Open diamonds indicate undetectable ctDNA. Below each individual patient graph is a summary of the patient's clinical course. Patient cases are ordered by time on treatment, matching the order in Figure 3.

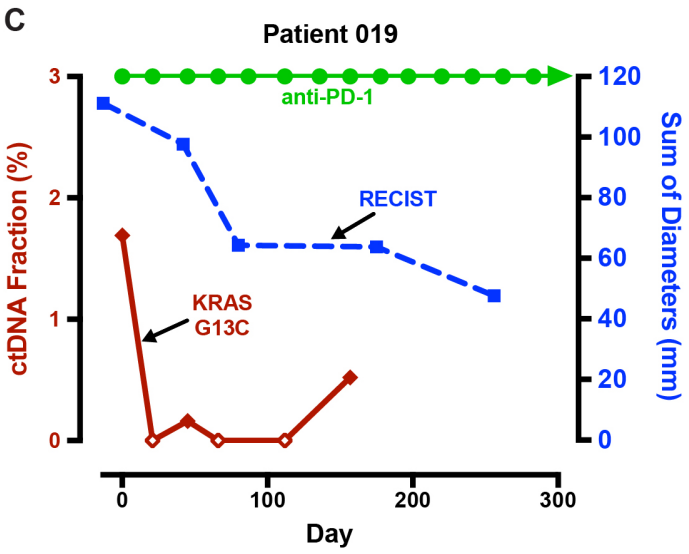
Fig. S1



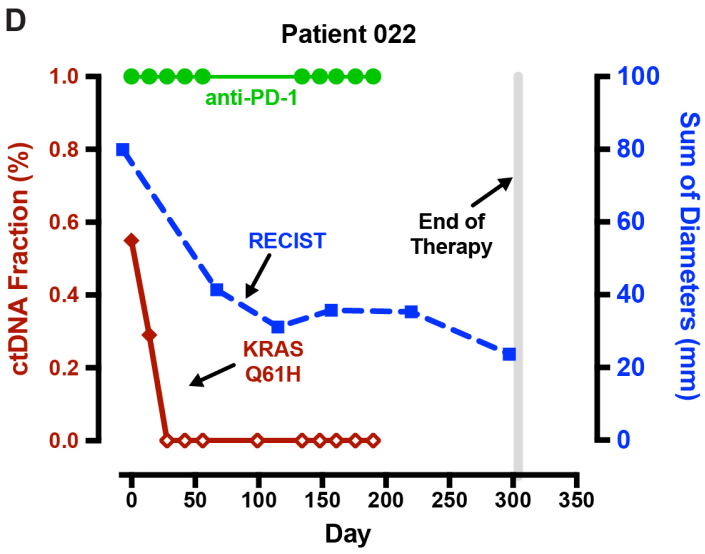
75 yo female with stage IV poorly differentiated lung adenocarcinoma. Anti-PD-L1 immunotherapy was started as first-line therapy (day 0). A ctDNA response was noted on day 19 and radiographic response was noted on day 37. She received a total of 28 cycles and was continuing therapy as of the time of analysis.



69 yo male with initial stage IIB lung squamous cell carcinoma. He was not a surgical candidate and received SBRT (stereotactic body radiotherapy). Metastatic disease was diagnosed approximately 2 years following initial diagnosis. Anti-PD-1/anti-CTLA-4 immunotherapy was initiated (day 0) as first-line treatment. A ctDNA response was seen on day 26 although radiographic response was not achieved. Therapy was stopped on the date of death (day 386), due to pulmonary embolism and perforated bowel.

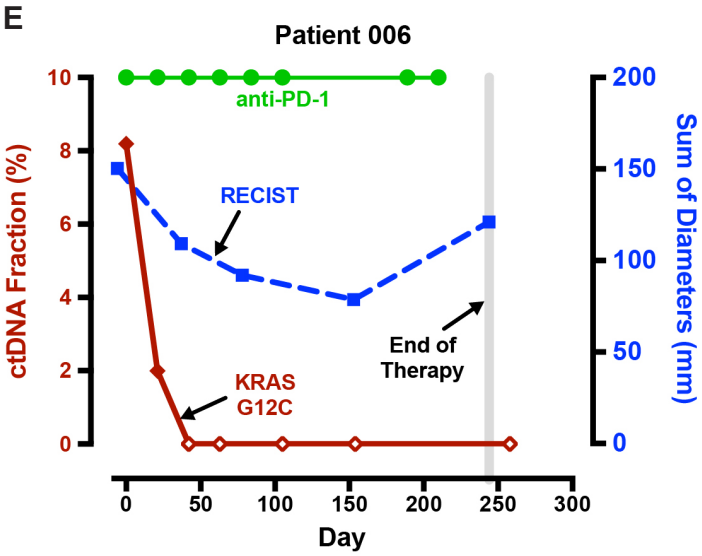


74 yo male initially diagnosed with stage I lung adenocarcinoma for which he underwent wedge resection. No adjuvant therapy was recommended. Ten years later he developed recurrence with metastatic disease. First-line anti-PD-1 immunotherapy was started (day 0). On day 21, ctDNA response was noted, followed by radiographic response on day 80. He remained on treatment as of the time of analysis.

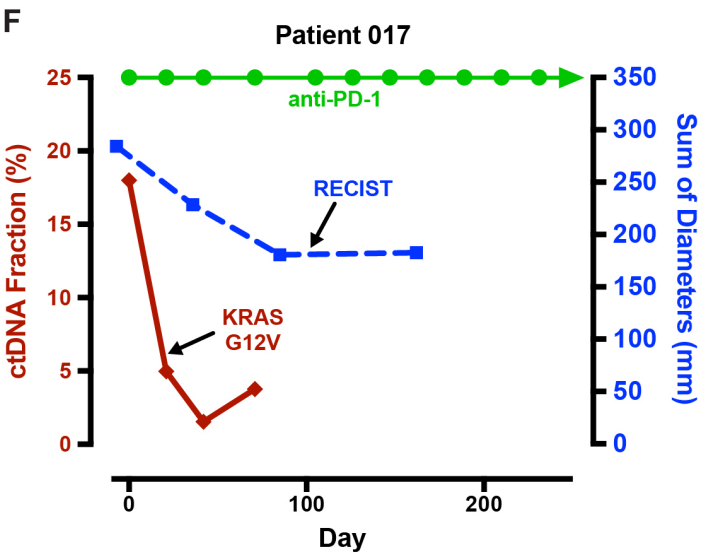


59 yo female with stage IV lung adenocarcinoma at diagnosis. Intracranial stereotactic radiosurgery was performed (day -34, not shown) followed by anti-PD-1/anti-CTLA-4 immunotherapy as first-line systemic therapy (day 0). A ctDNA response was noted on day 28 followed by radiographic response on day 67. The patient developed intracranial progression and immunotherapy was ultimately stopped on day 304.

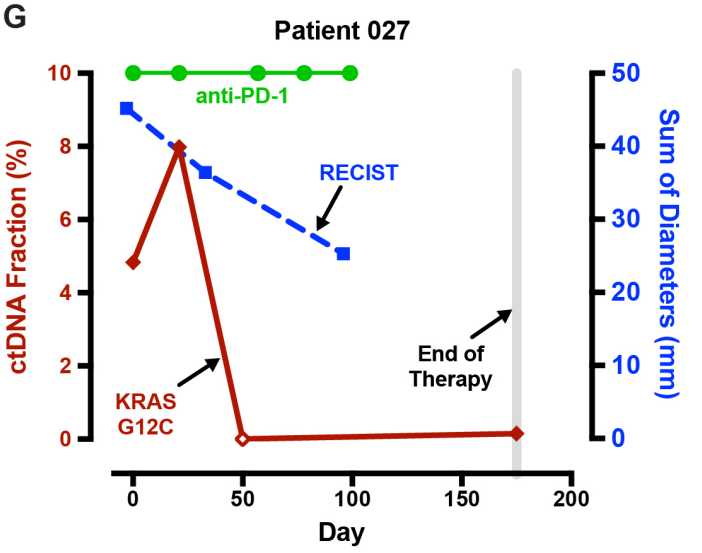
Fig. S1 (continued)



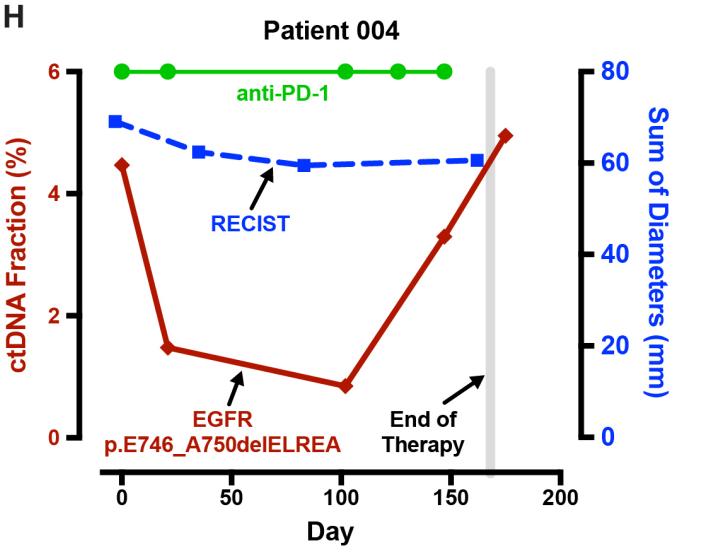
67 yo male with stage IV NSCLC. Anti-PD-1 immunotherapy was started as first-line therapy. CtDNA response was noted on day 21, followed by radiographic response on day 78. A total of 8 cycles were delivered. Intrathoracic disease progression was noted radiographically on day 244 and treatment was stopped.



76 yo male with stage IV poorly differentiated NSCLC. Anti-PD-1 immunotherapy was initiated as first-line therapy (day 0) and continued for 11 cycles without evidence of disease progression. A ctDNA response was noted on day 21 followed by radiographic response on day 85. Therapy was continuing as of the time of analysis.



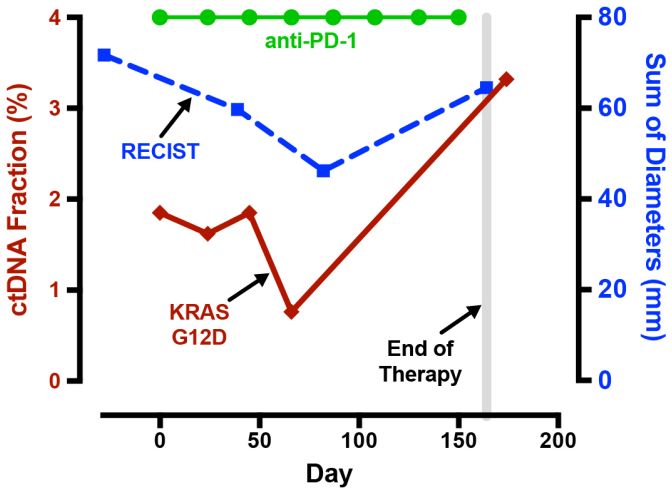
58 yo male with initial stage IIIB disease lung adenocarcinoma. After completing definitive chemoradiation he developed metastatic disease. Anti-PD-1 immunotherapy was started as first-line systemic therapy (day 0). CtDNA and radiographic response criteria were met on day 50 and day 96, respectively. He received a total of 5 cycles but developed colitis thought to be secondary to immunotherapy. Following a prolonged delay his therapy was ultimately discontinued (day 175).



60 yo female with stage IV lung adenocarcinoma with EGFR exon 19 deletion. She initially received multiple courses of systemic chemotherapy, targeted anti-EGFR therapy, and intracranial radiation. Anti-PD-1 immunotherapy was initiated (day 0) approximately 2 years after initial diagnosis. A ctDNA response was noted on day 21 without corresponding radiographic response. Anti-PD-1 therapy was given for a total of 5 cycles until intrathoracic disease progression was noted and therapy was stopped (day 168).

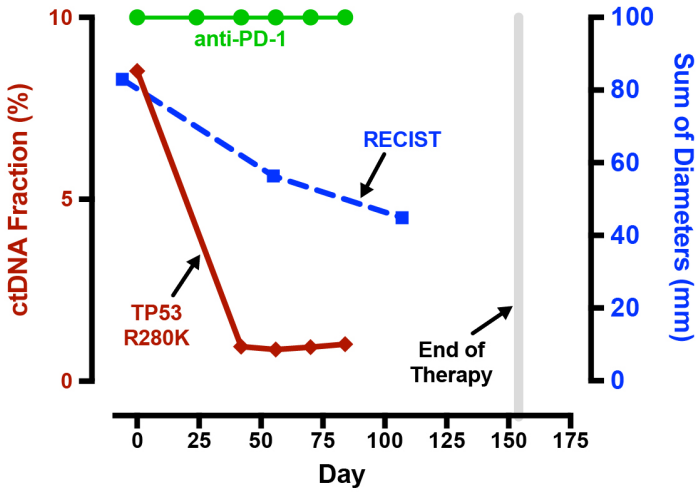
Fig. S1 (continued)

I



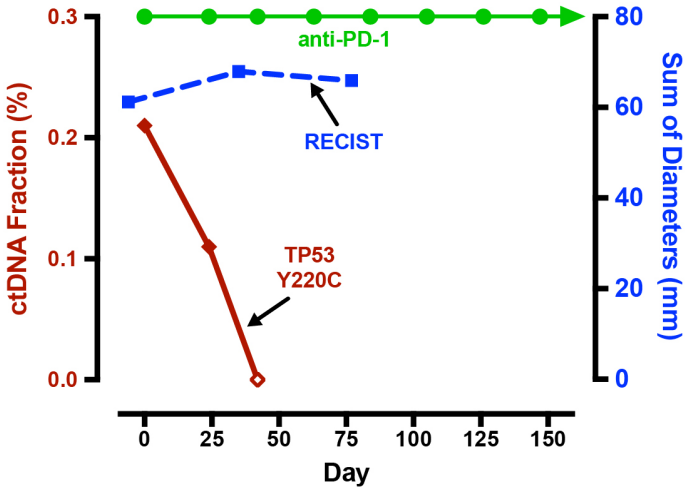
87 yo female with stage IV lung adenocarcinoma. Anti-PD-1 immunotherapy was started as first-line therapy (day 0). She received a total of 8 cycles without meeting ctDNA or radiographic response criteria. Progression was noted in multiple sites and therapy was stopped (day 164).

J



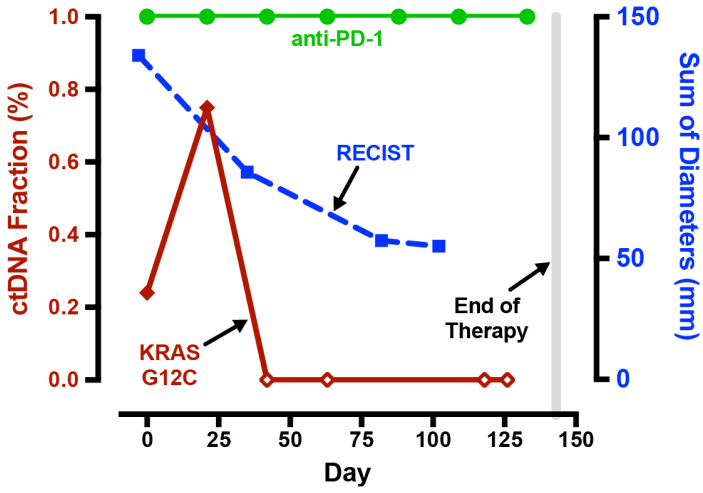
33 yo female with a long history of ALK-rearranged stage IV lung adenocarcinoma, initially diagnosed 6 years prior to the current study. She received multiple courses of systemic chemotherapy in combination with a tyrosine kinase inhibitor, as well as several courses of intracranial radiation therapy. Further disease progression was noted and anti-PD-1 immunotherapy was initiated (day 0). A ctDNA response was noted on day 42 followed by radiographic response on day 55. She developed progressive intracranial metastases and was ultimately taken off therapy (day 154).

K



73 yo male with stage IV lung adenocarcinoma. Anti-PD-1 immunotherapy was started as first-line therapy (day 0). Radiographic and ctDNA response criteria were not met (a confirmatory ctDNA measurement was not available). Treatment was continuing as of the time of analysis.

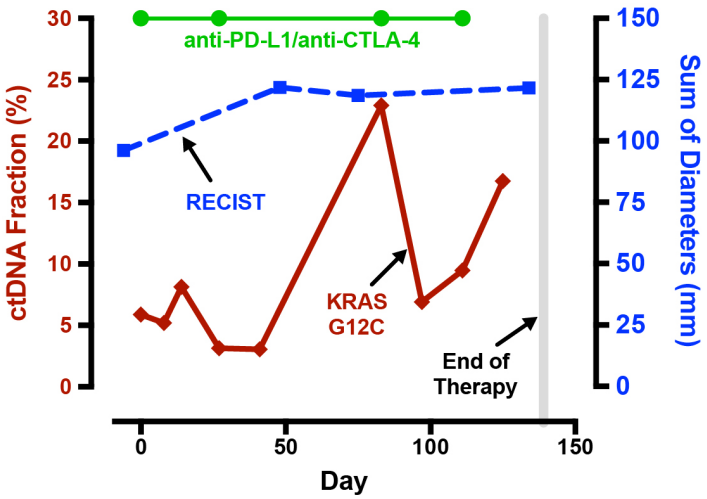
L



66 yo female with stage IV lung adenocarcinoma. Anti-PD-1 immunotherapy was initiated as first-line therapy (day 0). A radiographic response was noted on day 35 followed by ctDNA response on day 42. She received a total of of 7 cycles prior to being taken off treatment (day 143).

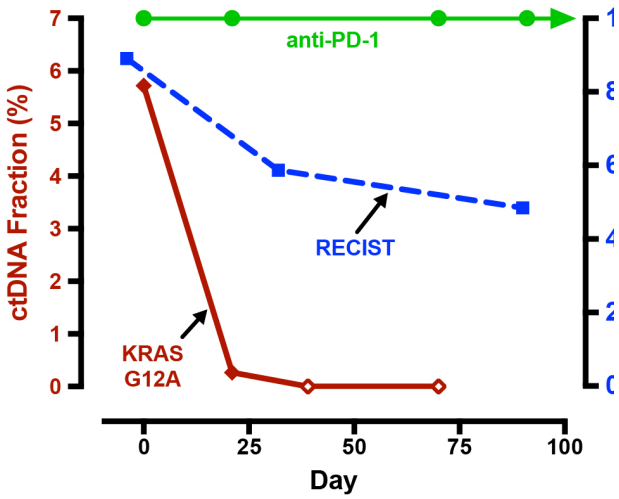
Fig. S1 (continued)

M



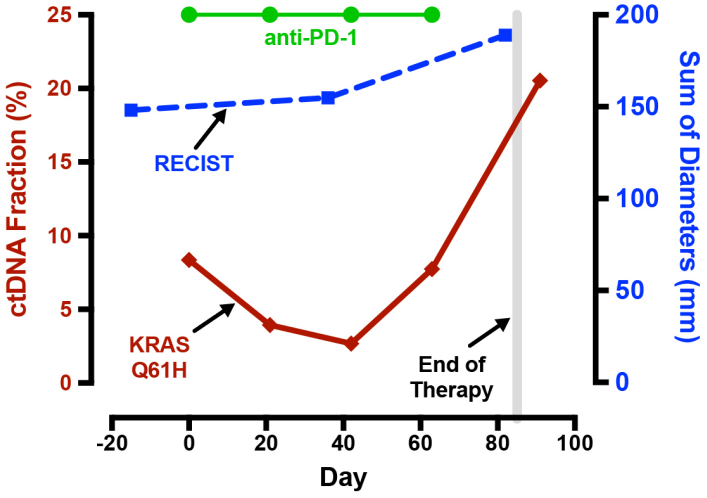
67 yo female initially diagnosed with stage IV lung adenocarcinoma. Initial therapy included two lines of systemic chemotherapy in combination with anti-VEGF therapy, followed by additional systemic chemotherapy alone. Approximately 2 years after diagnosis, she began anti-PD-L1/ anti-CTLA-4 immunotherapy (day 0). Immunotherapy was stopped on day 139 due to systemic and intracranial disease progression. There was no radiographic or ctDNA response during treatment.

N



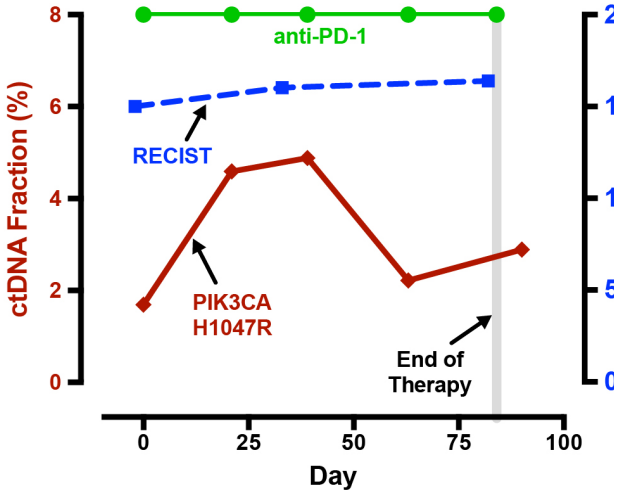
52 yo male with stage IV poorly differentiated NSCLC. H underwent stereotactic radiosurgery followed by anti-PD immunotherapy as first-line systemic therapy (day 0). A c response was noted on day 21, followed by radiographic response on day 32. Treatment was continuing as of the of analysis.

O



61 yo male initially diagnosed with stage IIIA lung adenocarcinoma, s/p definitive chemoradiation and consolidative chemotherapy who then developed metastases. He had further progression on chemotherapy and anti-PD-1 immunotherapy was initiated as second-line therapy (day 0). A total of 4 cycles were given. A short-lived ctDNA response was achieved on day 21 without corresponding radiographic response. CtDNA response ended prior to widespread disease progression (day 82).

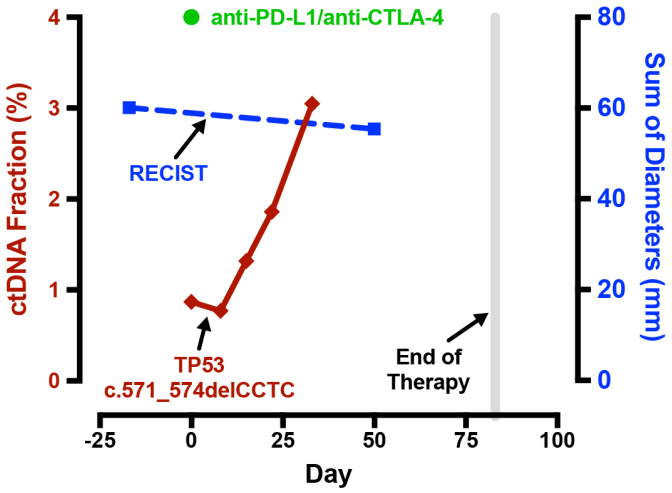
P



58 yo male with stage IV poorly differentiated lung adenocarcinoma. He began systemic chemotherapy and received palliative radiation. Following development of progressive disease, anti-PD-1 immunotherapy was star (day 0). Following 5 cycles of immunotherapy, there was questionable progression of his adrenal gland. He was ta therapy (day 84) without meeting criteria for ctDNA or radiographic response.

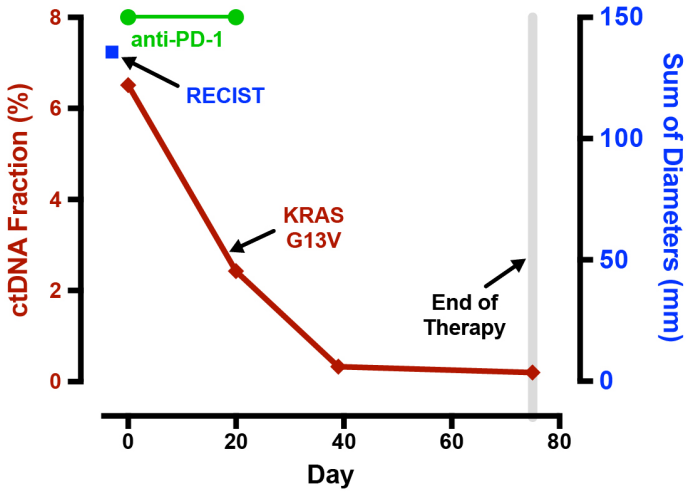
Fig. S1 (continued)

Q



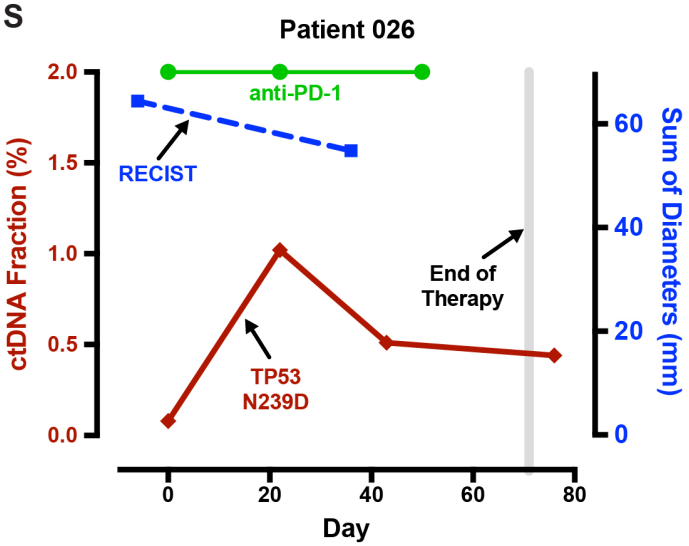
62 yo male initially diagnosed with stage IIIA lung adenocarcinoma approximately 2.5 years prior to the current study. He was treated with definitive chemoradiation followed by consolidative chemotherapy. One year later he developed a metastasis to the pancreas for which stereotactic body radiotherapy (SBRT) was recommended. Following SBRT, new metastases developed and anti-PD-L1/anti-CTLA-4 therapy was initiated. Only 1 cycle was given (day 0) due to development of pancreatitis followed by further disease progression. There was no evidence of ctDNA or radiographic response. Therapy was stopped on (day 83).

R



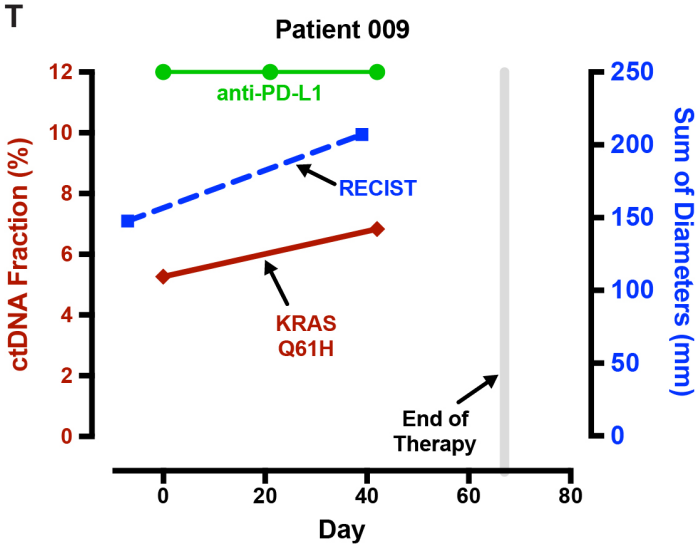
59 yo male with stage IV lung adenocarcinoma. Initial treatment included intracranial stereotactic radiosurgery and systemic chemotherapy, followed by repeat radiosurgery. At progression he began therapy with anti-PD-1 immunotherapy (day 0). A ctDNA response was noted on day 20. On restaging CT scan, a RECIST sum of tumor diameters was unable to be calculated due to difficulty measuring tumor size. Immunotherapy was stopped on day 75.

S



60 yo male with stage IV NSCLC who began anti-PD-1 immunotherapy (day 0) as first-line treatment. A total of 3 cycles were delivered but therapy was stopped (day 71) due to worsening skeletal involvement. Radiographic and ctDNA response criteria were not met.

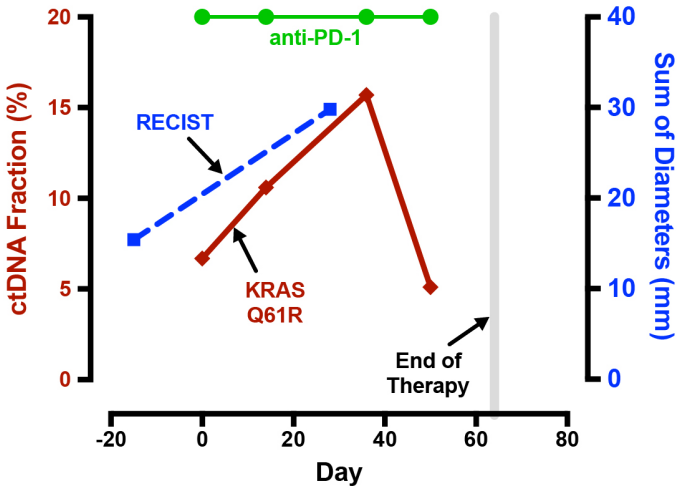
T



61 yo female with stage IV NSCLC. She initially received systemic chemotherapy and radiation to the cervical spine but her disease progressed. Approximately 6 months following diagnosis, anti-PD-L1 immunotherapy was initiated (day 0). Mediastinal progression was noted and therapy was stopped (day 67). Radiographic and ctDNA responses were not seen during therapy.

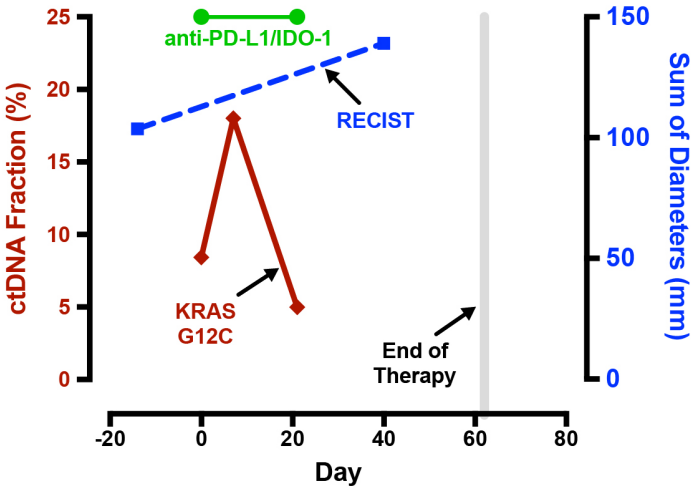
Fig. S1 (continued)

U



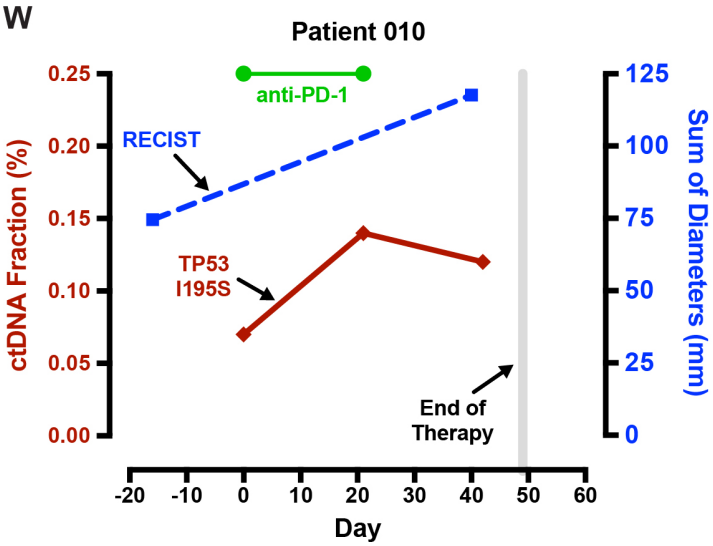
81 yo female with stage IV lung adenocarcinoma. She was initially treated with systemic chemotherapy and anti-PD-1 immunotherapy was initiated as second-line therapy (day 0). She did not meet criteria for radiographic or ctDNA response. Prior to her 4th cycle of immunotherapy, a single fraction of palliative RT was delivered to a painful rib lesion (day 43, not shown). Shortly thereafter she was noted to have further progression and immunotherapy was stopped (day 64).

V



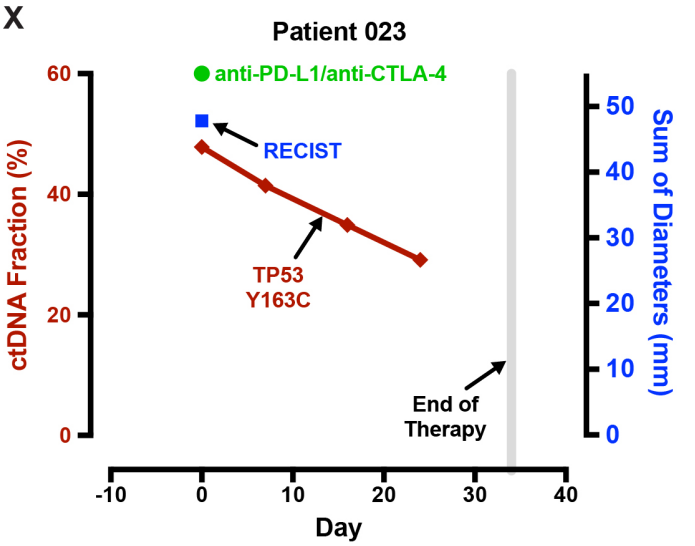
75 yo male initially diagnosed with stage IIIA NSCLC. He completed definitive concurrent chemoradiation and but developed metastatic disease and received systemic chemotherapy in combination with anti-VEGF therapy. Approximately 2 years following initial diagnosis, anti-PD-L1/ IDO-1 immunotherapy was given as second-line therapy (day 0). Two cycles were completed but disease progressed and therapy was stopped (day 62). Neither ctDNA nor radiographic responses were achieved.

W



67 yo female with stage IV moderately differentiated lung adenocarcinoma. She underwent intracranial stereotactic radiosurgery followed by systemic chemotherapy in combination with anti-VEGF therapy. Progressive intrathoracic disease was noted and anti-PD-1 immunotherapy was initiated as second-line therapy (day 0). She received 2 cycles of immunotherapy without evidence of ctDNA or radiographic response, followed by disease progression. She was taken off therapy on day 49.

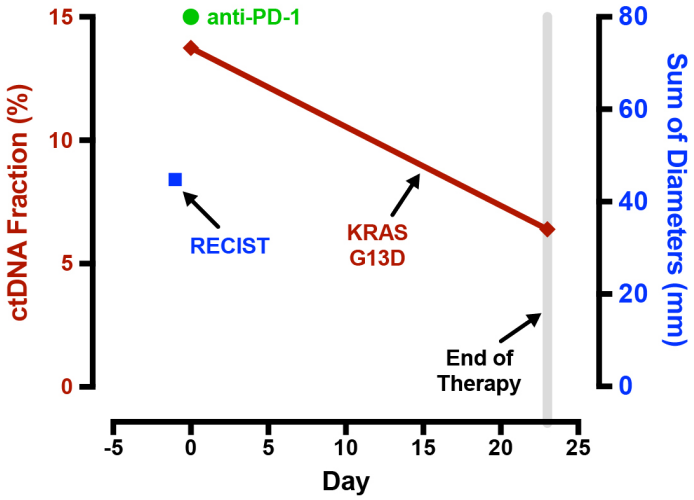
X



52 yo male with initial stage IIIB lung adenocarcinoma, status post definitive chemoradiation followed by SBRT (stereotactic body radiotherapy) for to a recurrent lung lesion. Widespread metastatic disease was later noted and systemic chemotherapy was initiated. Anti-PD-L1/anti-CTLA-4 immunotherapy was started following progression as second-line therapy (day 0) and given for 1 cycle. Only a baseline CT scan was performed, therefore radiographic response could not be formally assessed. CtDNA response criteria were not met. The patient expired on day 34.

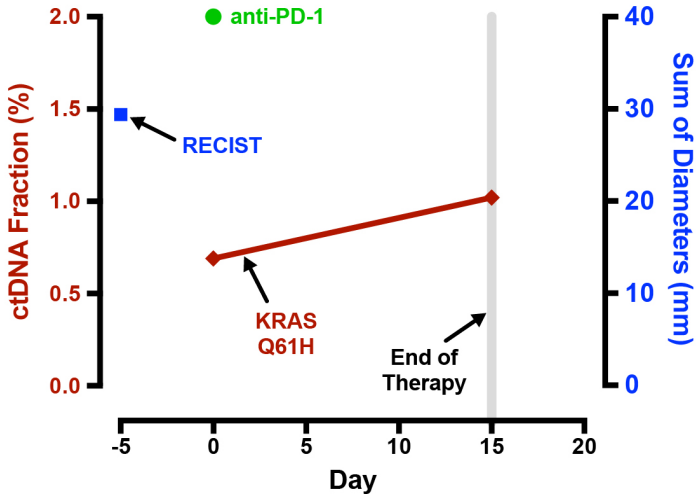
Fig. S1 (continued)

Y

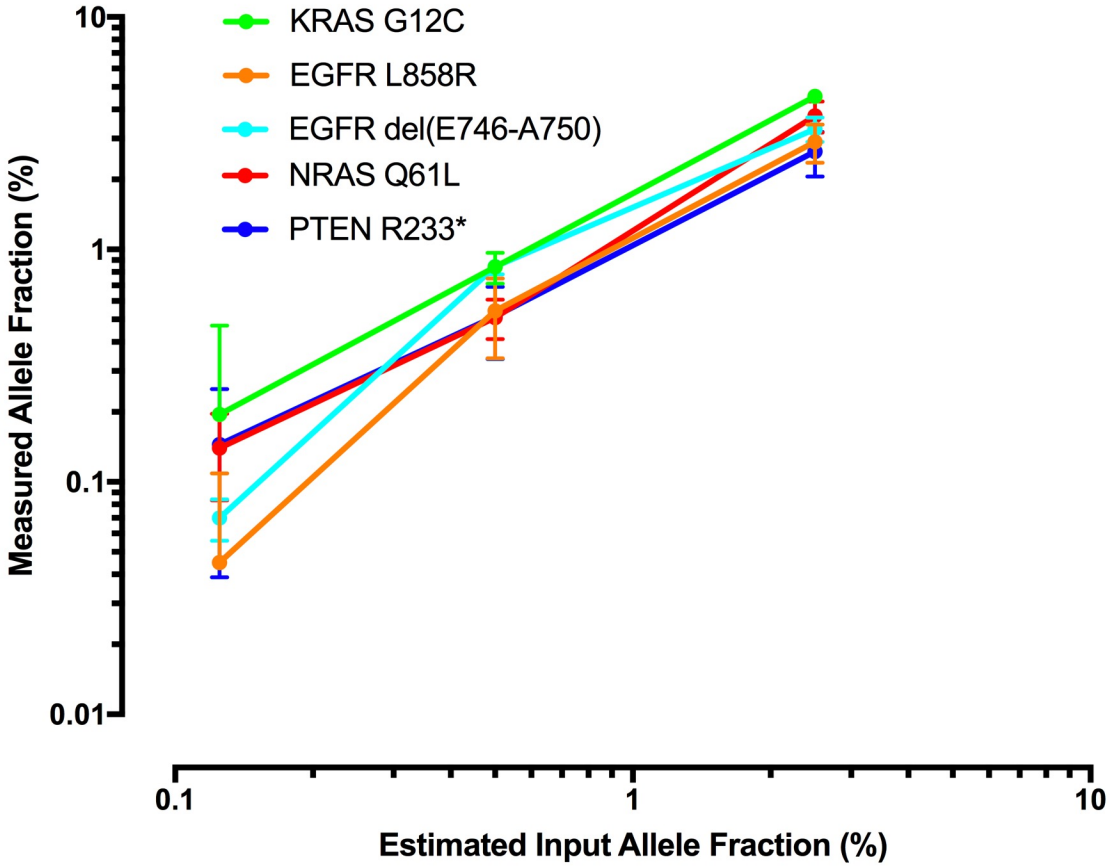


53 yo female with stage IV lung adenocarcinoma. She received 1 cycle of anti-PD-1 immunotherapy as first-line therapy (day 0) but shortly thereafter developed worsening respiratory status. She declined additional systemic treatment and was taken off therapy (day 23). Only a baseline CT scan was available. CtDNA response criteria were not met (a confirmatory ctDNA measurement was not available).

Z



77 yo female with stage IV lung adenocarcinoma. Systemic chemotherapy was initiated after which disease progression was noted. She received 1 cycle of anti-PD-1 immunotherapy (day 0) but developed symptomatic brain metastases and immunotherapy was stopped (day 15). CtDNA response criteria were not met. Only a baseline CT scan was obtained.



Supplementary Figure S2: Measurement of Serially Diluted Mutant DNA

DNA containing known mutations was spiked into 80 ng of wild-type DNA derived from human buffy coat, at predicted allele fractions of 0%, 0.125%, 0.5%, and 2.5%. The DNA was sheared using a Covaris ultrasonicator to an average fragment size of ~180 base pairs. The mutant allele fraction was then measured from duplicate samples using the enhanced Error Suppressed Deep Sequencing assay. Samples in which no mutant DNA was added all had measured allele fractions of 0 (this cannot be displayed on logarithmic axes). Mutant DNA was derived from commercially available DNA standards obtained from Horizon Discovery, HD-C318 (EGFR L858R), HD-C319 (KRAS G12C), HD-C320 (EGFR del[E746-A750]) and from cell lines SF-295 (PTEN R233*) and HL-60 (NRAS Q61L). Error bars indicate standard deviation. It should be noted that we used 80 ng of DNA to demonstrate the measurement of a broad range of allele fractions. However, because these test samples contained ~10-fold more DNA than the typical patient samples, the measurements cannot be directly extrapolated to the clinical data. We estimate that a typical 1 mL clinical sample would contain ~1500-3000 genome equivalents of cell-free DNA. Based on MLT thresholds set in our computational algorithm, we expect to be able to detect ~2-3 mutant molecules in such a sample. Thus, we estimate that the limit of detection for a 1 mL plasma sample would be in the range of ~0.1-0.5%.

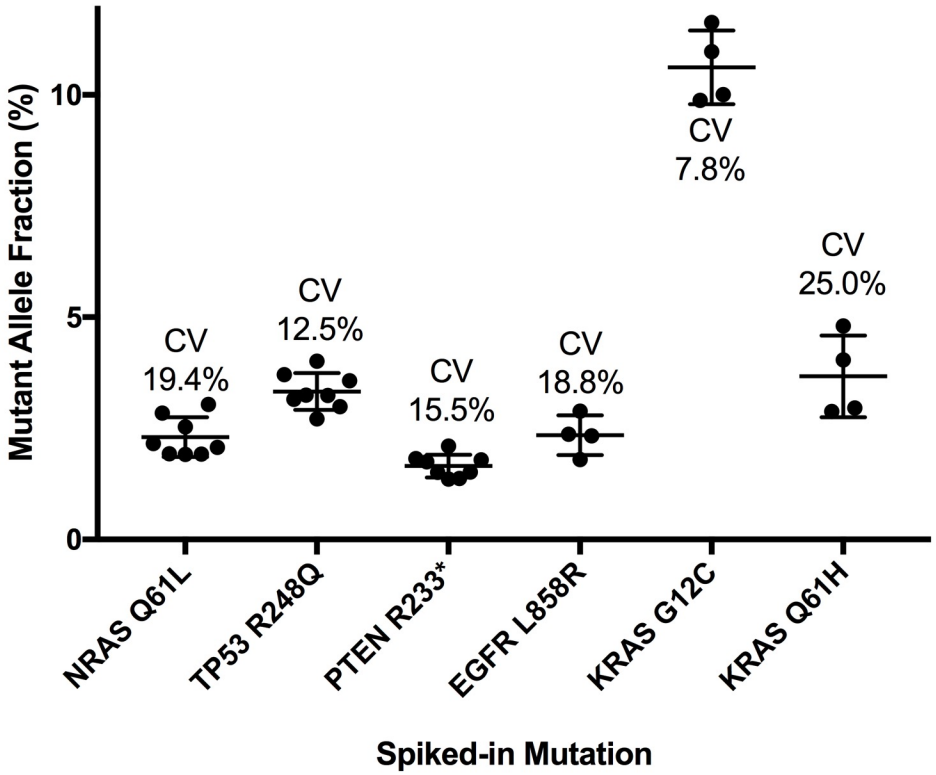
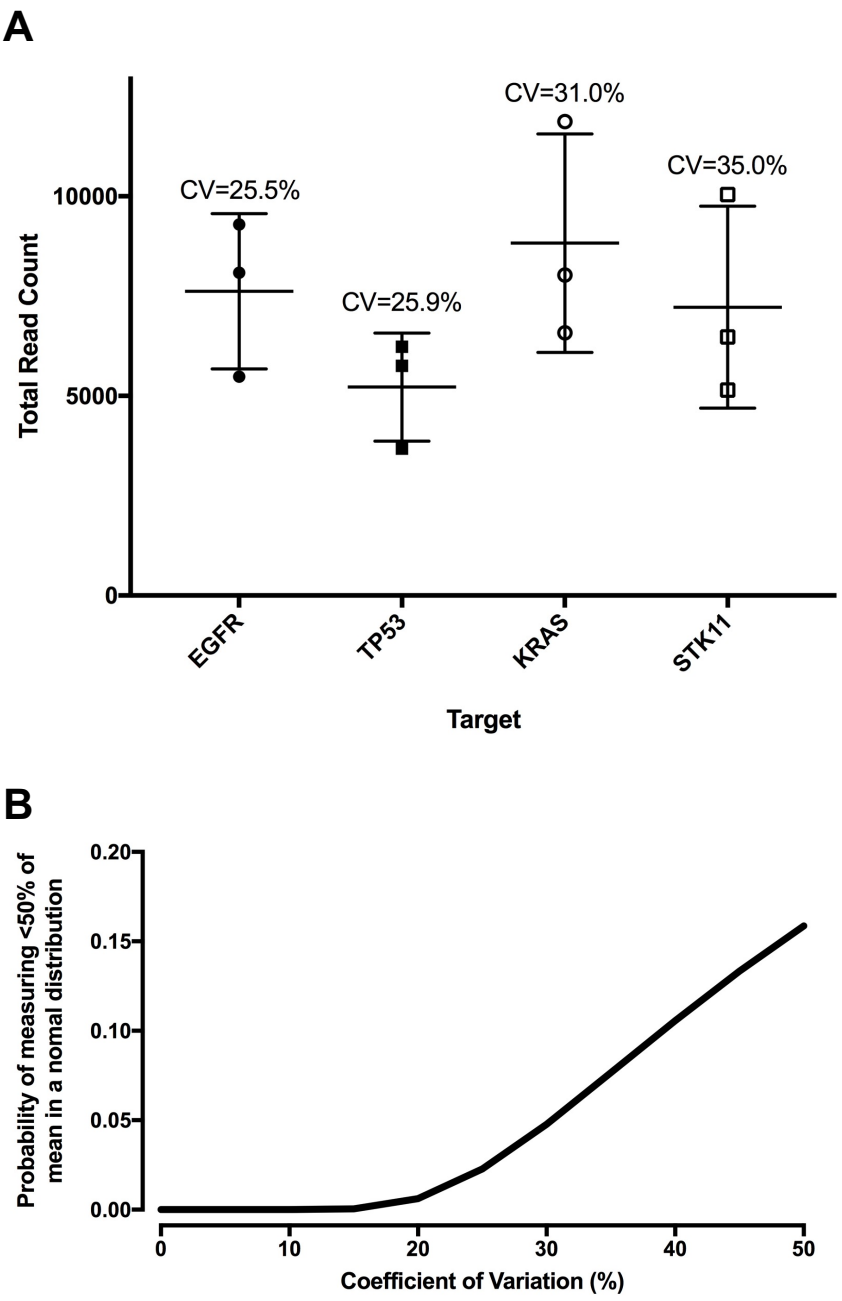


Fig S3. Technical Reproducibility of Assay

Mutant DNA derived from cell lines SF-295 and HL-60 (for mutations NRAS Q61L, TP53 R248Q, and PTEN R233*) or from DNA standards obtained from Horizon Discovery (for mutations EGFR L858R, KRAS G12C, and KRAS Q61H) was spiked into ~20 ng buffy coat DNA from a healthy volunteer which was sheared using a Covaris ultrasonicator to an average size of ~180 base pairs. The predicted allele fraction based on estimated quantities of wild-type and mutant DNA was 2.5% for NRAS, TP53, and PTEN; 3% for EGFR and KRAS Q61H; and 10% for KRAS G12C. Each dot represents a measurement of mutant allele fraction from a replicate sample. Mean and standard deviation are indicated for each set of replicate measurements. CV, coefficient of variation.



Supplementary Figure S4: Read Count Reproducibility of DNA Purification Replicates

(A) Total sequencing read counts are reported for 4 different targets measured from 3 replicate 1 mL human plasma samples (aliquots from a single blood draw of a healthy volunteer). DNA samples were extracted from each plasma aliquot separately using identical kits, were processed in parallel but with differently barcoded primers, and were sequenced on a single lane of an Illumina HiSeq flow cell. Mean and standard deviation are indicated for each set of replicate measurements. CV, coefficient of variation. Of note, CV values above are shown for total sequencing read counts (reflecting yield of cell-free DNA in purification replicates), whereas clinical samples are measured as mutant allele fractions. **(B)** A graph illustrating the relationship between coefficient of variation and the probability that a measurement could be less than 50% of the mean in a normally distributed data set (our threshold for ctDNA response).

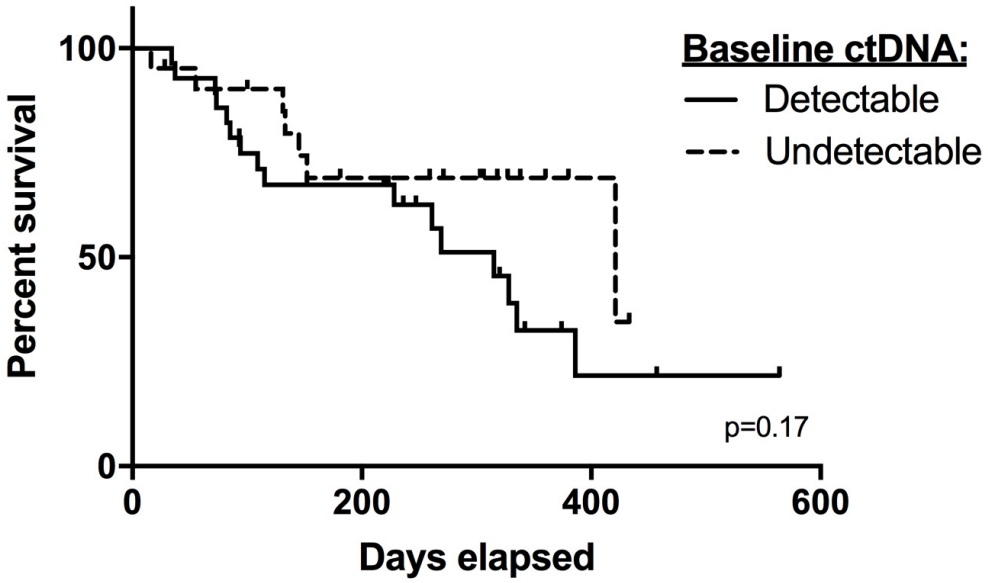
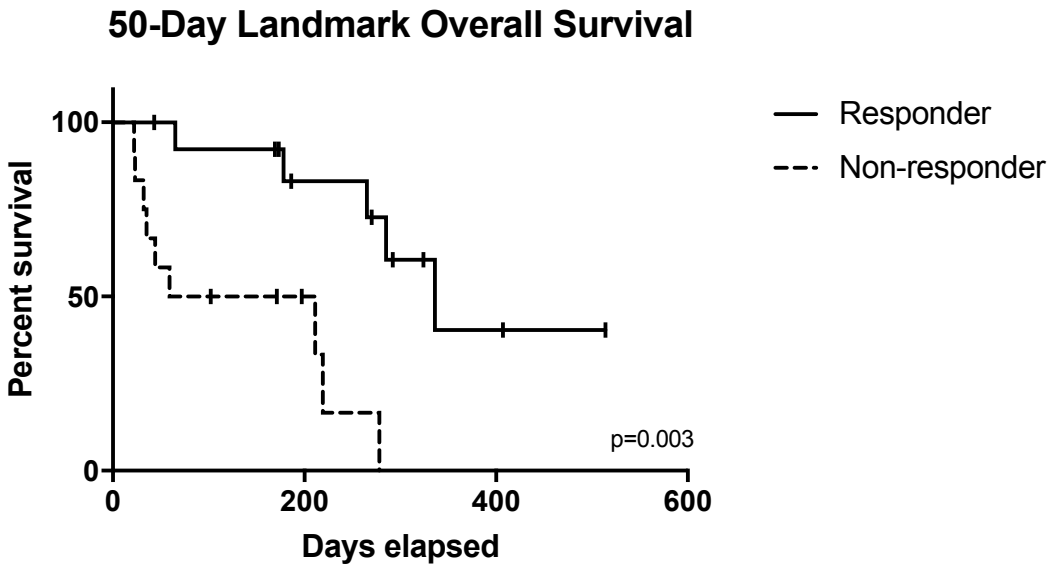


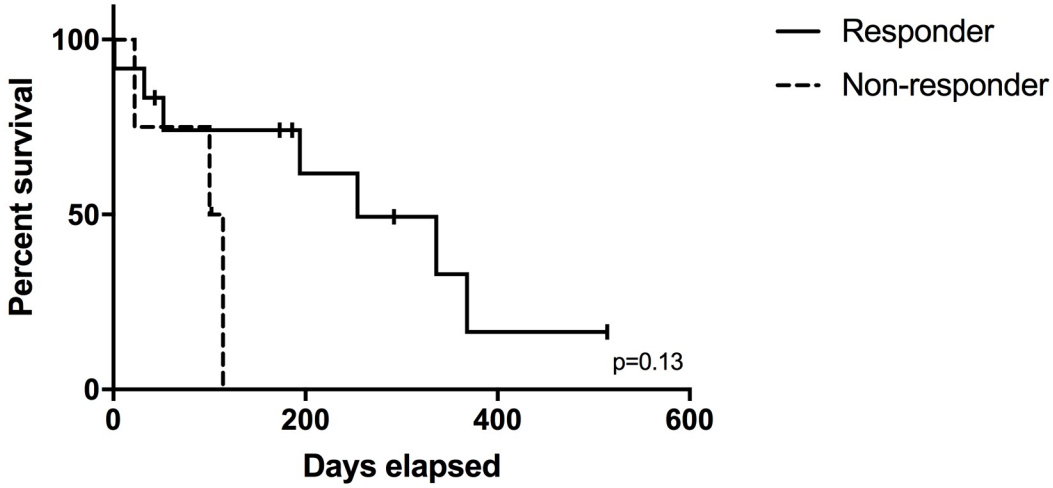
Fig S5. Comparison of Overall Survival Between Patients with Detectable vs. Undetectable Baseline ctDNA

Kaplan-Meier analysis of overall survival between patients with detectable baseline ctDNA who were included in the study (n = 28) and patients without detectable baseline ctDNA who were excluded (n = 21).

A



B



Supplementary Figure S6. Landmark Analysis of Overall and Progression-Free Survival According to ctDNA Response

Kaplan-Meier curves showing (A) overall survival and (B) progression-free survival using a landmark analysis. Patients with death or censoring during first 50 days were excluded (2 out of 28 excluded for OS and 12 out of 28 excluded for PFS analysis). Landmark status was determined by ctDNA change during first 50 days.

Supplementary Table S1. List of Genes and Corresponding Genomic Positions Queried

Gene Name	Chromosome	Genomic Position[a]
AKT1	Chr. 14	104780210 - 104780217
APC	Chr. 5	112839939 - 112839947
BRAF	Chr. 7	140753328 - 140753343
CDKN2A	Chr. 9	21971169 - 21971195
		21970993 - 21971037
CTNNB1	Chr. 3	41224605 - 41224647
EGFR	Chr. 7	55174764 - 55174801
		55191819 - 55191836
		55181370 - 55181382
EZH2	Chr. 7	148811633 - 148811655
FGFR3	Chr. 4	1806587 - 1806610
		1801828 - 1801845
		1804358 - 1804377
FLT3	Chr. 13	28018499 - 28018508
FOXL2	Chr. 3	138946309 - 138946329
GNAS	Chr. 20	58909358 - 58909370
HRAS	Chr. 11	534282 - 534307
		533862 - 533875
IDH1	Chr. 2	208248385 - 208248394
JAK2	Chr. 9	5073766 - 5073775
KIT	Chr. 4	54733150 - 54733165
		54727417 - 54727452
KRAS	Chr. 12	25245344 - 25245360
		25227327 - 25227343
MET	Chr. 7	116700202 - 116700217
		116783357 - 116783377
MYD88	Chr. 3	38141147 - 38141156
NRAS	Chr. 1	114716120 - 114716148
		114713887 - 114713909
PIK3CA	Chr. 3	179218290 - 179218310
		179234288 - 179234305
PPP2R1A	Chr. 19	52212716 - 52212734
		52213067 - 52213082
PTEN	Chr. 10	87933144 - 87933167
		87957910 - 87957928
STK11	Chr. 19	1207017 - 1207045
		1223101 - 1223128
TP53	Chr. 17	7675124 - 7675162
		7675064 - 7675095
		7674934 - 7674965
		7674871 - 7674896
		7674215 - 7674263
		7673763 - 7673806

[a] Based on human genome assembly GRCh38/hg38

Supplementary Table S2. Characteristics of Patients Undergoing Longitudinal ctDNA Measurement

Characteristic	N = 28
Age, median (range), years	67 (33-89)
Sex, No. (%)	
Female	14 (50)
Male	14 (50)
Smoking status, No. (%)	
Former	26 (93)
Never	2 (7)
Current	0
Tumor histology, No. (%)	
Non-squamous NSCLC	27 (96)
Squamous cell carcinoma	1 (4)
Group stage at initial diagnosis, No. (%)*	
I	1 (4)
II	1 (4)
III	5 (18)
IV	21 (75)
Time since initial diagnosis, median (range), days**	215 (25-3676)
Treatment, No. (%)	
Single-agent PD-1 or PD-L1 inhibitor	22 (79)
Combination of immunotherapeutic agents	6 (21)

Abbreviations: ctDNA, circulating tumor DNA; NSCLC, non-small cell lung cancer; PD-1, programmed cell death 1; PD-L1, programmed death ligand 1

* Percentages do not total 100 because of rounding.

** Day of diagnosis was unknown for two patients and was imputed as mid-month

Supplementary Table S3. (*see accompanying Excel spreadsheet*) Detailed Individual Characteristics of 49 Enrolled Patients

Supplementary Table S4. Comparison of Baseline Characteristics Between Patients with Detectable vs. Undetectable ctDNA

Characteristic	Detectable ctDNA (n=28)	Undetectable ctDNA (n=21)	Total (n=49)	p-value
Age, median (IQR)	66.50 (59.00 – 74.50)	69.00 (62.00 – 75.00)	67.00 (60.00 – 75.00)	0.52
Gender, n (%)				0.026
Female	14 (50.0%)	17 (81.0%)	31 (63.3%)	
Male	14 (50.0%)	4 (19.0%)	18 (36.7%)	
Histology, n (%)				1.00
Adenocarcinoma	27 (96.4%)	20 (95.2%)	47 (95.9%)	
Squamous cell carcinoma	1 (3.6%)	1 (4.8%)	2 (4.1%)	
Smoking Status, n (%)				0.36
Current	0 (0.0%)	1 (4.8%)	1 (2.0%)	
Former	26 (92.9%)	17 (81.0%)	43 (87.8%)	
Never	2 (7.1%)	3 (14.3%)	5 (10.2%)	
Smoking Pack-years, median (IQR)	30.00 (17.50 – 42.50)	25.00 (15.00 – 40.00)	30.00 (15.00 – 40.00)	0.56
RECIST tumor burden at baseline (mm), median (IQR)	77.25 (55.15 – 122.60)	54.00 (44.90 – 94.00)	70.40 (45.80 – 105.95)	0.12

* Wilcoxon rank sum test for continuous variables, Chi-square or Fisher's exact test for categorical variables
* One undetectable ctDNA participant missing RECIST tumor burden at baseline measurement

Supplementary Table S5. (*see accompanying Excel spreadsheet*) Table of Mutant Allele Fractions

Supplementary Table S6. Mutations Identified in Available Tumor Biopsy Specimens

Patient No.	Mutation
001	KRAS G12V
002	KRAS G12D
005	KRAS G12A
006	KRAS G12C
008	KRAS G12C
011	PIK3CA E542K
012	KRAS G13D
013	KRAS Q61R
016	KRAS G12C
017	KRAS G12V
018	KRAS Q61R
019	KRAS G13C
023	TP53 P72R; TP53 Y163C
027	KRAS G12C

Supplementary Table S7. List of Primers for First Round of PCR Amplification

Gene Name	Forward Primer Sequence (5' to 3')[a]	Reverse Primer Sequence (5' to 3')[b]
AKT1	X-TGAAGGTGCCATCATTCTTGAGGAG	X'-GCACCACCCGCACGTCTGTA
APC	X-CAGCTTGCTTAGGTCCACTCTCTC	X'-GACCTCCTCAAACAGCTCAAAC
BRAF	X-CTTACCATCCACAAAATGGATCCAGAC	X'-AACAGTAAAAATAGGTGATTTTGGTCTA
CDKN2A	X-CACCCTGGCTCTGACCATTCTG	X'-AGTTGGGCTCCGCGCCGTGGAGCA
	X-GGCATGGTTACTGCCTCTGGTGC	X'-GGCGCGGCTGGACGTGCGC
CTNNB1	X-AGGACTTGGGAGGTATCCACATCC	X'-CTGGCAGCAACAGTCTTACCT
EGFR	X-CCCACACAGCAAAGCAGAAAC	X'-CGGTGAGAAAGTTAAAATTCCCGTC
	X-AGCCACCTCCTTACTTTGCCTCC	X'-AAGCATGTCAAGATCACAGATTTTG
	X-GTCTTTGTGTTCCCGGACATAGTCC	X'-TGCCTCACCTCCACCGTGCA
EZH2	X-GTTAGTATATACAATGCCACCTGAATACAG	X'-TTTATCAAAGATCCTGTGCAGAA
FGFR3	X-GGGCTTGTCCATGCGGTGGCC	X'-ATGGGAGATCTTCACGCTGG
	X-CTCCACGTCGCTGCCCAGCACC	X'-TCCCTGAGCGTCATCTGCC
	X-CAGCCGCCACCACCAGGATGAAC	X'-CGCTGGTGGAGGCTGACGA
FLT3	X-GGAACGTGCTTGTCACCCACGG	X'-CCCTGACAACATAGTTGGAATCACTCA
FOXL2	X-CGGAAGGGCCTCTTCATGCGGC	X'-TGCAACTACTGGACGCTGGAC
GNAS	X-GGCTTACTGGAAGTTGACTTTGTCCAC	X'-CTCAATTTTGTTTCAGGACCTGCT
HRAS	X-GGGCAGGAGACCCTGTAGGAG	X'-GTGGTCAGCGCACTCTTGCCC
	X-CAAAAGACTTGGTGTTGTTGATGGCA	X'-TCATCCTGGATACCGCCGGC
IDH1	X-GGTCTTCAGAGAAGCCATTATCTGCA	X'-ACATGACTTACTTGATCCCCATAAGCA
JAK2	X-GAAGCAGCAAGTATGATGAGCAAGCT	X'-GCCTGTAGTTTTACTTACTCTCGTCTCC
KIT	X-CTTGGCAGCCAGAAATATCCTCCTTACTC	X'-GCGTTTCCTTTAACCACATAATTAGAATC
	X-CTCCCATTTGTGATCATAAGGAAGTTG	X'-GTTTTCCCTTTCTCCCCACAGA
KRAS	X-GTCACATTTTCATTATTTTTATTATAAGGCCTGC	X'-GTCGTCAAGGCACTCTTGCCT
	X-GTATTATTTATGGCAAATACACAAAGAAAGC	X'-CGATATTCTCGACACAGCAGGT
MET	X-CCTATTAAAGCAGTGCTCATGATTGG	X'-GTTCCCTATCAAATATGTCAACGACT
	X-CTTTCCAAAGCCATCCACTTCAC	X'-AATTTTGGTCTTGCCAGAGACA
MYD88	X-CAGACAGTGATGAACCTCAGGATGCTG	X'-CTTGCAGGTGCCCATCAGAAGC
NRAS	X-GATGTGGCTCGCCAATTAACCCTGA	X'-AGTCAGTGCGCTTTTCCCA
	X-CTTGCTATTATTGATGGCAAATACACAG	X'-TGACATACTGGATACAGCTGGA
PIK3CA	X-AAGAAACAGAGAATCTCCATTTTAGCAC	X'-TGCAATTTCTACACGAGATCCTCT
	X-TCAGTTCAATGCATGCTGTTTAATTGTG	X'-TCTTTGGAGTATTTCATGAAACAAATGA
PPP2R1A	X-CTGCTGCCTCAGGATCCCCGTCC	X'-ACCCAGCTTGGAGGCTGC
	X-GTGGGACAATGTCAACGGTCGCT	X'-AGCCAGGCCGCTGAAGACA
PTEN	X-CCCATAGAAATCTAGGGCCTCT	X'-GGCAATTCACTGTAAAGCTGGAAAG
	X-CTCTACTTTGATATCACCACACACAGG	X'-ATGAAGATATATTCCTCCAATTCAGGAC
STK11	X-GAGTCCAGCACCTCCTTCACCTTG	X'-TATCGACTCCACCGAGGTCA
	X-CGCCAGACTCACCGGGCACC	X'-ATACTTGGAGGACCTGCACG
TP53	X-CAAGATGTTTTGCCAACTGGCC	X'-CTGTGCTGTGACTGCTTG
	X-CCTGTCGTCTCTCCAGCCCCAG	X'-TAGCACATGACGGAGGTT
	X-GGCTGGTTGCCCAGGGTCCC	X'-ACAAATACTCCACACGCAAATT
	X-GCCACTGACAACCACCCTTAACC	X'-ATATTTGGATGACAGAAACACTT
	X-CCTCATCTTGGGCCTGTGTTATCT	X'-CTGTGATGATGGTGAGGATGG
	X-GGGCAGCTCGTGGTGAGGC	X'-CTGGGACGGAACAGCTTTGAG

[a] X = Biotin label

[b] X' = GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNN (N = degenerate position with approximately equal probability of having A, T, C, or G).

Supplementary Table S8. List of Forward Primers for Second Round of PCR Amplification

Gene Name	Primer Sequence (5' to 3')[a]
AKT1	X-GTGGCCGCCAGGTCTTGATG
APC	X-TTCAGCAGTAGGTGCTTTATTTTTAGGTAC
BRAF	X-CAAACTGATGGGACCCACTCCATC
CDKN2A	X-GTTCTCTCTGGCAGGTCATGATGATGG
	X-CAGGTACCGTGCGACATCGCGATG
CTNNB1	X-CCTCAGGATTGCCTTTACCACTCAG
EGFR	X-GAAACTCACATCGAGGATTTCCTTGTTG
	X-TGCATGGTATTCTTTCTCTTCCGCAC
	X-GAGGCAGCCGAAGGGCATGAG
EZH2	X-CAGGTTATCAGTGCCTTACCTCTCCAC
FGFR3	X-CAGCTTGAAGAGCTCCTCCACAG
	X-CCTGCAGGATGGGCCGGTG
	X-CCACCCCGTAGCTGAGGATGC
FLT3	X-GTGGTGAAGATATGTGACTTTGGATTGGCT
FOXL2	X-CGGTAGTTGCCCTTCTCGAACATG
GNAS	X-ACTTGGTCTCAAAGATTCCAGAAGTCAG
HRAS	X-CCTGAGGAGCGATGACGGAATATAAG
	X-ATGTACTGGTCCCGCATGGCG
IDH1	X-TGTGAGTGGATGGGTAAAACCTATCATCA
JAK2	X-TCTCACAAGCATTTGGTTTTAAATTATGGAGT
KIT	X-GGATCACAAAGATTTGTGATTTTGGTCTAGC
	X-GGTCTATGTAAACATAATTGTTTCCATTTATCT
KRAS	X-GCTGAAAATGACTGAATATAAACTTGTGGTA
	X-CAGTCCTCATGTACTGGTCCCTCATT
MET	X-GCTGGAGACATCTCACATTGTTTTTGTTG
	X-GCTTTGCACCTGTTTTGTTGTGTACAC
MYD88	X-GGAACTCTTTCTTCATTGCCTTGTACTTGATG
NRAS	X-TCTTGCTGGTGTGAAATGACTGAGTAC
	X-TCGCCTGTCCTCATGTATTGGTCT
PIK3CA	X-CACTTACCTGTGACTCCATAGAAAATCTTTC
	X-GAAGATCCAATCCATTTTTGTTGTCCAGC
PPP2R1A	X-CCGGAACCTGTGCTCAGATGACAC
	X-CTGTGAACTTGTCAGCCACCATGTAG
PTEN	X-CCTTTAAAAATTTGCCCCGATGTAATAAATATGC
	X-GGCTGAGGGAACTCAAAGTACATGAAC
STK11	X-CCCATCAGGTACTTGCCGATGAG
	X-CTGAGTGTAGATGATGTCATCCTCGATG
TP53	X-TGCAGCTGTGGGTTGATTCCAC
	X-CCAGCTGCTCACCATCGCTATCT
	X-TCCTCACTGATTGCTCTTAGGTCTGG
	X-CAAACCAGACCTCAGGCGGCTC
	X-GCTCTGACTGTACCACCATCCAC
	X-TCCCCTTTCTTGCGGAGATTCTCT

[a] X = ACACTCTTTCCCTACACGACGCTCTTCCGATCT[6 nucleotide barcode]