

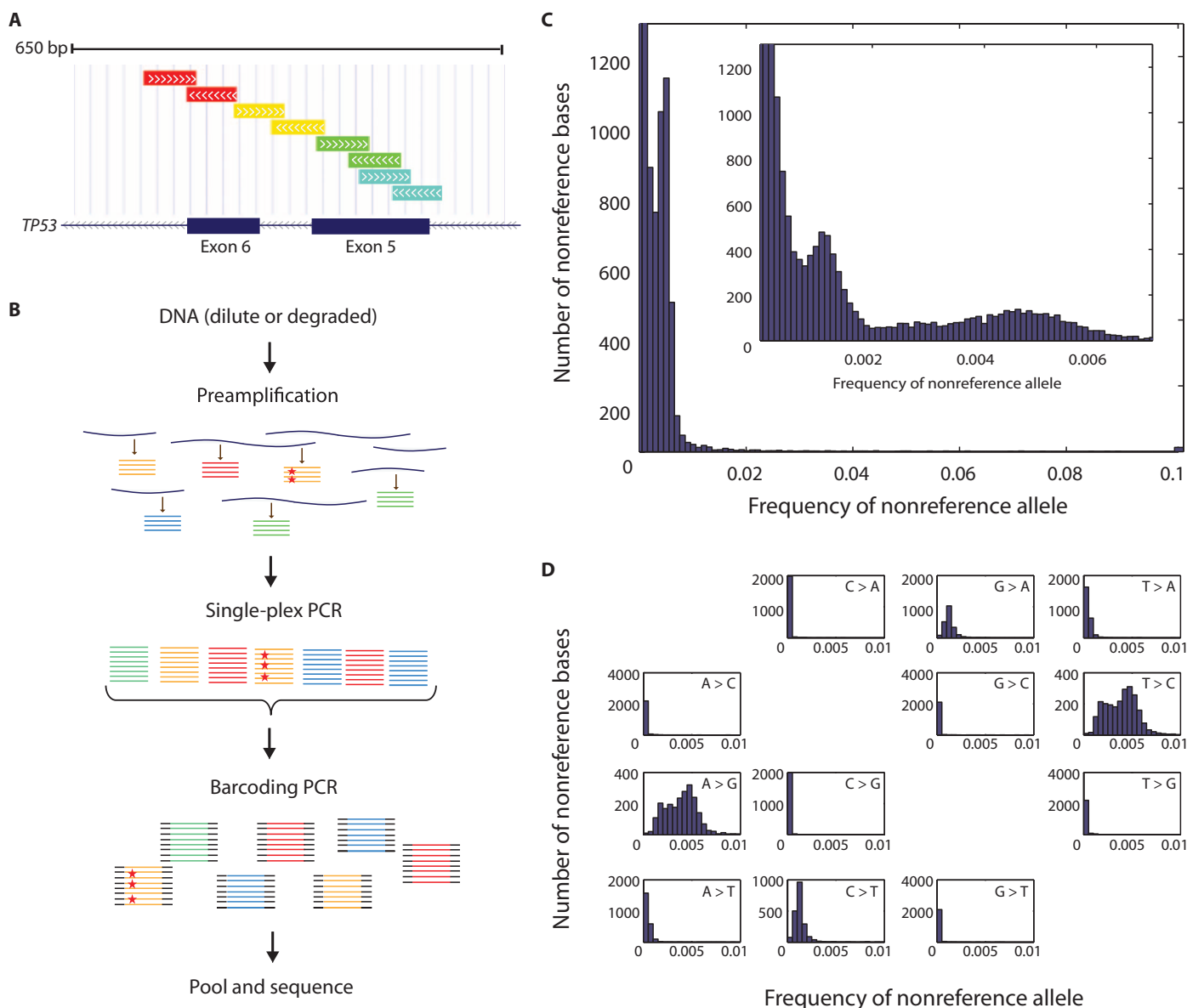


rare mutations in circulating DNA from blood plasma of ovarian and breast cancer patients. This sequencing approach allowed us to monitor changes in tumor burden by sampling only patient plasma over time. Combined with faster, more accurate sequencing technologies or rare allele amplification strategies, this approach could potentially be used for personalized medicine at point of care.

## RESULTS

### Targeted deep sequencing of fragmented DNA by TAM-Seq

To amplify and sequence fragmented DNA, we designed primers to generate amplicons that tile regions of interest in short segments of about 150 to 200 bases (Fig. 1A and table S1), incorporating universal



**Fig. 1.** Overview of tagged amplicon sequencing (TAM-Seq). **(A)** Illustration of amplicon design. Primers were designed to amplify regions of interest in overlapping short amplicons (table S1). Amplicon design is illustrated for a region covering exons 5 to 6 of *TP53*. Colored bars, segmented into forward and reverse reads, show regions covered by different amplicons (excluding primer regions). Sequencing adaptors are attached at either end, such that a single-end read generates separate sets of forward and reverse reads (fig. S1). Because amplicons are mostly shorter than 200 bp, the forward and reverse reads also partially overlap. Figure adapted from University of California, Santa Cruz, Genome Browser (<http://genome.ucsc.edu/>). **(B)** Workflow overview. Multiple regions were amplified in parallel. An initial preamplification step was

performed for 15 cycles using a pool of the target-specific primer pairs to preserve representation of all alleles in the template material. The schematic diagram shows DNA molecules that carry mutations (red stars) being amplified alongside wild-type molecules. Regions of interest in the preamplified material were then selectively amplified in individual (single-plex) PCR, thus excluding nonspecific products. Finally, sequencing adaptors and sample-specific barcodes were attached to the harvested amplicons in a further PCR. **(C)** Distribution of observed nonreference read frequencies, averaged over 47 FFPE samples, across all loci and all nonreference bases. Inset expands the low-frequency range. **(D)** Distribution of the observed background nonreference read frequencies averaged over 47 FFPE samples for the 12 different A/C/G/T base substitutions.





















## Noninvasive Identification and Monitoring of Cancer Mutations by Targeted Deep Sequencing of Plasma DNA

Tim Forshew, Muhammed Murtaza, Christine Parkinson, Davina Gale, Dana W. Y. Tsui, Fiona Kaper, Sarah-Jane Dawson, Anna M. Piskorz, Mercedes Jimenez-Linan, David Bentley, James Hadfield, Andrew P. May, Carlos Caldas, James D. Brenton and Nitzan Rosenfeld

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DOI: 10.1126/scitranslmed.3003726

### Deep Sequencing Tumor DNA in Plasma

Five liters of circulating blood contain millions of copies of the genome, broken into short fragments; in cancer patients, a small fraction is circulating tumor DNA (ctDNA). An even smaller number harbor mutations that affect cancer outcome. Looking for diagnostic answers in circulating DNA is a challenge, but Forshew, Murtaza, and colleagues have risen to the occasion by developing a tagged-amplicon deep sequencing (TAm-Seq) method that can amplify and sequence large genomic regions from even single copies of ctDNA. By sequencing such large regions, the authors were able to identify low-level mutations in the plasma of patients with high-grade serous ovarian carcinomas.

Forshew *et al.* designed primers to amplify 5995 bases that covered select regions of cancer-related genes, including *TP53*, *EGFR*, *BRAF*, and *KRAS*. In plasma obtained from 38 patients with high levels of ctDNA, the authors were able to identify mutations in *TP53* at allelic frequencies of 2% to 65%. In plasma samples from one patient, they also identified a de novo mutation in *EGFR* that had not been detected 15 months prior in the tumor mass itself. Finally, the TAm-Seq approach was used to sequence ctDNA in plasma samples collected from two women with ovarian cancer and one woman with breast cancer at different time points, tracking as many as 10 mutations in parallel. Forshew and coauthors showed that levels of mutant alleles reflected the clinical course of the disease and its treatment—for example, stabilized disease was associated with low allelic frequency, whereas patients at relapse exhibited a rise in frequency.

Through several experiments, the authors were able to show that TAm-Seq is a viable method for sequencing large regions of ctDNA. Although this provides a new way to noninvasively identify gene mutations in our blood, TAm-Seq will need to achieve a more sensitive detection limit (<2% allele frequency) to identify mutations in the plasma of patients with less advanced cancers. Nevertheless, once optimized, this "liquid biopsy" approach will be amenable to personalized genomics, where the level and type of mutations in ctDNA would inform clinical decision-making on an individual basis.

#### ARTICLE TOOLS

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#### SUPPLEMENTARY MATERIALS

<http://stm.sciencemag.org/content/suppl/2012/05/25/4.136.136ra68.DC1>

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## Supplementary Materials for

### Noninvasive Identification and Monitoring of Cancer Mutations by Targeted Deep Sequencing of Plasma DNA

Tim Forshew, Muhammed Murtaza, Christine Parkinson, Davina Gale, Dana W. Y. Tsui, Fiona Kaper, Sarah-Jane Dawson, Anna M. Piskorz, Mercedes Jimenez-Linan, David Bentley, James Hadfield, Andrew P. May, Carlos Caldas, James D. Brenton,\* Nitzan Rosenfeld\*

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#### The PDF file includes:

##### Methods

Fig. S1. PCR strategy and primer design.

Fig. S2. Sanger traces for mutations identified by tagged-amplicon sequencing.

Fig. S3. Background frequencies and detection limits for base substitutions.

Fig. S4. Replicate dilute Sanger sequencing of a mutation identified in plasma.

Table S1. Target-specific primers.

Table S2. Unique sequencing barcodes.

Table S3. Mutations identified in FFPE samples.

Table S4. SNPs identified in circulating DNA from two plasma control samples.

Table S5. Frequency of SNP alleles in dilution series of DNA from control plasma.

Table S6. Additional data for Table 2 for mutations identified in plasma samples.

Table S7. Mutations and amplicons studied in one breast cancer patient.

## SUPPLEMENTARY METHODS

### Sanger sequencing

Coding sequences of the *TP53* gene (exons 2 to 11) were amplified as described previously (36) with the following modifications: PCR reactions were performed in 25  $\mu$ l, universal primers M13 forward and M13 reverse were tagged onto both primers pairs and used to sequence in both the forward and reverse directions. To sequence exon 7, TP53-7F (CAGGTCTCCCCAAGGCGCAC) was used owing to a poly A tract downstream of the exon 7 forward primer described in (36): CATCCTGGCTAACGGTGAAAC. PCR products were sequenced on an ABI 3730 (Applied Biosystem). Mutational analysis was performed using Mutation Surveyor Software version 3.97 (SoftGenetics), using default settings (mutation score=5.00, mutation height=500, overlapping factor=0.20, dropping factor=0.20, SnRatio=1.00, mobility shift=imbedded algorithm). All exons were sequenced for 26 samples. For 12 samples, sequencing was performed for selected exons only.

### Digital PCR

The digital PCR method and the use of the BioMark system for digital PCR analysis have been described (7, 37). Samples were mixed with sequence-specific TaqMan probes, Master Mix, and 20 $\times$  GE Sample Loading Reagent (Fluidigm). This mixture was then added to the 12.765 Digital Array Chip (Fluidigm) where it was partitioned into 765 separate PCR reactions. These were PCR cycled on the Fluidigm BioMark and all positive reaction chambers counted. Amplifiable copies were calculated from the number of positive wells using a Poisson correction.

### Estimation of input DNA amounts

DNA amount was calculated as total amplifiable copies, including both mutant and germline alleles, as determined by digital PCR (median amplicon size 84 bp, range 58-177 bp), multiplied by the estimated amount of DNA in 1 haploid genome (3.3 pg). Amplifiable copies in control healthy plasma samples were estimated using the *FTH1* TaqMan assay Hs01694011\_s1 (Life Technologies), which has an amplicon size of 180 bp.

### Dilution of plasma DNA samples to determine allele quantification accuracy

After estimation of amplifiable copy numbers using digital PCR, both samples were diluted to 100 amplifiable copies per  $\mu$ l. A 5- $\mu$ l mix was prepared containing 500 amplifiable copies, of which 400 copies were contributed by plasmaDna1 and 100 copies were contributed by plasmaDna2 (table s4). This was serially diluted 2.5 $\times$  6 times (reaching 244 $\times$  dilution) in plasmaDna2. Each of the 7 mixes were then diluted 2.5 $\times$  in water a further six times until we had an expected 2 total amplifiable copies of DNA. Twenty-eight of these mixes (table s5) were amplified using the described primer set (table S1) in triplicate, barcoded, and sequenced. Minor allele frequencies expected for the dilutions can be calculated using heterozygosity status for each SNP in either sample, an example that applies to 3 of the 5 SNPs (rs1800899, rs1050171 and rs10241451) we studied is given in table s5.

### **Replicate dilute Sanger sequencing**

We performed replicate dilute Sanger Sequencing using the primers EGFR\_A\_F (CAGCAGGGTCTTCTCTGTTTCA) and EGFR\_A\_R (GGTGTTTTCCACCAGTACGTTCCT). A PCR mastermix was made containing 0.05 U/ $\mu$ l FastStart High Fidelity Enzyme Blend (Roche), 1 $\times$  FastStart High Fidelity Enzyme Buffer, 200  $\mu$ M each of dNTPs, 4.5 mM MgCl<sub>2</sub>, 5% DMSO, 50 nM of each primer, and 1X EvaGreen DNA binding dye (Biotium). Plasma DNA was quantified using digital PCR and diluted such that when partitioned in a 384-well plate, each reaction well would contain on average less than 1 amplifiable template. Forty-five cycles of PCR (50°C 2 min, 70°C 20 min, 95°C 10 min, 45 cycles of 95°C 15 s, 60°C 30 s, 72°C 1 min) were performed on a 7900HT Fast Real-Time PCR machine (Applied Biosystems).

### **TAm-Seq**

#### **Primer design for TAm-Seq**

Target-specific primers were designed with universal primer sequences (termed CS1 and CS2) appended at the 5'-end (table S1). Primers were designed using Primer3 with a T<sub>m</sub> range of 58-62°C, allowing homopolymer stretches no longer than 3 and keeping primer %GC less than 65%. Primer designs were screened against the hg19 reference genome to prevent cross-product generation, and were screened to prevent primer-dimer interactions and formation of intramolecular secondary structure. Primers were tested by amplifying from 10 ng human genomic DNA (GM17317, Coriell) in 5  $\mu$ l reaction volumes containing 0.05 U/ $\mu$ l FastStart High Fidelity Enzyme Blend (Roche), 1 $\times$  FastStart High Fidelity Enzyme Buffer, 200 $\mu$ M each of dNTPs, 4.5 mM MgCl<sub>2</sub>, 5% DMSO, 50 nM of each primer, and 1 $\times$  Access Array sample loading solution (Fluidigm) using 35 cycles of amplification: 50°C 2 min, 70°C 20 min, 95°C 10 min, 10 cycles of 95°C 15 s, 60°C 30 s, 72°C 60 s, 2 cycles of 95°C 15 s, 80°C 30 s, 60°C 30 s, 72°C 60 s, 8 cycles of 95°C 15 s, 60°C 30 s, 72°C 60 s, 2 cycles of 95°C 15 s, 80°C 30 s, 60°C 30 s, 72°C 60 s, 8 cycles of 95°C 15 s, 60°C 30 s, 72°C 60 s, 5 cycles of 95°C 15 s, 80°C 30 s, 60°C 30 s, 72°C 60 s, 1 cycle of 72°C for 3 min.

Barcode primers (table S2) comprised either the PE1 or PE2 sequences for Illumina cluster generation, a 10-bp barcode, followed by either CS1 or CS2 adaptor sequences. For example, one pair of barcode primers comprised 5'-PE1-CS1-3' + 5'-PE2-BC-CS2-3', the second pair comprised 5'-PE1-CS2-3' + 5'-PE2-BC-CS1-3'.

The sequencing reagent for read1 contained custom sequencing primers designed to anneal to both CS1 and CS2. The sequencing reagent for the index read contained custom sequencing primers designed to anneal to the reverse complements of CS1 and CS2.

#### **Pre-amplification for TAm-Seq**

Pre-amplification reactions were carried out in 10- $\mu$ l reaction volumes containing 50 nM of each forward and reverse target-specific primer. Target DNA (1 to 5  $\mu$ l) was added to a mastermix containing 0.5 U FastStart High Fidelity Enzyme Blend (Roche), 1 $\times$  FastStart High Fidelity Enzyme Buffer, 200  $\mu$ M each of dNTPs, 4.5 mM MgCl<sub>2</sub>, and 5% DMSO. Reactions were subjected to 15 cycles of amplification (95°C 10 min, 15 cycles of 95°C 15 s, 60°C 4 min). Following pre-amplification, 4  $\mu$ l Exo-SAP-it (Affymetrix) was added to each reaction and incubated for 15 min at 37°C, then for 15 min at 80°C. The pre-amplified samples were diluted 5-fold in PCR-grade water prior to amplification on the Access Array IFC system.

### **Target-specific amplification on the Access Array microfluidic system**

Individual primer pairs were loaded into the primer inlets of the Access Array IFC (Fluidigm) at a final concentration of 1  $\mu\text{M}$  (20 $\times$  the final concentration of 50 nM required for the PCR reaction) to account for the volume ratio of sample and primer chambers (20:1) in the Access Array IFC. One  $\mu\text{l}$  of each preamplified sample was added to 4  $\mu\text{l}$  pre-sample mix containing 0.05 U/ $\mu\text{l}$  FastStart High Fidelity Enzyme Blend (Roche), 1 $\times$  FastStart High Fidelity Enzyme Buffer, 200  $\mu\text{M}$  each of dNTPs, 4.5 mM  $\text{MgCl}_2$ , 5% DMSO, and 1 $\times$  Access Array sample loading solution (Fluidigm). Samples and primers were loaded into the IFC using an IFC-AX controller (Fluidigm). The volume of each sample chamber within the Integrated Fluidic Circuit (IFC) was 33 nl, containing 0.7% of the input reaction volume (5  $\mu\text{l}$ ). The IFC was then subjected to thermal cycling using a Biomark system (Fluidigm): 35 cycles of amplification (50 $^\circ\text{C}$  2 min, 70 $^\circ\text{C}$  20 min, 95 $^\circ\text{C}$  10 min, 10 cycles of 95 $^\circ\text{C}$  15 s, 60 $^\circ\text{C}$  30 s, 72 $^\circ\text{C}$  60 s, 2 cycles of 95 $^\circ\text{C}$  15 s, 80 $^\circ\text{C}$  30 s, 60 $^\circ\text{C}$  30 s, 72 $^\circ\text{C}$  60 s, 8 cycles of 95 $^\circ\text{C}$  15 s, 60 $^\circ\text{C}$  30 s, 72 $^\circ\text{C}$  60 s, 2 cycles of 95 $^\circ\text{C}$  15 s, 80 $^\circ\text{C}$  30 s, 60 $^\circ\text{C}$  30 s, 72 $^\circ\text{C}$  60 s, 8 cycles of 95 $^\circ\text{C}$  15 s, 60 $^\circ\text{C}$  30 s, 72 $^\circ\text{C}$  60 s, 5 cycles of 95 $^\circ\text{C}$  15 s, 80 $^\circ\text{C}$  30 s, 60 $^\circ\text{C}$  30 s, 72 $^\circ\text{C}$  60 s, 1 cycle of 72 $^\circ\text{C}$  for 3 min). Harvesting solution (0.05% Tween-20) was loaded onto the IFC prior to harvesting on an IFC-AX controller. One  $\mu\text{l}$  of harvested product was then transferred to a clean PCR plate containing 99  $\mu\text{l}$  PCR-free water.

### **Sequencing adaptor and barcode primer addition**

For each sample, 1  $\mu\text{l}$  of the 100-fold diluted PCR products was added to each of two PCR plates containing 15  $\mu\text{l}$  pre-sample mastermix containing 0.05 U/ $\mu\text{l}$  FastStart High Fidelity Enzyme Blend (Roche), 1 $\times$  FastStart High Fidelity Enzyme Buffer, 200  $\mu\text{M}$  each of dNTPs, 4.5 mM  $\text{MgCl}_2$ , and 5% DMSO. In the first plate, 4  $\mu\text{l}$  of one pair of primers containing an individual 10-base barcode (BC) sequence, and sequence tags for reading in one direction (PE1-BC-CS1 + PE2-CS2) were added to each well. In the second plate, 4  $\mu\text{l}$  of primers containing (PE1-BC-CS2 + PE2-CS1) were added to each well. The corresponding wells in both plates contained primers with the same barcode sequence (e.g. plate 1, well A1 = Barcode FLD0001, plate 2, well A1 = barcode FLD0001). Reaction products in plates were amplified for 15 cycles: 95 $^\circ\text{C}$  10 min, 15 cycles of 95 $^\circ\text{C}$  15 s, 60 $^\circ\text{C}$  30 s, 72 $^\circ\text{C}$  4 min, 1 cycle of 72 $^\circ\text{C}$  for 3 min.

### **Quantification and clean up of DNA library**

After PCR products were barcoded, they were analyzed using Agilent 2100 BioAnalyzer to ensure expected insert size (~180 bp) was obtained. They were then pooled together and purified using AMPure XP beads using a bead to amplicon ratio of 1.8:1. The library was quantified by Agilent BioAnalyzer and subjected to Illumina cluster generation. Single-end sequencing of 100 bases was performed on an Illumina GAIIx sequencer followed by a 10-base indexing (barcode) read, using custom sequencing primers targeted to the CS1 and CS2 tags for both read1 and the index read according to manufacturer's recommendations.

## Analysis of sequencing data

***De-multiplexing and alignment.*** Reads generated by the primary Illumina pipeline were demultiplexed using a known list of barcodes (table S2), allowing one base mismatch out of 10 bases. Each set of reads was aligned independently to the hg19 reference genome using bwa-short in the single-end mode. Using expected genomic positions, each set of aligned reads was separated further into its constituent amplicons. A pileup was generated for each amplicon using samtools v1.12a. Using a base quality and a mapping quality cut-off of 30, observed frequencies of non-reference alleles for every sequenced locus across all amplicons and barcodes were calculated.

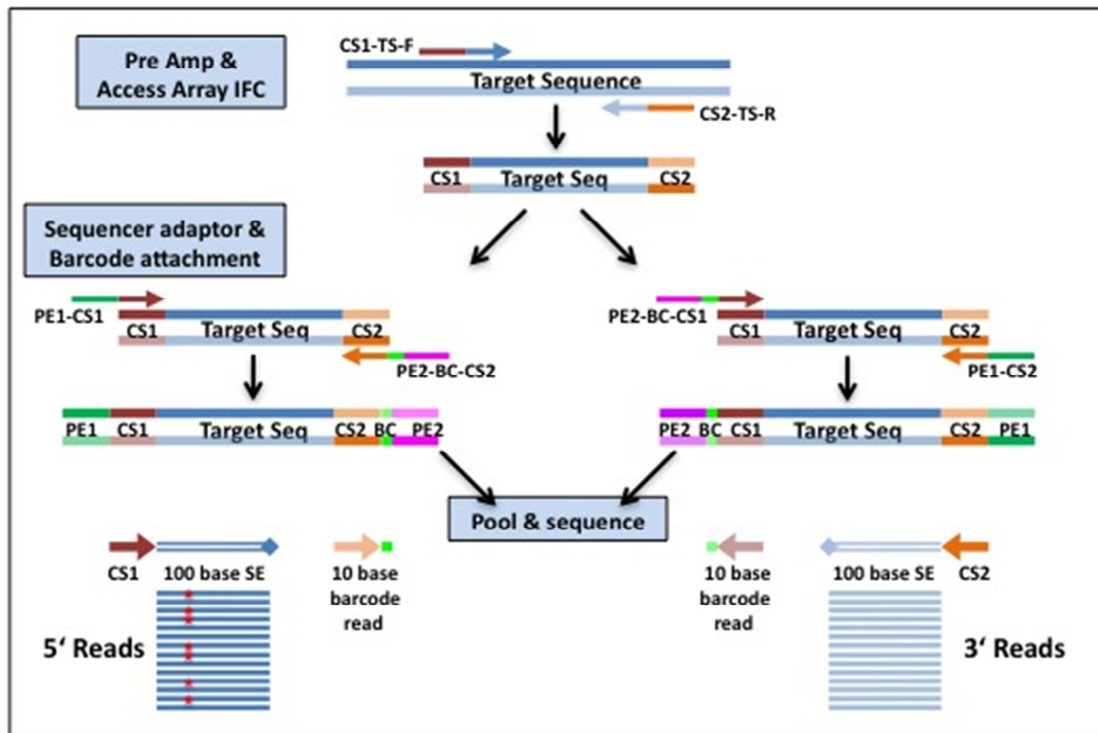
***Mutation identification.*** For each locus and base, the distribution of non-reference background allele frequencies/reads was modeled as a Normal distribution and as a Poisson distribution, fitting to a Normal distribution and using the mean number of observed non-reference reads in a set of representative barcodes as the parameter for the Poisson distribution. For each barcode, the probability of obtaining the observed frequency/number of reads (or greater) was calculated in both models, and the lower value was retained. Putative substitutions that passed a probability cut-off (confidence margin) of 0.9995 were kept for further analysis as candidate mutations.

For each barcode, all loci that passed the probability cut-off were ranked by observed frequency, corrected for the median frequency (over all samples) for each locus/base. Known SNPs obtained from the 1000 Genomes project and regions covering amplification primers were discarded, leaving 17,934 possible base calls per sample. Indels are not analyzed in the current calling algorithm. A point mutation was called in a sample if it ranked among the 6 loci with highest non-reference frequency in both duplicates, and was represented by at least 10 reads in each. If a mutation was called, it was also discarded from the pool and the process was repeated by testing the 6 highest remaining frequencies. Mutations that occurred at low frequency may have fallen outside the list of top-ranked loci, especially for more noisy samples, and may have therefore been missed. For each sample, we estimated the threshold for mutation calling by the highest background-corrected non-reference allele frequency that did not get into the top-ranked list and may have been missed by the calling algorithm. For mutations called in multiple (overlapping) amplicons, the amplicon showing best concordance between duplicates was retained.

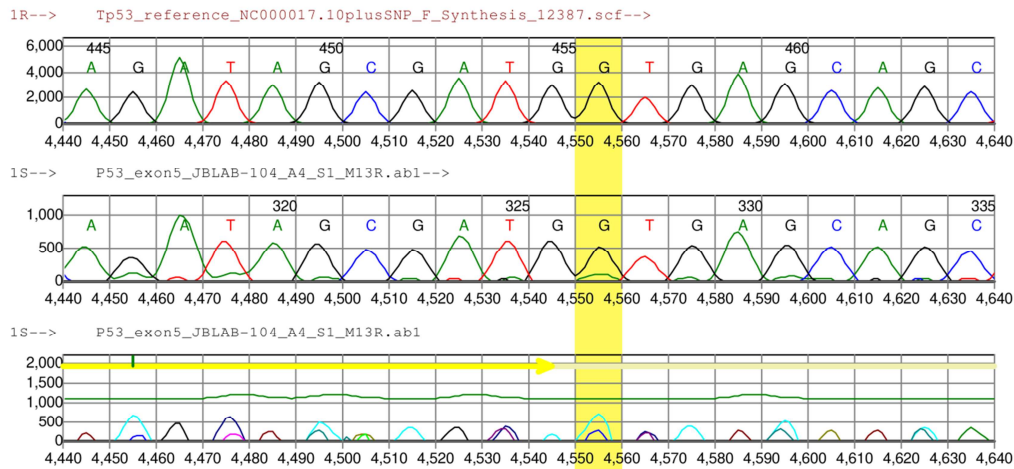
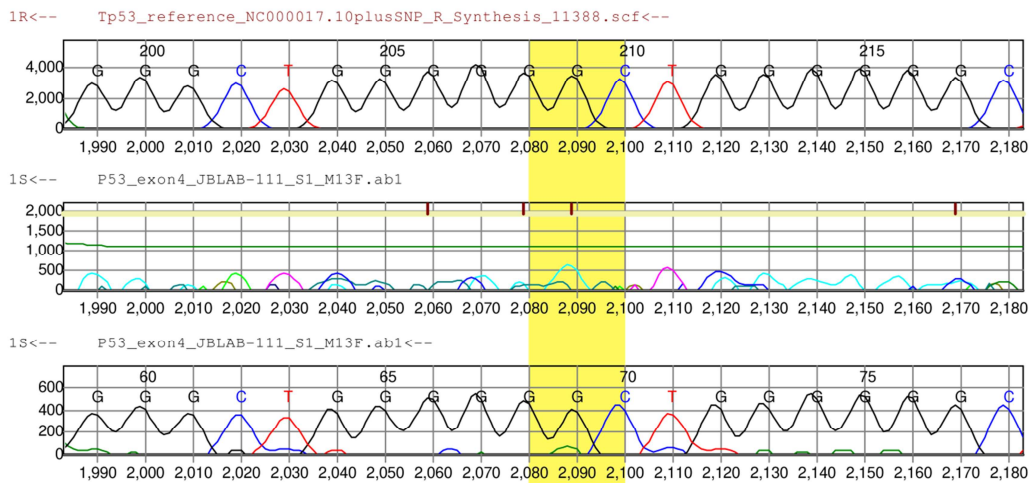
For the sequencing data obtained from a mix of FFPE samples, we performed an additional lower-stringency analysis using a probability cut-off of 0.9995 keeping the 12 loci with highest non-reference frequency to enhance sensitivity. For the data obtained from plasma sequencing, we performed an additional higher-stringency analysis using a probability cut-off of 0.9999 keeping the 6 loci with highest non-reference frequency to enhance sensitivity.

***Mutation detection.*** When applying TAM-Seq to measure a pre-defined mutation (as opposed to screening nearly 18,000 possible substitutions), the frequency of the mutant allele can be read out directly from the data at the desired locus. We used confidence margins of  $\sim 0.95$  using a Normal distribution model for each examined substitution, and requiring a minimum of 10 representative reads, in at least one amplified library per sample. When a mutation was positively detected, its allele frequency was estimated by averaging the allele frequencies in all replicates after subtracting substitution-specific background frequencies.

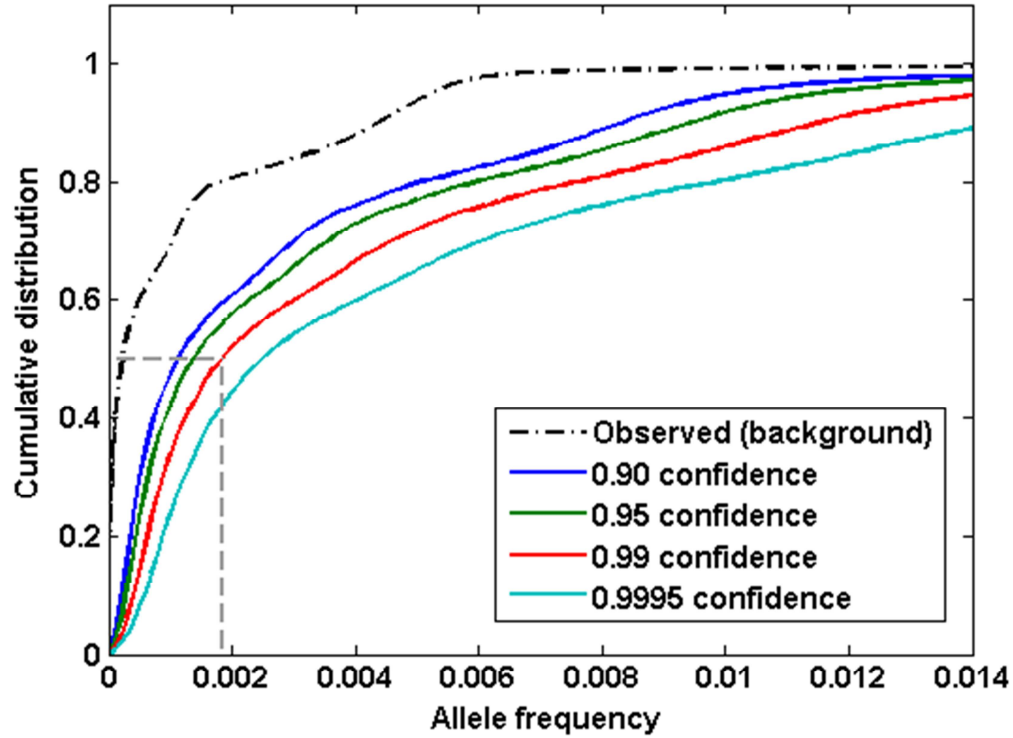
## SUPPLEMENTARY FIGURES



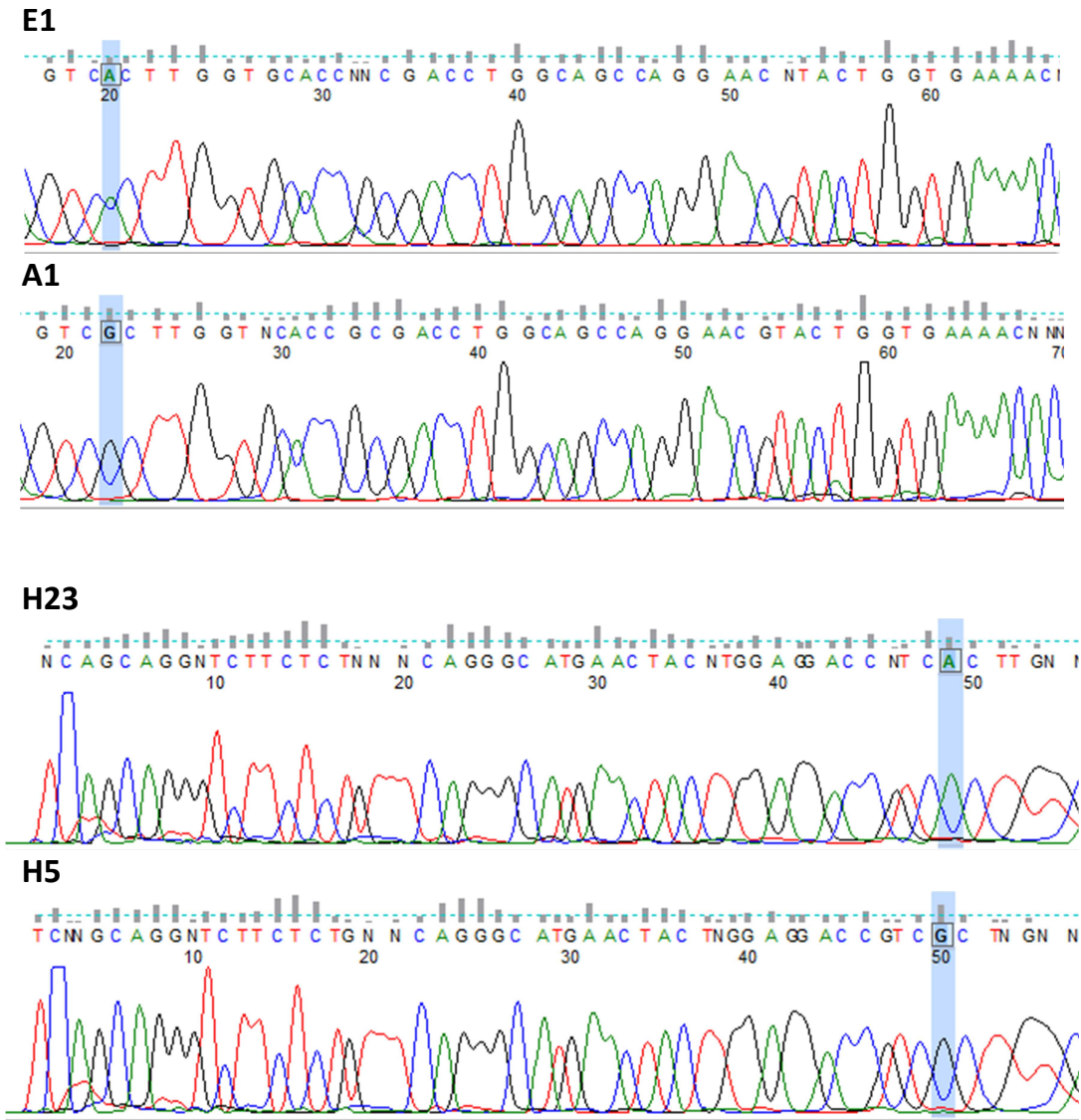
**Figure S1: PCR strategy and primer design.** Target-specific primers were synthesised with common adaptor sequences at their 5' ends (CS1 and CS2). These were used during the preamplification and single-plex stages to amplify the selected regions. Sequencing platform-specific adaptors and unique sample barcodes were attached during an additional round of PCR. The barcoding primers consist of Illumina sequencer adaptors (PE1 and PE2) at their 3' end, a unique 10 base barcode, and the common CS1 and CS2. Permutations of the universal tags and the sequencing adaptors enabled construction of bidirectional barcoded sequencing libraries. Using custom sequencing primers directed to the two tag sequences, sequence reads representing both strands of the amplicon pool were obtained from a single-end run. Both combinations were used in order to barcode and read amplicons in both directions. All samples were then pooled, cleaned, and then sequenced.

**A****B**

**Figure S2: Sanger traces for mutations identified by tagged-amplicon sequencing.** Mutational analysis of Sanger traces was performed by Mutation Surveyor Software version 3.97 (SoftGenetics) using default settings. The *TP53* gene was on the reverse strand, therefore mutations seen in the sequencing traces were the reverse complement of the mutations as indicated by the hg19 coordinates. **(A)** Sanger sequencing of sample #104. Highlighted locus shows a mutation that was identified by TAM-Seq (17:7578370C>T), but not Sanger sequencing. The Sanger sequencing trace for the sample (middle panel) shows a weak signal above the reference (top panel), but this was at background noise levels and did not pass detection thresholds (lower panel). **(B)** Sanger sequencing of sample #111. Highlighted locus shows a mutation that was identified by TAM-Seq (17:7579642C>T), but not Sanger sequencing. The Sanger sequencing trace (lower panel) showed a weak signal above the reference (top panel), but this did not pass detection thresholds (middle panel).



**Figure S3: Background frequencies and detection limits for base substitutions.** For each one of the possible base substitutions in the sequenced amplicons, we calculated the mean background read rate in the 124 barcoded libraries generated from the set of 62 plasma samples (cumulative distribution shown in black), and the standard deviation. Colored lines show the cumulative distribution of frequencies that would exceed the indicated confidence margins, using a Normal distribution model for each base substitution, given its measured mean and standard deviation. Substitutions that were covered by more than one amplicon or read direction were included multiple times, once for each amplicon/direction in which they were sequenced. Substitutions that had zero reads in all barcodes had undefined confidence limits and were omitted ( $n = 589$ ). Dashed grey lines indicate the median value for 0.99 confidence margin.



**Figure S4: Replicate dilute Sanger sequencing of a mutation identified in plasma.** Replicate Sanger sequencing of highly dilute template was used to validate the *EGFR* mutation that was identified *de novo* in plasma and was not found in the corresponding tumour sample from the same patient (Patient #27, Table 1). The panels show examples of mutant and wild type reads in either direction, with the mutated base (chr7:55259437G>A, genome build hg19 coordinates) highlighted in blue. E1 is an example of mutant forward read, and A1 is a matched wild-type. H23 is an example of mutant reverse read, and H5 a matched wild-type.

## SUPPLEMENTARY TABLES

**Table S1: Target-specific primers.** Each target-specific primer consists of a universal 5' end and a target-specific 3' end as follows:

5'-ACACTGACGACATGGTTCTACA-[Target Specific-Forward]-3'.

5'-TACGGTAGCAGAGACTTGGTCT-[Target Specific-Reverse]-3'

| Gene name and Amplicon ID | Target Specific Primer – Forward | Target Specific Primer- Reverse | Chr | Amplicon Start | Amplicon End |
|---------------------------|----------------------------------|---------------------------------|-----|----------------|--------------|
| PIK3CA_E00001077674 *     | CAGAGGGGAAAAATATGACAAA           | AACAGAGAATCTCCATTTTAGCAC        | 3   | 178935943      | 178936150    |
| PIK3CA_E00001139987       | TGAGCAAGAGGCTTTGGAGT             | GGTCTTTGCCTGCTGAGAGT            | 3   | 178952038      | 178952227    |
| EGFR_Exon19               | TCACAATTGCCAGTTAACGTCT           | CCACACAGCAAAGCAGAAAC            | 7   | 55242373       | 55242537     |
| EGFR_E00001601336_1       | GCGTCTTCACCTGGAAGGG              | CCGGACATAGTCCAGGAGG             | 7   | 55248902       | 55249111     |
| EGFR_E00001601336_2       | GCGTGGACAACCCCCAC                | GGCTCCTTATCTCCCCTCC             | 7   | 55249005       | 55249213     |
| EGFR_E00001681524_1       | GGATGCAGAGCTTCTTCCCA             | TTCTCTTCCGCACCCAG               | 7   | 55259352       | 55259542     |
| EGFR_E00001681524_2       | GGTCTTCTCTGTTTCAGGGCAT           | GCTGACCTAAAGCCACCTCC            | 7   | 55259395       | 55259591     |
| EGFR_E00001631695_1       | GTGTCACTCGTAATTAGGTCCA           | GGCCTCAGTACAACTCATTAGC          | 7   | 55260366       | 55260575     |
| EGFR_E00001779947_1       | TGTTCAATTCATGATCCCACTGC          | CCACCAGTCACTCACACTTG            | 7   | 55266362       | 55266571     |
| EGFR_E00001779947_2       | TCCCTGCCAGCGAGAT                 | AGGGATGCAAAGGCCTCA              | 7   | 55266461       | 55266641     |
| EGFR_E00001790701_1       | GCCTTCTTTAAGCAATGCCATCTTTAT      | CAATGGAAGCACAGACTGCAA           | 7   | 55267932       | 55268134     |
| EGFR_E00001801208_1       | CCCCTGCTCCTATAGCCAA              | ATGAGGTACTCGTCGGCATC            | 7   | 55268806       | 55268987     |
| EGFR_E00001801208_2       | ACTTCTACCGTGCCCTGA               | GTTCAAATGAGTAGACACAGCTT         | 7   | 55268921       | 55269101     |
| EGFR_E00001773562_1       | TACCCTCCATGAGGCACAC              | GGAGAGCTGTAAATTCTGGCTT          | 7   | 55269336       | 55269516     |
| BRAF_E00002324725         | TCATAATGCTTGCTCTGATAGGA          | CTGATGGGACCCACTCCAT             | 7   | 140453108      | 140453256    |
| PTEN_E00001456562_1       | GCAGCTTCTGCCATCTCTCT             | TCCGTCTACTCCCACGTTCT            | 10  | 89624175       | 89624372     |
| PTEN_E00001156351_1       | TGCTGCATATTTAGATATTTCTTTCTTA     | ATGAAAACACAACATGAATATAAACATCAAT | 10  | 89653738       | 89653927     |
| PTEN_E00001156344_1       | AATCTGTCTTTTGGTTTTTCTTGATAGT     | AATAGTTGTTTTAGAAGATATTTGCAAGC   | 10  | 89685172       | 89685367     |
| PTEN_E00001156337_4       | TATATCACTTTTAACTTTTCTTTTAGTTGTGC | CTCGATAATCTGGATGACTCATTATTGTT   | 10  | 89690776       | 89690940     |
| PTEN_E00001156330_1       | TTCTTATTCTGAGGTTATCTTTTACCAC     | TCATTACACCAGTTTCGTCCCT          | 10  | 89692739       | 89692919     |

|                       |                             |                               |    |          |          |
|-----------------------|-----------------------------|-------------------------------|----|----------|----------|
| PTEN_E00001156330_3   | TGACCAATGGCTAAGTGAAGATGA    | TCCAGGAAGAGGAAAGGAAAAACA      | 10 | 89692840 | 89693048 |
| PTEN_E00001156327_1   | TCTTAAATGGCTACGACCCAG       | TCCAGATGATTCTTTAACAGGTAGC     | 10 | 89711775 | 89711942 |
| PTEN_E00001156327_4   | CAGTCAGAGGCGCTATGTGT        | TCTAGATATGGTTAAGAAAAGTGTCCA   | 10 | 89711889 | 89712077 |
| PTEN_E00001156321_1   | TGACAGTTTGACAGTTAAAGGCAT    | CACACACAGGTAACGGCTGA          | 10 | 89717547 | 89717726 |
| PTEN_E00001156321_2   | TGTGGTCTGCCAGCTAAAGG        | TCTCCAATGAAAGTAAAGTACAAACC    | 10 | 89717620 | 89717802 |
| PTEN_E00001156321_4m  | TCCACAAACAGAACAAGATGCT      | GGCCTTTTCCTTCAAACAGGATT       | 10 | 89717748 | 89717956 |
| PTEN_E00001156315_1m  | GCAACAGATAACTCAGATTGCCTT    | GTTTCCTCTGGTCTGGTATGA         | 10 | 89720457 | 89720706 |
| PTEN_E00001156315_5 * | AGGACAAAATGTTTCACCTTTGGGTAA | ACTAGATATTCCTTGTCAATTATCTGCAC | 10 | 89720649 | 89720799 |
| PTEN_E00001156315_7   | CCTCAGAAAAAGTAGAAAATGGAAGTC | ACAAGTCAACAACCCCCACA          | 10 | 89720706 | 89720915 |
| PTEN_E00001456541_1   | AGATGAGTCATATTTGTGGGTTTTCA  | TCTGGATCAGAGTCAGTGGT          | 10 | 89724997 | 89725180 |
| PTEN_E00001456541_2 * | GTAGAGGAGCCGTCAAATCCA       | TTCATGGTGTTTTATCCCTCTTGA      | 10 | 89725068 | 89725264 |
| KRAS_E00000936617     | GCCTGCTGAAAATGACTGAA        | AGAATGGTCTGCACCAGTAA          | 12 | 25398163 | 25398329 |
| TP53_E00001757276_1   | GACCCAAAACCCAAAATGGC        | TCCCTGCTTCTGTCTCCTAC          | 17 | 7572850  | 7573030  |
| TP53_E00001728015_1   | GGAATCCTATGGCTTTCCAACC      | CCCCCTCCTCTGTTGCTG            | 17 | 7573859  | 7574054  |
| TP53_00001404886_13   | TCTGTATCAGGCAAAGTCATAGAA    | GCCTCAAAGACAATGGCTCC          | 17 | 7576584  | 7576734  |
| TP53_E00001789298_1   | AGAAAACGGCATTTTGAGTGT       | AAGGGTGCAGTTATGCCTCA          | 17 | 7576786  | 7576983  |
| TP53_E00001789298_2   | CTGGTGTGTTGGGCAGT           | ATCTCCGCAAGAAAGGGGAG          | 17 | 7576908  | 7577075  |
| TP53_E00001789298_3   | TGTCCTGCTTGCTTACCTCG        | GCCTCTTGCTTCTCTTTTCT          | 17 | 7577003  | 7577187  |
| TP53_E00001665758_1   | GGGGTCAGAGGCAAGCAG          | CTTGGGCTGTGTTATCTCC           | 17 | 7577432  | 7577631  |
| TP53_E00001255919_1   | GAGAAAGCCCCCTACTGC          | AGCATCTTATCCGAGTGGAAGG        | 17 | 7578091  | 7578274  |
| TP53_E00001255919_3   | TCCAAATACTCCACACGCAAA       | GCTGCCCCACCATGAG              | 17 | 7578229  | 7578406  |
| TP53_E00001255919_5   | AGCTGCTCACCATCGCTA          | CCAAGTGGCCAAGACCT             | 17 | 7578361  | 7578525  |
| TP53_E00001255919_6   | TGTGCTGTGACTGCTTGTAG        | TGCCCTGACTTTCAACTCTGT         | 17 | 7578425  | 7578594  |
| TP53_E00001612188_1   | ATACGGCCAGGCATTGAAGT        | CCTCCTGGCCCCTGTC              | 17 | 7579260  | 7579421  |
| TP53_E00001612188_2   | GGAAACCGTAGCTGCCCTG         | AAGACCCAGGTCCAGATGAA          | 17 | 7579359  | 7579520  |
| TP53_E00002359670     | CAGCCTCTGGCATTCTGG          | CCTGGTCTCTGACTGCTCT           | 17 | 7579479  | 7579626  |
| TP53_E00002419584     | TCAAATCATCCATTGCTTGG        | CCATGGGACTGACTTTCTGC          | 17 | 7579557  | 7579754  |
| TP53_E00001596491_1   | TTTCGCTTCCCACAGGTCTC        | CAGCCAGACTGCCTTCCG            | 17 | 7579758  | 7579940  |

\*Each of these 3 primer pairs potentially amplifies an additional non-target region, owing to large regions of homology and the short amplicon size. This was taken into account in data analysis.

**Table S2: Unique sequencing barcodes.** Platform-specific adaptors and barcodes are attached through PCR following the single-plex amplification step. The primers consisted of the PE1 and PE2 sequences for Illumina cluster generation, a 10-bp barcode, and the CS1 and CS2 adaptors, used in pairs: PE1-CS1 with PE2-BC-CS2, and PE1-CS2 with PE2-BC-CS1.

PE1: AATGATACGGCGACCACCGAGATCT.

PE2: CAAGCAGAAGACGGCATAACGAGAT.

| Barcode name | Barcode sequence |         |            |         |             |
|--------------|------------------|---------|------------|---------|-------------|
| FLD0001      | GTATCGTCGT       | FLD0060 | GCACGTAGCT | FLD0121 | CAGAGCTAGT  |
| FLD0002      | GTGTATGCGT       | FLD0061 | TCACGCTATG | FLD0122 | CGCAGAGCAT  |
| FLD0003      | TGCTCGTAGT       | FLD0062 | CGTACTACGT | FLD0123 | TGTACAGCGA  |
| FLD0004      | GTCGTGCTCT       | FLD0063 | CAGCTGAGTA | FLD0124 | ACGTACAGTAT |
| FLD0005      | GTGCGTGTGT       | FLD0064 | GAGATCAGTC | FLD0125 | TCACAGCATA  |
| FLD0006      | GCGTCGTGTA       | FLD0065 | TACTGAGCTG | FLD0126 | ACTGCGTGTCT |
| FLD0007      | GTCGTGTA         | FLD0066 | TAGTAGCGCG | FLD0127 | CGATCGACTG  |
| FLD0008      | GATGTAGCGT       | FLD0067 | GACGTCTGCT | FLD0128 | GCGAGATGTA  |
| FLD0009      | GAGTGATCGT       | FLD0068 | TACTCGCGA  | FLD0129 | CTGATGCAGA  |
| FLD0010      | CGCTATCAGT       | FLD0069 | TCTGAGCGCA | FLD0130 | GTGACGTACG  |
| FLD0011      | CGCTGTAGTC       | FLD0070 | TAGACGTGCT | FLD0131 | CGACGCTGAT  |
| FLD0012      | GCTAGTGAGT       | FLD0071 | GTGACTCGTC | FLD0132 | CTACGATCAG  |
| FLD0013      | GAGCTAGTGA       | FLD0072 | TCGAGTAGCG | FLD0133 | GCATGCAGCA  |
| FLD0014      | CGTGCTGTCA       | FLD0073 | CGTATGATGT | FLD0134 | CTAGCAGATG  |
| FLD0015      | GATCGTCTCT       | FLD0074 | TAGTCTGTCA | FLD0135 | CATGATACGC  |
| FLD0016      | GTGCTGTCGT       | FLD0075 | TGTCCTATC  | FLD0136 | GCAGCTGTCA  |
| FLD0017      | TGAGCGTGCT       | FLD0076 | CTAGAGTATC | FLD0137 | ACGTATCATC  |
| FLD0018      | CATGTCGTCA       | FLD0077 | TATCATGTGC | FLD0138 | AGTATCGTAC  |
| FLD0019      | TCAGTGTCTC       | FLD0078 | CATGAGTGTA | FLD0139 | GATACACTGA  |
| FLD0020      | GTGCTCATGT       | FLD0079 | TGTCGTCATA | FLD0140 | GACTAGTCAG  |
| FLD0021      | CGTATCTCGA       | FLD0080 | TATCTCATGC | FLD0141 | GATGACTACG  |
| FLD0022      | GTCATGCGTC       | FLD0081 | TGTGTCACTA | FLD0142 | CAGAGAGTCA  |
| FLD0023      | CTATGCGATC       | FLD0082 | TATCGATGCT | FLD0143 | TCGATCGACA  |
| FLD0024      | TGCTATGCTG       | FLD0083 | TAGAGTCTGT | FLD0144 | ACTGATGTAG  |
| FLD0025      | TGTGTGCATG       | FLD0084 | CATGCATCAT | FLD0145 | ACTCGATAGT  |
| FLD0026      | GAGTGTCACT       | FLD0085 | TGATCAGTCA | FLD0146 | GACGATCGCA  |
| FLD0027      | TAGTCTCGT        | FLD0086 | CGTCTATGAT | FLD0147 | TCATCATGCG  |
| FLD0028      | GAGTGCATCT       | FLD0087 | GTGATACTGA | FLD0148 | ACATGTCTGA  |
| FLD0029      | TGCGTAGTCG       | FLD0088 | CTAGATCTGA | FLD0149 | AGTCATCGCA  |
| FLD0030      | CTGTGTCGTC       | FLD0089 | TATCAGTCTG | FLD0150 | TAGCATAACG  |
| FLD0031      | CTGTAGTGCG       | FLD0090 | TCAGATGCTA | FLD0151 | AGAGTCGCGT  |
| FLD0032      | GTGCGCTAGT       | FLD0091 | TATGTACGTG | FLD0152 | TCTACGACAT  |
| FLD0033      | TGTGCTCGCA       | FLD0092 | CTATACAGTG | FLD0153 | CACGAGATGA  |
| FLD0034      | GATGCGAGCT       | FLD0093 | TGATACTCTG | FLD0154 | ACGCACATAT  |
| FLD0035      | CTGTACGTGA       | FLD0094 | TCAGCGATAT | FLD0155 | ACGTGCTCTG  |
| FLD0036      | GCGATGATGA       | FLD0095 | CTACTGATGA | FLD0156 | ACGATCACAT  |
| FLD0037      | TGTGAGTCA        | FLD0096 | GCTATACACA | FLD0157 | AGTGTACTCA  |
| FLD0038      | GTCTACTGTC       | FLD0097 | TGCTACATCA | FLD0158 | TGATGTATGT  |
| FLD0039      | CAGTCAGAGT       | FLD0098 | AGTGTGTCTA | FLD0159 | GATATATGTC  |
| FLD0040      | CGCAGTCTAT       | FLD0099 | TCATATCGCG | FLD0160 | TAGTACTAGA  |
| FLD0041      | GTATGAGCAC       | FLD0100 | TACGTATAGC | FLD0161 | TATAGAGATC  |
| FLD0042      | CGAGTGCTGT       | FLD0101 | CAGCTATAGC | FLD0162 | TCGATATCTA  |
| FLD0043      | TATAGCACGC       | FLD0102 | TCGATGCGCT | FLD0163 | TACATGATAG  |
| FLD0044      | TCATGCGCGA       | FLD0103 | GCACGCGTAT | FLD0164 | TGAGATCATA  |
| FLD0045      | TATGCGCTGC       | FLD0104 | GCAGTATGCG | FLD0165 | CTACATACTA  |
| FLD0046      | TCTCTGTGCA       | FLD0105 | TGATAGAGAG | FLD0166 | ATCAGTGTAT  |
| FLD0047      | CTATCGCGTG       | FLD0106 | GCTACTAGCG | FLD0167 | ATCATATCTC  |
| FLD0048      | TACGCTGCTG       | FLD0107 | TGCGAGACGT | FLD0168 | AGTAGATCAT  |
| FLD0049      | CTGCATGATC       | FLD0108 | CGATGACAGA | FLD0169 | ACATAGTATC  |
| FLD0050      | CGCGTATCAT       | FLD0109 | GACTCATGCT | FLD0170 | ATGTATAGTC  |
| FLD0051      | GTATCTCTCG       | FLD0110 | GTCTGATACG | FLD0171 | ACAGTCATAT  |
| FLD0052      | GCTCATATGC       | FLD0111 | ACTAGCTGTC | FLD0172 | ACATATACGT  |
| FLD0053      | CACTATGTGCG      | FLD0112 | CGGTAGACGA | FLD0173 | AGCATCTATA  |
| FLD0054      | TAGCGCGTAG       | FLD0113 | CTCAGCAGTG | FLD0174 | AGACTATATC  |
| FLD0055      | CGTCACAGTA       | FLD0114 | CAGTCTACAT | FLD0175 | CAGCATCTAG  |
| FLD0056      | TCGCGTGAGA       | FLD0115 | TACTGCAGCG | FLD0176 | CGAGACGACA  |
| FLD0057      | TACATCGCTG       | FLD0116 | TACACAGTAG | FLD0177 | ATCACTCATA  |
| FLD0058      | GTGAGAGACA       | FLD0117 | CACATACAGT | FLD0178 | AGCTCTGTGA  |
| FLD0059      | GACTGTACGT       | FLD0118 | CACAGTGATG | FLD0179 | ATGTATGCTG  |
|              |                  | FLD0119 | CGAGCTAGCA | FLD0180 | GCTGACAGAG  |
|              |                  | FLD0120 | GAGACTATGC | FLD0181 | ATACAGTCTC  |

|         |             |         |            |         |            |
|---------|-------------|---------|------------|---------|------------|
| FLD0182 | CATAGACGTG  | FLD0250 | GAAGGAGATA | FLD0318 | GAGAGGACAT |
| FLD0183 | AGAGATATCA  | FLD0251 | CGAATGTATG | FLD0319 | GAGCACGGAA |
| FLD0184 | ATGCTGCGCT  | FLD0252 | TCGTGAATGA | FLD0320 | GCTCTAACAT |
| FLD0185 | AGTCAGACGC  | FLD0253 | GAATAGCTGA | FLD0321 | TGCTGGCTTG |
| FLD0186 | ACGATACACT  | FLD0254 | TTGTCACATC | FLD0322 | TGCATGGAGC |
| FLD0187 | AGCGAGTATG  | FLD0255 | TTGGAGGCTA | FLD0323 | GTACTAAGAG |
| FLD0188 | ATCGCTACAT  | FLD0256 | TGTCAGCTTA | FLD0324 | GAAGTCAAGC |
| FLD0189 | ATGCTAGAGA  | FLD0257 | GTTCTTCGTA | FLD0325 | GCGCATTATG |
| FLD0190 | AGCAGTACTC  | FLD0258 | TTACACGTTT | FLD0326 | GTCCAGACAT |
| FLD0191 | ATCTAGATCA  | FLD0259 | GTAGCCAGTA | FLD0327 | GAGACCTCTA |
| FLD0192 | ATCGCATAGA  | FLD0260 | TGAGAAGGTA | FLD0328 | TTGCACTCAG |
| FLD0193 | TTGTTGCTGT  | FLD0261 | CCATATGATC | FLD0329 | TGCGGCGATA |
| FLD0194 | GTGTGGTTGT  | FLD0262 | CGATCCTATA | FLD0330 | TTGCTCAGAT |
| FLD0195 | TAGGTGGAAT  | FLD0263 | TGACTAGCTT | FLD0331 | AGGATTGAGG |
| FLD0196 | TGTAGGTGGA  | FLD0264 | TAACCTGCT  | FLD0332 | CCAGAACAGA |
| FLD0197 | TTAGTGGTGA  | FLD0265 | TCGAATGTGC | FLD0333 | CGTCAAGCAT |
| FLD0198 | GTGAAGGTAA  | FLD0266 | TGCGTGAACA | FLD0334 | TTGTCGAGAC |
| FLD0199 | TGTTGTGGTA  | FLD0267 | GCGTTATTGC | FLD0335 | GACAGGTGAC |
| FLD0200 | GTTGATGAGT  | FLD0268 | GAACATCAC  | FLD0336 | CTGACAAGTG |
| FLD0201 | GGTCAGTGTA  | FLD0269 | TCGAGGTA   | FLD0337 | CACGAAGAGC |
| FLD0202 | GTAATGGAGT  | FLD0270 | TGCGGATGGT | FLD0338 | CATACCTGAT |
| FLD0203 | CTCGTTATTC  | FLD0271 | TTCGAGCTAT | FLD0339 | GACGTGCTTC |
| FLD0204 | GGAAGTAAGG  | FLD0272 | GGTCTGGTGT | FLD0340 | ATTGTGGAGT |
| FLD0205 | CGGTGTGTGT  | FLD0273 | CTAAGTCATG | FLD0341 | TCTGGTCTCA |
| FLD0206 | CGTCTTCTTA  | FLD0274 | TTGCAGATCA | FLD0342 | AAGTAAGAGG |
| FLD0207 | TGTGAATCTC  | FLD0275 | CTGCGAATGT | FLD0343 | TCCTGACAGA |
| FLD0208 | CTAATCGTGT  | FLD0276 | CTGTTCTAGC | FLD0344 | GCACTGTTGC |
| FLD0209 | CTCTTAGTTC  | FLD0277 | CACCTGTGTG | FLD0345 | ACCATGAGTC |
| FLD0210 | GGATAGGATC  | FLD0278 | TGGATGACAT | FLD0346 | AATGCAGTGT |
| FLD0211 | GGTGTCTTGT  | FLD0279 | GATCCTGAGC | FLD0347 | ATATGGTGGG |
| FLD0212 | GATGGTTGTA  | FLD0280 | GTGCGTCTGA | FLD0348 | ACTCAGTTAC |
| FLD0213 | CCTCGTTGTT  | FLD0281 | TGTTACGATC | FLD0349 | AAGTGCGATG |
| FLD0214 | GGTTGGAGTT  | FLD0282 | GTCTTGCTC  | FLD0350 | CCACAGAGTG |
| FLD0215 | TGGTGTCCGT  | FLD0283 | GGTCGTGCAT | FLD0351 | AGTGGTGATC |
| FLD0216 | CGTTAGCGTA  | FLD0284 | CAGGCTCAGT | FLD0352 | ACTTCTTAGC |
| FLD0217 | TACTAGGATC  | FLD0285 | TAGCTTCACT | FLD0353 | GCCACATATA |
| FLD0218 | GTCTCAATGT  | FLD0286 | TGATGTCCT  | FLD0354 | AATGCAGTGT |
| FLD0219 | GATGAGGTAT  | FLD0287 | TTACGCAGTG | FLD0355 | AATATGCTGC |
| FLD0220 | GGTGTTAGTG  | FLD0288 | TTCGTTCCTG | FLD0356 | AAGCGTAGAA |
| FLD0221 | CATTCTCTGA  | FLD0289 | CACCTGCTGA | FLD0357 | GACAGCAAGC |
| FLD0222 | CATCTGGAGT  | FLD0290 | TCTAGCGTGG | FLD0358 | CTGACCGAGA |
| FLD0223 | GAATGGAAGA  | FLD0291 | GCATAATCGC | FLD0359 | CGGCACTTGT |
| FLD0224 | GGCTGTGATC  | FLD0292 | GTGTAACAC  | FLD0360 | CATCAACATG |
| FLD0225 | TGGTGCTGGA  | FLD0293 | GAGATTGCTA | FLD0361 | TGGCTACGCT |
| FLD0226 | TATGGTAAGG  | FLD0294 | GGACAGATGG | FLD0362 | ACGCGACTA  |
| FLD0227 | GTTTCGATTGT | FLD0295 | CTTACGTTGC | FLD0363 | AGAGGTCCGA |
| FLD0228 | GGTAGAATGA  | FLD0296 | GTGTTGCGTC | FLD0364 | AATCGAGCGT |
| FLD0229 | TTCTCATCGT  | FLD0297 | CTCAAGAAGC | FLD0365 | AAGTACACTC |
| FLD0230 | CTCAATCGTA  | FLD0298 | TCTCGGATAG | FLD0366 | AGCTGAATGA |
| FLD0231 | CGCTAATGTA  | FLD0299 | CTCTGGACGA | FLD0367 | ATGCCTATCA |
| FLD0232 | GCGTCTGAAT  | FLD0300 | CGAGCATTGT | FLD0368 | ACTGTAGGAC |
| FLD0233 | TTCTGTTGCC  | FLD0301 | CCAAGAAGAA | FLD0369 | ATAGCCGTGT |
| FLD0234 | TTGTCCTTGC  | FLD0302 | TCCTTGTCT  | FLD0370 | TCACGACGAA |
| FLD0235 | CCTGTGTAGA  | FLD0303 | GTAACGATGT | FLD0371 | ATCTGTCCAT |
| FLD0236 | GATAAGAAGG  | FLD0304 | TGGACTCAGA | FLD0372 | ACTTAGAGAG |
| FLD0237 | CAGGTCACAT  | FLD0305 | GGCATCATGC | FLD0373 | AGTGGCAGGT |
| FLD0238 | GCCATGTCAT  | FLD0306 | GTATAACGCT | FLD0374 | ATGAGGTCGT |
| FLD0239 | TCTGCCTATA  | FLD0307 | GCAGATAAGT | FLD0375 | AGGAGAAGGA |
| FLD0240 | CTTAGTTCGC  | FLD0308 | GTGCGCTCTA | FLD0376 | ACAACGCAA  |
| FLD0241 | CGTAATGAGC  | FLD0309 | TTGATAGCA  | FLD0377 | ATTAGCGAGT |
| FLD0242 | TTGCTTAGTC  | FLD0310 | GTCTAGCAGG | FLD0378 | ACAACGAACA |
| FLD0243 | TCTTGTTCAC  | FLD0311 | GGAACACAGG | FLD0379 | AGAGCGCCAA |
| FLD0244 | GTGGCTTCGT  | FLD0312 | TGGTTCGCTG | FLD0380 | AGGTAGCTCA |
| FLD0245 | TGTTTCGATAG | FLD0313 | GCAATTAGCG | FLD0381 | AACGCCAAGA |
| FLD0246 | TCATTCAAGT  | FLD0314 | GAAGCGCACT | FLD0382 | AAGGTATGAG |
| FLD0247 | GTGGAGAGCT  | FLD0315 | GATGCCAGT  | FLD0383 | ATGGAGCACT |
| FLD0248 | GTAGAAGTGG  | FLD0316 | GGAGACTGTA | FLD0384 | ACGGTGCTAG |
| FLD0249 | TGGAGCATGT  | FLD0317 | TCGAAGTGA  |         |            |

**Table S3: Mutations identified in FFPE samples.** Diagnosis: type (FT, fallopian tube; O, ovarian; PP, primary peritoneal), histiotype (END, endometrioid, grade III; HGSOC, high-grade serous ovarian carcinoma; S/CC, mixed serous and clear cell; S/END, mixed serous papillary and endometrioid, high grade), and stage. FFPE source: sample type (B, biopsy; IDS, interval-debulking surgery; PS, primary surgery), sample tissue (FT, fallopian tube; O, ovary; OM, omentum; P, peritoneum; SC, sigmoid colon; U, uterus). Cellularity: estimated from H&E-stained slide of the FFPE block. Identified by Sanger sequencing: I, identified; FAIL, samples where one duplicate had a technical fault; Indel, indel identified (indels were not analyzed by the calling algorithm); M, missed; U, unsequenced region or sample. S-135 and S-165 were from the same patient, but collected at different timepoints. S-140 and S-106 were from the same block, cut in different batches. S-111, S-143, and S-122 had two mutations called each. S-133, S-169, and S-147 had known indels, and did not have any point mutations called. S-119 had no mutations called. S-176, S-177, and S-178 failed harvesting in one of the repeats and calling algorithm was not applied.

| Patient ID | Age at diagnosis | Diagnosis: type, histiotype, and stage. | Sample ID | FFPE source | Cellularity | Gene | Mutation and base change (genome build hg19) | cDNA change | Protein change | TAm-Seq Allele frequency (repeat 1) | TAm-Seq Allele frequency (repeat 2) | Identified by Sanger sequencing |
|------------|------------------|---|-----------|-------------|-------------|------|--|-------------|----------------|-------------------------------------|-------------------------------------|---------------------------------|
| 1          | 60               | O HGSOC III                             | S-131     | PS O        | 60%         | TP53 | 17:7577570 C>T                               | c.711G>A    | p.M237I        | 0.72                                | 0.70                                | I                               |
| 2          | 63               | O HGSOC III                             | S-142     | IDS O       | 40%         | TP53 | 17:7578203 C>A                               | c.646G>T    | p.V216L        | 0.42                                | 0.43                                | I                               |
| 3          | 54               | O HGSOC III                             | S-150     | IDS O       | 70%         | TP53 | 17:7577538 C>T                               | c.743G>A    | p.R248Q        | 0.87                                | 0.91                                | I                               |
| 4          | 46               | O HGSOC I                               | S-167     | PS O        | 70%         | TP53 | 17:7578290 C>T                               | c.560-1G>A  | p.?            | 0.73                                | 0.73                                | I                               |
| 5          | 38               | O HGSOC III                             | S-174     | PS O        | 90%         | TP53 | 17:7578268 A>G                               | c.581T>C    | p.L194P        | 0.69                                | 0.72                                | I                               |
| 6          | 63               | O HGSOC III                             | S-102     | IDS FT      | 60%         | TP53 | 17:7578271 T>C                               | c.578A>G    | p.H193R        | 0.39                                | 0.34                                | I                               |
| 7          | 68               | O HGSOC III                             | S-112     | IDS O       | 60%         | TP53 | 17:7578263 G>A                               | c.586C>T    | p.R196*        | 0.30                                | 0.28                                | I                               |
| 8          | 60               | O HGSOC III                             | S-132     | PS O        | 90%         | TP53 | 17:7577120 C>T                               | c.818G>A    | p.R273H        | 0.74                                | 0.75                                | I                               |
| 9          | 62               | PP HGSOC IV                             | S-151     | PS SC       | 70%         | TP53 | 17:7578479 G>A                               | c.451C>T    | p.P151S        | 0.39                                | 0.38                                | I                               |
| 10         | 58               | PP HGSOC IV                             | S-168     | IDS O       | 90%         | TP53 | 17:7578508 C>T                               | c.422G>A    | p.C141Y        | 0.82                                | 0.86                                | I                               |
| 11         | 55               | O HGSOC III                             | S-104     | IDS O       | 15%         | TP53 | 17:7578370 C>T                               | c.559+1G>A  | p.?            | 0.12                                | 0.12                                | M                               |
| 12         | 62               | O HGSOC IV                              | S-115     | IDS O       | 70%         | TP53 | 17:7577579 G>T                               | c.702C>A    | p.Y234*        | 0.57                                | 0.59                                | I                               |
| 13         | 45               | O HGSOC IV                              | S-123     | IDS O       | 80%         | TP53 | 17:7577574 T>C                               | c.707A>G    | p.Y236C        | 0.53                                | 0.55                                | I                               |
| 14         | 58               | O HGSOC III                             | S-153     | IDS O       | 95%         | TP53 | 17:7578212 G>A                               | c.637C>T    | p.R213*        | 0.56                                | 0.56                                | I                               |
| 15         | 61               | O HGSOC IV                              | S-116     | IDS O       | 98%         | TP53 | 17:7577121 G>A                               | c.817C>T    | p.R273C        | 0.76                                | 0.75                                | I                               |
| 16         | 65               | O HGSOC III                             | S-127     | IDS O       | 90%         | TP53 | 17:7577094 G>A                               | c.844C>T    | p.R282W        | 0.76                                | 0.74                                | I                               |
| 17         | 64               | O HGSOC III                             | S-145     | PS FT       | 60%         | TP53 | 17:7578176 C>T                               | c.672+1G>A  | p.?            | 0.52                                | 0.55                                | I                               |
| 18         | 63               | FT HGSOC III                            | S-161     | PS O        | 95%         | TP53 | 17:7578534 C>G                               | c.396G>C    | p.K132N        | 0.86                                | 0.88                                | I                               |
| 19         | 68               | PP HGSOC III                            | S-170     | IDS U       | 50%         | TP53 | 17:7579312 C>A                               | c.375G>T    | p.T125T        | 0.22                                | 0.20                                | I                               |

|    |    |    |       |     |       |     |    |     |      |             |     |            |         |      |      |       |
|----|----|----|-------|-----|-------|-----|----|-----|------|-------------|-----|------------|---------|------|------|-------|
| 20 | 67 | PP | HGSOC | III | S-108 | IDS | OM | 20% | TP53 | 17:7578431  | G>A | c.499C>T   | p.Q167* | 0.74 | 0.93 | I     |
| 21 | 64 | O  | HGSOC | I   | S-117 | PS  | O  | 95% | TP53 | 17:7574012  | C>A | c.1015G>T  | p.E339* | 0.70 | 0.70 | I     |
| 22 | 65 | O  | HGSOC | III | S-128 | IDS | O  | 95% | TP53 | 17:7579521  | C>A | c.166G>T   | p.E56*  | 0.68 | 0.67 | I     |
| 23 | 54 | O  | HGSOC | III | S-138 | IDS | OM | 95% | TP53 | 17:7577569  | A>C | c.712T>G   | p.C238G | 0.79 | 0.79 | I     |
| 24 | 63 | FT | HGSOC | III | S-146 | PS  | O  | 60% | TP53 | 17:7577559  | G>T | c.722C>A   | p.S241Y | 0.62 | 0.62 | I     |
| 25 | 61 | PP | HGSOC | III | S-162 | IDS | OM | 40% | TP53 | 17:7578404  | A>T | c.526T>A   | p.C176S | 0.11 | 0.19 | I     |
| 26 | 72 | PP | HGSOC | III | S-171 | IDS | OM | 50% | TP53 | 17:7578265  | A>T | c.584T>A   | p.I195N | 0.71 | 0.70 | I     |
| 27 | 68 | O  | HGSOC | III | S-179 | IDS | O  | 90% | TP53 | 17:7578262  | C>G | c.587G>C   | p.R196P | 0.83 | 0.86 | I     |
| 28 | 46 | O  | HGSOC | III | S-109 | IDS | O  | 50% | TP53 | 17:7577548  | C>T | c.733G>A   | p.G245S | 0.40 | 0.39 | I     |
| 29 | 82 | O  | HGSOC | III | S-118 | IDS | OM | 80% | TP53 | 17:7577568  | C>G | c.713G>C   | p.C238S | 0.74 | 0.75 | I     |
| 30 | 69 | FT | HGSOC | III | S-130 | PS  | O  | 80% | TP53 | 17:7579389  | G>A | c.298C>T   | p.Q100* | 0.75 | 0.79 | I     |
| 31 | 64 | O  | HGSOC | III | S-172 | IDS | OM | 70% | TP53 | 17:7578406  | C>T | c.524G>A   | p.R175H | 0.48 | 0.42 | I     |
| 32 | 71 | O  | HGSOC | III | S-101 | IDS | P  | 90% | TP53 | 17:7578370  | C>T | c.559+1G>A | p.?     | 0.95 | 0.94 | I     |
| 33 | 73 | O  | HGSOC | III | S-144 | IDS | OM | 70% | TP53 | 17:7577557  | A>C | c.724T>G   | p.C242G | 0.44 | 0.47 | I     |
| 34 | 54 | O  | HGSOC | IV  | S-165 | IDS | O  | 95% | TP53 | 17:7578527  | A>G | c.403T>C   | p.C135R | 0.47 | 0.46 | I     |
|    |    |    |       |     | S-135 | B   | OM | 80% | TP53 | 17:7578527  | A>G | c.403T>C   | p.C135R | 0.57 | 0.60 | I     |
| 35 | 55 | PP | S/END | III | S-106 | PS  | O  | 90% | TP53 | 17:7574021  | C>A | c.1006G>T  | p.E336* | 0.96 | 0.96 | I     |
|    |    |    |       |     | S-140 |     |    | 90% | TP53 | 17:7574021  | C>A | c.1006G>T  | p.E336* | 0.96 | 0.96 | I     |
| 36 | 52 | O  | HGSOC | III | S-111 | PS  | O  | 95% | TP53 | 17:7578502  | A>T | c.428T>A   | p.V143E | 0.45 | 0.44 | I     |
|    |    |    |       |     |       |     |    | 95% | TP53 | 17:7579642  | C>T | Intron     | Intron  | 0.22 | 0.28 | M     |
| 37 | 45 | O  | END   | II  | S-143 | PS  | O  | 95% | TP53 | 17:7577538  | C>T | c.743G>A   | p.R248Q | 0.75 | 0.77 | I     |
|    |    |    |       |     |       |     |    | 95% | PTEN | 10:89720778 | A>G | c.929A>G   | p.D310G | 0.59 | 0.52 | I     |
| 38 | 55 | O  | END   | IV  | S-122 | IDS | O  | 80% | PTEN | 10:89692791 | A>G | c.275A>G   | p.D92G  | 0.19 | 0.18 | M     |
|    |    |    |       |     |       |     |    | 80% | PTEN | 10:89685287 | A>G | c.182A>G   | p.H61R  | 0.17 | 0.17 | I     |
| 39 | 54 | O  | HGSOC | III | S-119 | PS  | OM | 80% |      |             |     |            |         |      |      | U     |
| 40 | 71 | O  | HGSOC | III | S-133 | IDS | OM | 80% |      |             |     |            |         |      |      | Indel |
| 41 | 66 | O  | HGSOC | III | S-169 | IDS | OM | 50% |      |             |     |            |         |      |      | Indel |
| 42 | 68 | FT | S/CC  | III | S-147 | IDS | O  | 90% |      |             |     |            |         |      |      | Indel |
| 43 | 49 | FT | HGSOC | III | S-176 | PS  | O  | 95% |      |             |     |            |         |      | FAIL |       |
| 44 | 73 | PP | HGSOC | IV  | S-177 | PS  | OM | 50% |      |             |     |            |         |      | FAIL |       |
| 45 | 44 | O  | HGSOC | III | S-178 | IDS | OM | 30% |      |             |     |            |         |      | FAIL |       |

**Table S4: SNPs identified in circulating DNA from two plasma control samples.**

| <b>SNP ID</b> | <b>Genomic location<br/>(genome build hg19)<br/>and genotypes</b> | <b>Normal plasma DNA 1<br/>("plasmaDna1")</b> | <b>Normal plasma DNA 2<br/>("plasmaDna2")</b> |
|---------------|---|---|---|
| rs1625895     | chr17:7578115 (T/C)   | TT  | TC  |
| rs1800899     | chr17:7576841 (A/G)   | AG  | AA  |
| rs17337360    | chr7:55260440 (C/T)   | CC  | TT  |
| rs1050171     | chr7:55249063 (G/A)   | GA  | GG  |
| rs10241451    | chr7:55248926 (T/C)   | TC  | TT  |

**Table S5: Frequency of SNP alleles in dilution series of DNA from control plasma.**

| Total amplifiable<br>copies | Minor SNP percentage         |       |       |       |       |       |       |
|-----------------------------|------------------------------|-------|-------|-------|-------|-------|-------|
|                             | 200.00                       | 80.00 | 32.00 | 12.80 | 5.12  | 2.05  | 0.82  |
| 2.05                        |                              |       |       |       |       | 40.00 | 16.00 |
| 5.12                        |                              |       |       |       |       | 40.00 | 16.00 |
| 12.80                       |                              |       |       |       | 40.00 | 16.00 | 6.40  |
| 32.00                       |                              |       |       | 40.00 | 16.00 | 6.40  | 2.56  |
| 80.00                       |                              |       | 40.00 | 16.00 | 6.40  | 2.56  | 1.02  |
| 200.00                      | 40.00                        | 16.00 | 6.40  | 2.56  | 1.02  | 0.41  | 0.16  |
| 500.00                      | 40.00                        | 16.00 | 6.40  | 2.56  | 1.02  | 0.41  | 0.16  |
|                             | 200.00                       | 80.00 | 32.00 | 12.80 | 5.12  | 2.05  | 0.82  |
|                             | Minor SNP amplifiable copies |       |       |       |       |       |       |

**Table S6: Additional data for Table 2 for mutations identified in plasma samples.** Patient ID links to patient ID in table S3 (for patients 46 to 56, sequencing of tumour FFPE DNA was not included in table S3). Age: at diagnosis. Time: elapsed since surgery (months); chemotherapy lines: number of previous lines of chemotherapy. Vol: Plasma per amplification reaction (μL). DNA: DNA amount per amplification (in ng) was calculated from the number of total amplifiable copies (both mutant and germline allele) determined by digital PCR. Mean depth: number of sequencing reads covering this locus. AF, TAM-Seq: mean allele frequency using TAM-Seq, with the freq in each replicate given in parenthesis. Mutation call: FP, called using TAM-Seq, but not detected in FFPE DNA, and not called using more stringent calling criteria; M, mutation found in FFPE DNA and detected at allele frequency >2% in the plasma sample by digital PCR, but only found by TAM-Seq in one of the two duplicate amplicon sets; V, validated in FFPE tumour sample from the same patient (or, in the case of *EGFR* mutation in patient 27, using replicate Sanger sequencing of dilute template) and by digital PCR in plasma DNA; V\*, sample failed amplification in the first run owing to a technical fault, but was reanalyzed and the mutation validated in FFPE tumour sample from the same patient and by digital PCR in plasma DNA.

| Sample number | Patient ID | Age | Time (months); chemotherapy lines | Vol | DNA (ng) | Gene        | Location (build hg19) and base change |     | Protein change | Mean depth | AF, TAM-Seq                                     | Mean AF, digital PCR | Mutation call |
|---------------|------------|-----|-----------------------------------|-----|----------|-------------|---------------------------------------|-----|----------------|------------|---|----------------------|---------------|
| 1             | 15         | 61  | 35; 4                             | 70  | 0.9      | <i>TP53</i> | 17:7577121                            | G>A | p.R273C        | 640        | 0.26 (0.227, 0.292)                             | 0.167                | V             |
| 2             | 3          | 54  | 44; 3                             | 160 | 4.2      | <i>TP53</i> | 17:7577538                            | C>T | p.R248Q        | 340        | 0.244 (0.245, 0.242)                            | 0.150                | V             |
| 3             | 3          | 54  | 59; 6                             | 160 | 5.7      | <i>TP53</i> | 17:7577538                            | C>T | p.R248Q        | 640        | 0.507 (0.49, 0.524)                             | 0.410                | V             |
| 4             | 14         | 58  | 62; 5                             | 120 | 9.9      | <i>TP53</i> | 17:7578212                            | G>A | p.R213X        | 810        | 0.059 (0.056, 0.062)                            | 0.035                | V             |
| 5             | 10         | 58  | 48; 3                             | 120 | 1.4      | <i>TP53</i> | 17:7578508                            | C>T | p.C141Y        | 680        | 0.021 (0.017, 0.024)                            | 0.013                | V             |
| 6             | 10         | 58  | 54; 4                             | 120 | 2.1      | <i>TP53</i> | 17:7578508                            | C>T | p.C141Y        | 720        | 0.044 (0.044, 0.044)                            | 0.038                | V             |
| 7             | 26         | 72  | 13; 1                             | 190 | 17.9     | <i>TP53</i> | 17:7578265                            | A>T | p.I195N        | 800        | 0.091 (0.081, 0.101)                            | 0.081                | V             |
| 8             | 31         | 64  | 21; 2                             | 160 | 14.8     | <i>TP53</i> | 17:7578406                            | C>T | p.R175H        | 510        | 0.608 (0.614, 0.602)                            | 0.627                | V             |
| 9             | 31         | 64  | 21; 2                             | 160 | 10.7     | <i>TP53</i> | 17:7578406                            | C>T | p.R175H        | 550        | 0.526 (0.529, 0.523)                            | 0.604                | V             |
| 10            | 31         | 64  | 21; 3                             | 160 | 6.1      | <i>TP53</i> | 17:7578406                            | C>T | p.R175H        | 530        | 0.651 (0.705, 0.597)                            | 0.682                | V             |
| 11            | 31         | 64  | 22; 3                             | 160 | 4.9      | <i>TP53</i> | 17:7578406                            | C>T | p.R175H        | 490        | 0.526 (0.548, 0.504)                            | 0.581                | V             |
| 13            | 47         | 54  | 12; 2                             | 160 | 2.8      | <i>TP53</i> | 17:7578527                            | A>G | p.C135R        | 480        | 0.039 (0.034, 0.045)                            | 0.045                | V             |
| 14            | 47         | 54  | 18; 2                             | 160 | 2.5      | <i>TP53</i> | 17:7578527                            | A>G | p.C135R        | 610        | 0.046 (0.047, 0.045)                            | 0.120                | V             |
| 15            | 47         | 54  | 20; 3                             | 160 | 3        | <i>TP53</i> | 17:7578527                            | A>G | p.C135R        | 470        | 0.091 (0.133, 0.05)                             | 0.068                | V             |
| 16            | 27         | 68  | 25; 3                             | 130 | 3.7      | <i>TP53</i> | 17:7578262                            | C>G | p.R196P        | 1070       | 0.088 (0.093, 0.084)                            | 0.135                | V             |
| 16            | 27         | 68  | 25; 3                             | 130 | 3.7      | <i>EGFR</i> | 7:55259437                            | G>A | p.R832H        | 614        | 0.048 (0.055, 0.068, 0.008, 0.062) <sup>†</sup> | 0.050                | V             |

|    |    |    |       |     |      |      |             |     |            |      |                      |       |    |
|----|----|----|-------|-----|------|------|-------------|-----|------------|------|----------------------|-------|----|
| 17 | 25 | 61 | 18; 2 | 160 | 4.2  | TP53 | 17:7578404  | A>T | p.C176S    | 580  | 0.113 (0.106, 0.12)  | 0.432 | V  |
| 18 | 25 | 61 | 24; 3 | 160 | 4.4  | TP53 | 17:7578404  | A>T | p.C176S    | 620  | 0.029 (0.024, 0.033) | 0.108 | V  |
| 20 | 46 | 56 | 48; 6 | 140 | 5.2  | TP53 | 17:7578406  | C>T | p.R175H    | 650  | 0.201 (0.205, 0.197) | 0.226 | V  |
| 21 | 46 | 56 | 49; 6 | 140 | 3.6  | TP53 | 17:7578406  | C>T | p.R175H    | 650  | 0.085 (0.083, 0.087) | 0.074 | V  |
| 22 | 46 | 56 | 50; 6 | 140 | 4.1  | TP53 | 17:7578406  | C>T | p.R175H    | 630  | 0.081 (0.053, 0.109) | 0.125 | V  |
| 23 | 46 | 56 | 51; 6 | 140 | 3.7  | TP53 | 17:7578406  | C>T | p.R175H    | 710  | 0.074 (0.045, 0.103) | 0.106 | V  |
| 24 | 46 | 56 | 52; 6 | 140 | 7.1  | TP53 | 17:7578406  | C>T | p.R175H    | 760  | 0.269 (0.276, 0.261) | 0.286 | V  |
| 25 | 8  | 60 | 12; 1 | 130 | 3.9  | TP53 | 17:7577120  | C>T | p.R273H    | 750  | 0.094 (0.082, 0.106) | 0.099 | V  |
| 26 | 48 | 47 | 20; 2 | 160 | 5.7  | TP53 | 17:7577094  | G>A | p.R282W    | 640  | 0.048 (0.059, 0.036) | 0.061 | V  |
| 27 | 55 | 68 | 11; 2 | 150 | 3.6  | TP53 | 17:7578508  | C>T | p.C141Y    | 480  | 0.321 (0.342, 0.3)   | 0.364 | V  |
| 29 | 50 | 71 | 0; 1  | 150 | 9.5  | TP53 | 17:7577509  | C>T | p.E258K    | 190  | 0.548 (0.534, 0.563) | 0.253 | V  |
| 31 | 51 | 62 | 0; 1  | 160 | 3.6  | TP53 | 17:7578526  | C>T | p.C135Y    | 620  | 0.04 (0.058, 0.021)  | 0.034 | V  |
| 32 | 22 | 65 | 12; 1 | 140 | 2.4  | TP53 | 17:7579521  | C>A | p.E56X     | 1480 | 0.137 (0.147, 0.127) | 0.122 | V  |
| 33 | 18 | 63 | 48; 3 | 160 | 13.2 | TP53 | 17:7578534  | C>G | p.K132N    | 740  | 0.216 (0.238, 0.194) | 0.206 | V  |
| 34 | 18 | 63 | 49; 4 | 60  | 5.3  | TP53 | 17:7578534  | C>G | p.K132N    | 570  | 0.151 (0.173, 0.13)  | 0.201 | V  |
| 36 | 18 | 63 | 56; 4 | 160 | 5.8  | TP53 | 17:7578534  | C>G | p.K132N    | 620  | 0.191 (0.18, 0.202)  | 0.275 | V  |
| 37 | 18 | 63 | 57; 5 | 160 | 9.4  | TP53 | 17:7578534  | C>G | p.K132N    | 530  | 0.287 (0.245, 0.328) | 0.362 | V  |
| 38 | 18 | 63 | 58; 5 | 160 | 10.1 | TP53 | 17:7578534  | C>G | p.K132N    | 590  | 0.275 (0.267, 0.283) | 0.331 | V  |
| 39 | 18 | 63 | 59; 5 | 160 | 16.4 | TP53 | 17:7578534  | C>G | p.K132N    | 700  | 0.315 (0.302, 0.328) | 0.323 | V  |
| 40 | 18 | 63 | 60; 5 | 160 | 19.7 | TP53 | 17:7578534  | C>G | p.K132N    | 830  | 0.435 (0.439, 0.43)  | 0.482 | V  |
| 41 | 18 | 63 | 61; 6 | 160 | 15   | TP53 | 17:7578534  | C>G | p.K132N    | 730  | 0.452 (0.461, 0.442) | 0.445 | V  |
| 42 | 18 | 63 | 62; 6 | 160 | 8.5  | TP53 | 17:7578534  | C>G | p.K132N    | 560  | 0.185 (0.2, 0.17)    | 0.245 | V  |
| 43 | 56 | 53 | 15; 2 | 150 | 3.6  | TP53 | 17:7578290  | C>T | Splicing   | 680  | 0.143 (0.166, 0.12)  | 0.121 | V  |
| 30 | 52 | 55 | 49; 4 | 170 | 5.2  | TP53 | 17:7577569  | A>G | p.C238R    | 1543 | 0.071(0.064, 0.077)  | 0.073 | V* |
| 35 | 18 | 63 | 50; 4 | 60  | 2.7  | TP53 | 17:7578534  | C>G | p.K132N    | 620  | 0.015 (0.002, 0.029) | 0.073 | M  |
| 12 | 53 | 51 | 23; 3 | 160 | 17   | PTEN | 10:89720799 | T>G | Intergenic | 1200 | 0.013 (0.014, 0.012) | -     | FP |
| 37 | 18 | 63 | 57; 5 | 160 | 9.4  | TP53 | 17:7579790  | G>A | Intronic   | 380  | 0.031 (0.031, 0.03)  | -     | FP |

†The mutation locus in the *EGFR* gene was measured four separate times, in two overlapping amplicons in each of the two duplicates

**Table S7: Mutations and amplicons studied in one breast cancer patient.** To identify multiple mutations, genomic libraries from tumour and matched normal tissues were prepared using the standard Illumina paired-end sample preparation kit according to the manufacturer's instructions. DNA fragments of 300 bp were sequenced using paired-end 100-bp reads on an Illumina HiSeq 2000 sequencer to 30× genomic coverage. Sequencing reads were aligned to the hg19 reference genome using ELAND (Illumina) and subsequent data analysis was performed using the CASAVA 1.8 analysis pipeline (Illumina) to identify somatic mutations. We selected 10 mutations and designed an amplicon of <120 bp to cover each mutation. We validated these mutations by TAM-Seq in the tumour material and then followed them in serial plasma samples collected from the same patient.

| Gene            | Genomic location of amplicon<br>(genome build hg19) | Genomic location of mutation<br>(genome build hg19) | Reference allele | Mutant allele |
|-----------------|---|---|------------------|---------------|
| <i>CD1A</i>     | chr1:158226710-158226810                            | chr1:158226762                                      | C                | T             |
| <i>BUB1</i>     | chr2:111430288-111430374                            | chr2:111430343                                      | G                | A             |
| <i>IQCA1</i>    | chr2:237402413-237402499                            | chr2:237402439                                      | G                | A             |
| <i>PIK3CA</i>   | chr3:178936048-178936130                            | chr3:178936091                                      | G                | A             |
| <i>ARAP3</i>    | chr5:141041694-141041773                            | chr5:141041739                                      | G                | A             |
| <i>MET</i>      | chr7:116398593-116398675                            | chr7:116398635                                      | C                | T             |
| <i>GIMAP5</i>   | chr7:150439702-150439805                            | chr7:150439730                                      | C                | T             |
| <i>ZFYVE21</i>  | chr14:104195437-104195539                           | chr14:104195486                                     | G                | A             |
| <i>KIAA0406</i> | chr20:36641162-36641252                             | chr20:36641201                                      | C                | T             |
| <i>ADAMTS1</i>  | chr21:28214239-28214357                             | chr21:28214274                                      | C                | G             |