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Analytical Validation and Application of a Targeted Next-Generation Sequencing Mutation-Detection Assay for Use in Treatment Assignment in the NCI-MPACT Trial

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Robust and analytically validated assays are essential for clinical studies. We outline an analytical validation study of a targeted next-generation sequencing mutation-detection assay used for patient selection in the National Cancer Institute Molecular Profiling–Based Assignment of Cancer Therapy (NCI-MPACT) trial (NCT01827384). Using DNA samples from normal or tumor cell lines and xenografts with known variants, we assessed the sensitivity, specificity, and reproducibility of the NCI-MPACT assay in five variant types: single-nucleotide variants (SNVs), SNVs at homopolymeric (HP) regions (≥3 identical bases), small insertions/deletions (indels), large indels (gap ≥4 bp), and indels at HP regions. The assay achieved sensitivities of 100% for 64 SNVs, nine SNVs at HP regions, and 11 large indels, 83.33% for six indels, and 93.33% for 15 indels at HP regions. Zero false positives (100% specificity) were found in 380 actionable mutation loci in 96 runs of haplotype map cells. Reproducibility analysis showed 96.3% to 100% intraoperator and 98.1% to 100% interoperator mean concordance in detected variants and 100% reproducibility in treatment selection. To date, 38 tumors have been screened, 34 passed preanalytical quality control, and 18 had actionable mutations for treatment assignment. The NCI-MPACT assay is well suited for its intended investigational use and can serve as a template for developing next-generation sequencing assays for other cancer clinical trial applications. *(J Mol Diagn 2016, 18: 51–67; http://dx.doi.org/10.1016/j.jmoldx.2015.07.006)*

Cancer is a genetic disease in which accumulating somatic mutations eventually lead to the deregulation of cell proliferation and survival-signaling pathways. In some tumors, there is crucial dependence of cancer cell growth on somatic driver mutations. These deleterious mutations provide an opportunity for developing cancer-specific targeted therapies.[1,2] The development of U.S. Food and Drug Administration (FDA)–approved targeted cancer therapeutics has demonstrated that drug efficacy is dependent on the accurate detection of the presence of the target in tumor tissue. Such therapies require analytically validated diagnostic assays for the selection of patients for treatments.[3–6] Existing companion diagnostic assays are mainly single-analyte assays that require a dedicated biopsy specimen. Because pharmaceutical agents may target driver mutations that occur only in a small subset of cancers, and because each tumor may have different driver mutations, it

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is clear that, as more targeted treatments become available, the use of multianalyte assays that test for multiple mutations will be required for screening patients for the best possible treatment. This approach will add value in making the most efficient use of patients' biopsy samples.

The emergence of next-generation sequencing (NGS) technologies has dramatically transformed cancer research by aiding in the rapid discovery of genetic aberrations in a large number of tumors.[7–10] NGS technologies continue to evolve and are becoming comprehensive platforms for the development of diagnostic assays and for use in screening tumor specimens for somatic mutations in the clinical setting. Recent advances in NGS permit larger amounts of the genome to be sequenced in a single run, with lower-input DNA, faster turnaround time, and reduced costs, resulting in rapid adoption by laboratories.[7–12]

The Division of Cancer Treatment and Diagnosis, National Cancer Institute (NCI), is conducting a randomized pilot trial, the Molecular Profiling-Based Assignment of Cancer Therapy (NCI-MPACT), to assess the utility of applying sequencing data to the selection of treatment in cancer patients whose disease has progressed despite the administration of standard treatment (*https://clinicaltrials.gov/ct2/home*; trial number NCT01827384).[13] Apart from a few notable examples, it has not yet been established whether making therapeutic choices based on molecular profiling provides superior clinical benefit compared with treatment selection based on best clinical judgment. In NCI-MPACT, patients with actionable mutations will be randomly assigned, in a 2:1 ratio, to receive either a predefined targeted treatment agent based on their corresponding mutation status, or therapy from the complementary set of drugs (from the same predefined set of agents) not prospectively identified to target one of their mutations. The objectives of this trial are to assess whether the response rate (primary objective) or 4-month progression-free survival rate (secondary objective) will be improved in the treatment arm in which treatment was chosen based on sequencing results compared with the arm in which treatment was not selected based on sequencing results (Supplemental Figure S1).

In this trial, core needle biopsy samples obtained from patients' tumors on trial entry will be assayed on the Ion Torrent PGM (Thermo Fisher Scientific, Waltham, MA) using a customized AmpliSeq panel that interrogates genetic variants in targeted genomic regions. The FDA has the authority to review assays used for assigning treatment or stratifying patients (termed *integral assays*) in clinical trials for the necessity of an Investigational Device Exemption (Treatment Use of an Investigational Device, *http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=812.36*, last accessed July 2, 2015).[14,15] To do so, information on the validation of the NCI-MPACT assay, as well as the risk posed to the patient, were discussed with the FDA in presubmission meetings. We believe that this article will be of value to others and could serve as a template for preparing an NGS assay for clinical use.

Recent public discussions led by the FDA indicated a desire for all clinical laboratories using laboratory-developed NGS tests to follow some form of design control to ensure that tests are sufficiently robust for their intended clinical use. The intended use of the NCI-MPACT assay is to screen patients' tumor biopsy samples for actionable mutations of interest (aMOI), which would then be used for assigning a study treatment in the NCI-MPACT clinical study. To assign treatment arms in a timely manner, the NCI-MPACT assay workflow was streamlined to ensure a 7- to 10-day turnaround in reporting results (Figure 1). Patients eligible for this trial have histologically confirmed solid tumors that have progressed after at least one line of standard therapy, or for which there is no standard therapy that prolongs survival. This intended use of the assay that directed the desired criteria for the assay performance was agreed on by the study physicians (B.A.C. and A.P.C.) and the sequencing laboratory. In addition, it was agreed that the assessment of the NCI-MPACT assay result would require a high specificity threshold to prevent a patient from entering the study through a false-positive (FP) result.

Before the validation study, an assay-feasibility study was conducted to assess assay performance, to identify potential FP loci, to empirically determine assay quality-control (QC) metrics, and to establish and lock standard operating procedures. An initial feasibility study was performed by sequencing well-characterized specimens so that a preliminary assessment of assay sensitivity and specificity could be determined in combination with setting and examining different thresholds of assay quality metrics. An analytical validation plan was then developed that included the experimental design and expected performance criteria. A total of 191 NCI-MPACT assay runs were performed in this analytical validation study. The results of sensitivity, specificity, accuracy, and reproducibility assessments suggested that the NCI-MPACT assay was well-suited for the intended investigational use of the NCI-MPACT trial. This is the first report that details the analytical validation process of an NGS-based assay for use as an integral assay in a clinical study.

Materials and Methods

Cell Lines and Tumor Specimens

Cell lines (Supplemental Table S1) obtained from Frederick National Laboratory for Cancer Research (Frederick, MD), American Type Culture Collection (Manassas, VA), and Coriell Institute for Medical Research (Camden, NJ) were cultured using vendor-recommended conditions. A minimum of two core needle biopsy specimens (18 gauge) were obtained from each patient with cancer metastasis at the NIH Clinical Center after the completion of an informed-consent form using an Institution Review Board–approved protocol. Biopsy specimens were shipped in neutral buffered formalin and embedded in paraffin within

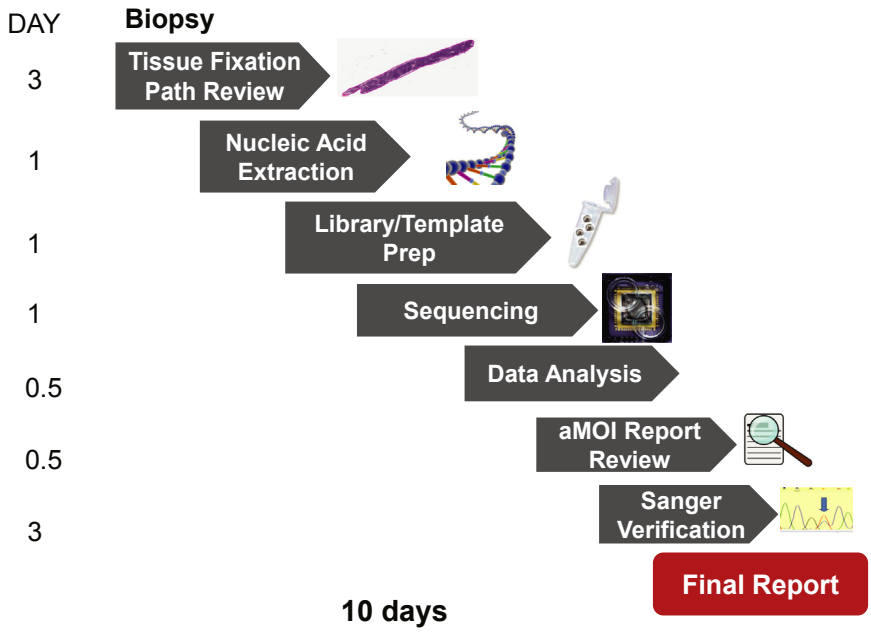

Figure 1 Workflows and turnaround time of the NCI-MPACT assay. Seven steps from receipt of biopsy to report are displayed in arrow-shaped boxes. The estimated turnaround times of each step and of the entire process are indicated at the left and bottom, respectively. aMOI, actionable mutations of interest.

48 hours of collection. Tumor content was assessed from a hematoxylin and eosin–stained, 4-μm section of the specimen. In each biopsy, the area of viable tumor and stroma was estimated by a certified pathologist. The estimated tumor content of the biopsy sample was calculated as (total tumor cellular content)/(tumor content + stromal cellular content). If tumor content was found to be <50% of the total cellular content in the section, a manual macrodissection was performed to enrich for tumor cells before further processing. Xenograft tissue samples established from cell lines were gifted by the Biological Testing Branch of the Developmental Therapeutics Program, NCI. Formalin-fixed, paraffin-embedded (FFPE) xenograft core needle biopsies and cell-line pellets were made using the same method.

Preparation of Control Plasmid Spike-in CEPH DNA

A positive-control DNA sample was generated by spiking 27 plasmids that carry MOIs into fresh-frozen (FF) haplotype map (HapMap) CEPH (Utah residents with ancestry from northern and western Europe) genomic DNA (NA12878) at 10% copy number ratio [control plasmid spike-in CEPH (CPSC) at 10%]. Control plasmids were constructed by inserting a 1000-bp genomic DNA fragment with the MOI (eg, *BRAF*, p.V600E) in the middle (approximately 500 bp from the ends) into a pUC19-based vector. A six-base (ACATCG) molecular barcode was introduced within 20 bp, but no closer than 5 bp, from the MOI as an identifier of the plasmid, to distinguish plasmid-borne mutations from endogenous mutations in the genome. The plasmids were constructed, using the gene-synthesis method, by a custom service company (DNA2.0, Menlo Park, CA). The full-length insert sequence was verified by Sanger sequencing. Plasmid DNA samples were linearized by a single-cut restriction enzyme on the plasmid backbone (*Sca*I or *Bgl*II; New England BioLabs, Ipswich, MA), and the completion of linearization was verified by a Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) and quantitated by NanoDrop (Thermo Fisher Scientific) and TaqMan real-time quantitative PCR using custom primers and probes (Thermo Fisher Scientific) against the vector backbone sequences. The quantitated, linearized plasmid DNA samples were pooled at an equal molar ratio, and the pooled plasmids were spiked in FF CEPH NA12878 DNA at 10% (plasmid versus CEPH) copy number ratio (CPSC at 10%). Detailed information of the mutations in the 27 control plasmids used in this study is listed in Supplemental Table S2.

DNA Extraction

FF cell-pellet DNA was extracted using the AllPrep DNA/RNA mini kit (Qiagen, Valencia, CA). FFPE cell pellet, xenograft, and clinical specimen DNA was extracted using the Qiagen AllPrep DNA/RNA FFPE kit. The entire core biopsy was used for nucleic acid extraction, with the exception of a section on the leading edge, which was used for hematoxylin and eosin staining. Briefly, the entire core needle biopsy sample (if tumor content was >50%) or fragments of an enriched tumor portion were removed from the paraffin blocks and deparaffinized by xylene and ethanol, followed by homogenization with 1.4-mm ceramic beads in 2-mL Ruptor tubes (Omni International, Kennesaw, GA). After proteinase K digestion, the genomic DNA samples were extracted using Qiagen columns per the vendor's instruction manual. DNA samples were quantitated by a NanoDrop spectrophotometer (Thermo Fisher Scientific).

Selection of Genes and Mutations Used for Treatment Assignment

Four therapeutic regimens targeting three different pathways are being evaluated in the NCI-MPACT trial (Table 1): i) trametinib, a MEK kinase inhibitor to target tumors with *RAS/RAF/MEK* pathway activation[16]; ii) everolimus, an mammalian target of rapamycin (mTOR) inhibitor, to target *AKT/PI3K/MTOR* pathway activation[17,18]; iii) veliparib, a poly(ADP-ribose) polymerase inhibitor[19] in combination with temozolomide to target DNA repair pathways with loss-of-function mutations; and iv) AZD1775, a WEE1 kinase inhibitor in combination with carboplatin to target DNA-repair pathways with loss-of-function mutations, especially in *TP53*.[20] A total of 20 genes (Table 1) were identified for use in treatment selection because these genes are either direct drug targets or targets upstream to the drug-targeted genes, and they have a mutation frequency of >5% in the Catalog of Somatic Mutation in Cancer database version 61 (COSMICv61).[21,22] These genes were categorized into two groups: gain-of-function mutations, which are predicted to activate gene function (eg, kinases and *RAS* genes), and loss-of-function mutations, which are predicted to inactivate gene function (eg, *PTEN* and *NF1* pathway inhibitors and DNA repair).

Because the NCI-MPACT trial was designed as a hypothesis-testing study, rules were developed for selecting variants considered to be aMOIs by an NCI-MPACT Molecular Tumor Board, which is composed of molecular biologists and clinical researchers using their best knowledge and logical assumptions. Gain-of-function gene variants were selected for which there was published evidence [in functional studies from either preclinical models or clinical studies (one case was acceptable)] of gain of function (literature rule), or for which there were two or more specimens reported in the COSMICv61 and <5% population frequency in the 1000 Genome Project (COSMIC rule). Loss-of-function gene variants were selected for which there was published evidence [in functional studies in preclinical models or clinical studies (one case was acceptable)] of loss of function (literature rule), or for which there was the creation of a stop codon, frameshift, or loss of the start codon (loss-of-function rule). References supporting those variants selected by the literature rule are appended in Supplemental Table S3. By applying these rules, 380 unique aMOIs with known COSMIC identifiers were found in 17 of the 20 genes. Three of the 20 genes, *MLH1*, *PARP2*, and *ERCC1*, contained no *a priori*—known aMOIs based on the criteria above. There were 262 single-nucleotide variants (SNVs), 7 SNVs at homopolymeric (HP) regions, 67 indels, 15 indels at HP, and 29 large indels in 380 aMOIs (Figure 2 and Supplemental Table S3[23–46]). Those 380 aMOIs plus any novel loss-of-function mutations in 13 tumor-suppressor and DNA-repair genes (*NF1, PTEN, FBXW7, ATM, ATR, MLH1, MSH2, NBN, RAD51, ERCC1, TP53, PARP1,* and *PARP2*) were defined as the reportable range for the NCI-MPACT assay. The aMOI-selection rules were implemented in GeneMed,[47] a web-based informatics system developed at NCI for identifying and annotating aMOIs from the Torrent Suite (TS) bioinformatics data—analysis pipeline Variant Call Format (VCF) files and then assigning the targeted treatment. GeneMed can also be used for identifying novel aMOIs (not reported in COSMICv61) that meet the above rules (eg, a novel stop codon in a DNA-repair gene).

Primer Design and Synthesis

The 380 aMOIs contained in the 20 selected genes were added to the hotspot list of a commercially available

Table 1 Drugs, Targeted Pathways, and Genes in the NCI-MPACT Trial

Drug	Pathway	Gain of function*	Loss of function†
Trametinib	RAS/RAF/MEK	*BRAF* *KRAS* *NRAS* *HRAS*	*NF1*
Everolimus	AKT/PI3K/MTOR	*AKT1* *PIK3CA* *MTOR*	*PTEN* *FBXW7*
Veliparib + temozolomide AZD1775 + carboplatin	DNA repair		*ATM* *ATR* *ERCC1* *MLH1* *MSH2* *NBN* *PARP1* *PARP2* *RAD51* *TP53*

*Genes whose actionable mutations lead to functional activation.
†Genes whose actionable mutations lead to functional inactivation.

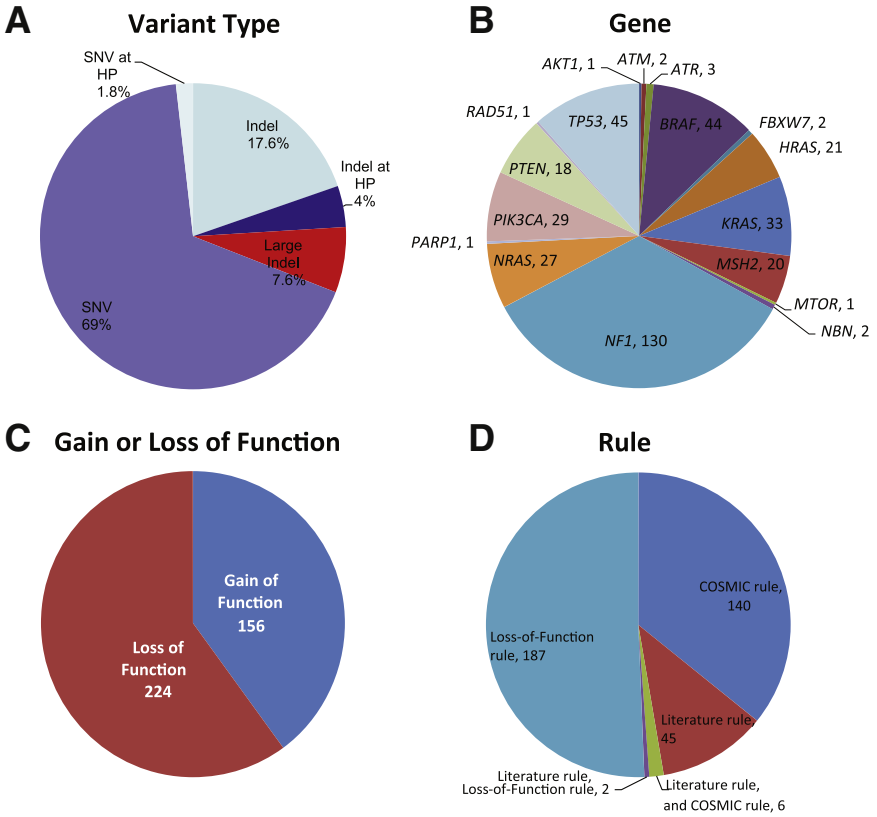

Figure 2 Distribution of 380 actionable mutations detected validation studies. Distribution of 380 actionable mutations of interest (aMOI) is categorized by the variant type (**A**), gene (**B**), gain or loss of function (**C**), and rules of aMOI selection (**D**). Numbers or percentages of variant counts are indicated. COSMIC, Catalog of Somatic Mutation in Cancer database[21,22]; HP, homopolymeric; SNV, single-nucleotide variant.

46-gene Ion AmpliSeq Cancer Hotspot Panel version 1 (Thermo Fisher Scientific) to generate a target list for amplicon design. After the omission of redundant genes, a total of 62 unique genes were assigned in the final NCI-MPACT panel (Supplemental Table S4). The primers in the NCI-MPACT AmpliSeq panel were designed and synthesized by the Ion AmpliSeq Designer. The resulting primer pools of 383 amplicons were divided into two tubes with an approximately equal number of amplicons. Supplemental Table S5 lists the Browser Extensible Data file for the NCI-MPACT panel.

AmpliSeq Library Preparation and Sequencing Reaction

Amplicon libraries were prepared from 20 ng of genomic DNA derived from patients' tumor biopsy samples, cell lines, CEPH NA12878 (the assay negative control), or CPSC at 10% (the assay positive control), following the protocol from Ion AmpliSeq Library kit version 2.0, with subtle modifications during library preparation to accommodate the two primer pools of the panel; the reaction PCR volume was reduced to 10 μL for each primer pool. All reagents used in the AmpliSeq library preparation, template preparation, and sequencing reaction were purchased from Thermo Fisher Scientific. Two half-reactions consisting of 10 ng of genomic DNA, 2 μL of 5X Ion AmpliSeq HiFi Master Mix, 5 μL of either 2X NCI-MPACT Primer Pool 1 or Pool 2, and 1 μL of nuclease-free water were subjected to 20 cycles (for FFPE DNA samples) of PCR following the manufacturer's recommended cycling conditions (17 cycles for DNA samples extracted from fresh or frozen cells). In addition to the DNA-containing reactions, a library no-template control (LibNTC) reaction containing water in place of the genomic DNA was run with each library preparation to detect PCR contamination. After this target-specific PCR reaction, the two half-reactions for each sample were combined and were digested with the FuPa enzyme from the AmpliSeq kit following the times and temperatures recommended. To each library, 2 μL of a single barcoded adaptor was ligated from the Ion Xpress Barcode Adaptors Kit (Thermo Fisher Scientific), and the adapted libraries were purified using Agencourt AMPure XP Beads (Beckman Coulter Genomics) per the manufacturer's recommendations. A 1:500 dilution aliquot of each library was prepared and subjected to real-time quantitative PCR with the Ion Library Quantitation kit, to quantify the libraries and to assess the LibNTC for PCR contamination. At least 10 μL of a 10-pmol/L dilution of each library was prepared, and up to five libraries, including the controls, were pooled together and subjected to emulsion PCR after the Ion PGM 200 Xpress Template Prep kit User Guide (Thermo Fisher Scientific). The templated Ion Sphere Particles from the emulsion PCR were loaded onto an Ion 316 chip and sequenced on the PGM using the Ion PGM 200 Sequencing Kit (Thermo Fisher Scientific), following the manufacturer's recommendations. The positive and negative controls prepared during the library preparation were run on each chip containing test samples, whereas the LibNTC was only run once per library batch.

Data Analysis

Sequencing data generated on the PGM were base called and aligned to the hg19 human reference sequence using the Ion TS version 3.2.1 (TS3.2.1; Thermo Fisher Scientific). The default parameters in TS3.2.1 were used without any further adjustment. The lower limit of detection was set at 4% for SNV, 15% for indel variants, and 2% for SNV-type aMOIs. Due to a known software issue that results in missing variants when an SNV and indel were detected in overlapping positions, the individual SNV and indel VCF files of a sample were merged by a script developed internally (Supplemental Script S1), and the merged VCF file was submitted to GeneMed to identify and annotate aMOIs[47]; GeneMed also reports variants that generate nonsynonymous substitution, frameshift, and loss of stop/start codon mutations using the SnpEff program.[48]

Assay Quality Metrics

To ensure the quality of the results obtained from the NCI-MPACT assay and to determine reportable results, documentation and QC systems were developed in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory. As a part of the specimen preanalytics, the following QC metrics were applied: i) >50% of the cells in the section were to have been tumor cells, or macrodissection of the remaining core was performed to enrich the tumor content; ii) a minimum of 20 ng of DNA was to have been recovered from a specimen; otherwise additional material was prepared (when possible); iii) the adaptor-ligated amplicon library yield was to have been >10 pmol/L in 50 μL of volume; and iv) the LibNTC was not to have shown a signal indicating contamination of the reagents used for preparing the libraries. If any of the preanalytics QC criteria failed, the specimen was processed a second time starting from DNA; however, if the second attempt failed to meet QC criteria, no further attempts were made. The following QC metrics were applied to the sequence results to ensure robust, high-quality results: i) >80% of test fragment A, Ion Sphere Particles containing a known sequence with varying sequence context provided in the Ion PGM Sequencing 200 Kit, was to have been of 50AQ17 quality (a Phred quality score of at least 17 for reads of >50 bp in length); ii) the read-length histogram was to have demonstrated that the mean read length of the fragment was >90 bp; iii) >80% of amplicons was to have had a read depth of at least 450×; iv) all plasmids composing the positive-control CPSC sample described above were to have been detected (see *Preparation of Control Plasmid Spike-in CEPH DNA*); v) no aMOIs were to have been detected in the negative control, CEPH NA12878; and vi) the total number of reads of the LibNTC were to have been <2% of mean reads of the rest of the libraries, or the mean read length was to have been <120 bp. The thresholds of the QC metrics were determined empirically and are summarized in Table 2. The TS3.2.1 data pipeline is known to produce spurious calls in indel types and in variants adjacent to a HP region.[49] Therefore, the expected acceptance criteria of the NCI-MPACT assay performance in sensitivity, specificity, accuracy, and reproducibility were set at 95% for SNV variants, 80% for the other four types of variants, and 90% for all types.

Table 2 Quality-Control Metrics and Thresholds in the NCI-MPACT Assay

Quality-control metric	Threshold
Tissue specimen review	>50% tumor nuclei present in section
DNA quantity	>20 ng
Library yield	>10 pmol/L in 50 μL
Test fragments	TF-A > 50AQ17, mean read length >90 bp
Amplicon coverage	≥80% of amplicons coverage >450×
Positive (CPSC) control	Detect and call all plasmid variants
Negative (CEPH) control	Detect no false-positive aMOIs
LibNTC sequence data	Total reads <2% mean reads of libraries or <120 bp mean read length

aMOIs, actionable mutations of interest; CEPH, Utah residents with ancestry from northern and western Europe; CPSC, control plasmid spike-in CEPH; LibNTC, library no-template control; TF-A, testing fragment A.

aMOI Verification and Review Processes

Variants identified as aMOIs by GeneMed were further reviewed by the laboratory staff. The aMOIs were rejected if the following criteria were met: i) the variant allele frequency was <5% for SNVs and <15% for indels; ii) the Bayesian score as calculated by TS3.2.1 was <5; and/or iii) the variant had been identified as being out of the reportable range (defined below) of the assay. An aMOI was classified as out of the reportable range if one or more of the following criteria was met: i) its position was within the amplicon primer region or <5 bp from the end of an amplicon, with the exception of *AKT1* p.E17K in amplicon AMPL391607825; ii) it was identified in CEPH NA12878 but was not found in the National Institute of Standards and Technology highly confident variant calls[50]; and/or iii) it had been verified, using Sanger sequencing during prevalidation feasibility study, as a false call (Supplemental Table S6). If the read depth of an aMOI was <450× (the threshold established by statistical modeling of the ability to detect a variant at 5% allele frequency with 95% confidence), the aMOI was within or immediately adjacent to a homopolymer region, or the aMOI generated an insertional tandem repeat, then the aMOI was verified by Sanger sequencing. If there was insufficient DNA for verification using Sanger sequencing, the NCI-MPACT assay results did not report the variant as positive.

Treatment Assignment Rules

The NCI-MPACT Molecular Tumor Board determined the rules to be used for treatment selection based on the identification of aMOIs matched to the various treatments. The

rules stated that if only one aMOI was detected, then that aMOI would determine the matched treatment. If more than one aMOI was identified and verified by the assay, then the frequency of the allele was assessed, and the aMOI with an allele frequency of ≥15% compared with that of the other aMOIs was used for treatment selection. If the difference in the allele frequencies of two or more aMOIs was <15%, then the treatment cohort with fewer patients assigned was to have been selected. These rules were programed into the GeneMed system and applied to the sequencing results in an automated fashion.

Analytical Validation Plan

Experiments were performed to assess NCI-MPACT assay-performance metrics, including amplicon read depth, sensitivity, specificity, accuracy, reproducibility, proficiency testing, and fit for purpose. The lower limits of detection of different variant types were not planned to be addressed in this validation plan because the TS3.2.1 data-analysis pipeline had a set lower limit of detection of 4% for SNVs and 15% for indels in general, and 2% for SNV-type aMOIs. In addition, the implementation of the enrichment of tumor cells in specimens with <50% tumor content mitigated the issue of low-level mutations due to tissue heterogeneity. A total of 191 DNA samples, consisting of 120 cell lines (28 unique), four CPSC (1 unique), 17 xenografts (eight unique), and 50 clinical specimens (30 unique), were used in this analytical validation study. Of these, 73 samples were in FFPE format and 118 samples were in FF format (Supplemental Table S1). Although clinical specimens should be the most relevant sample type used in the

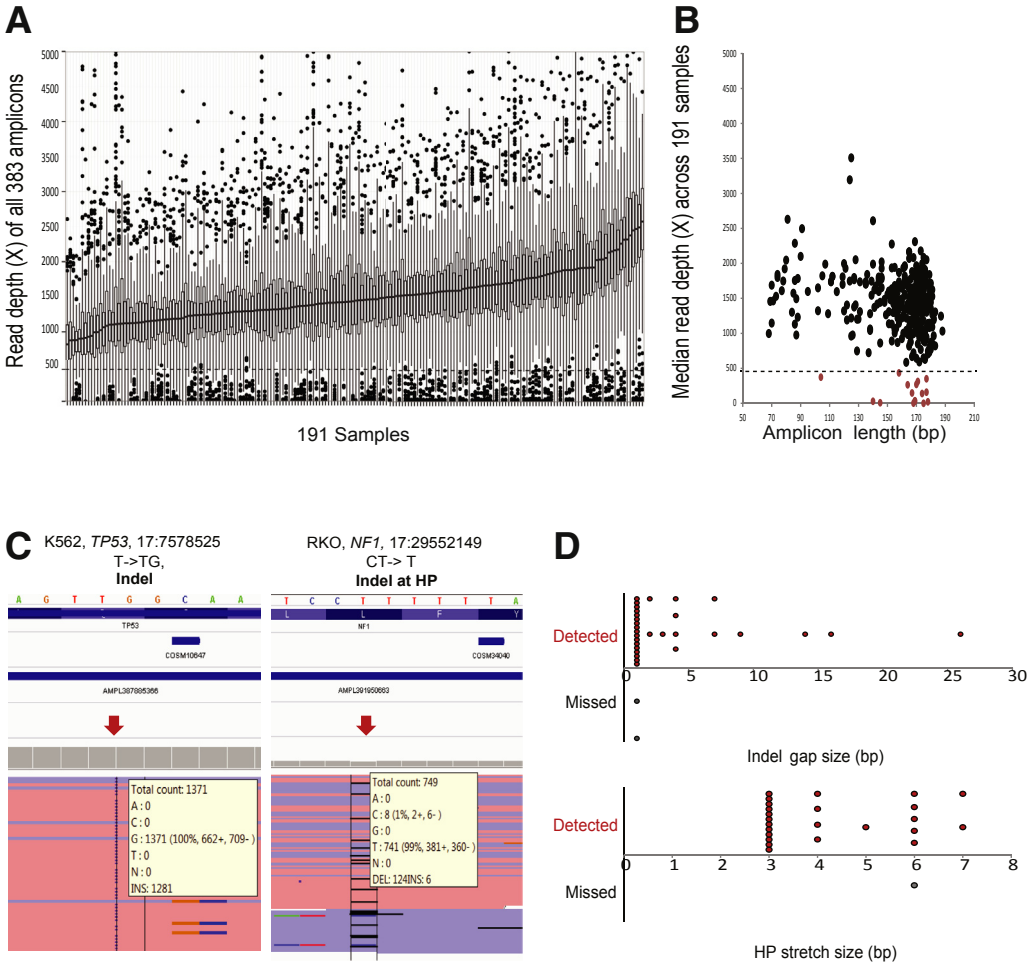

Figure 3 Amplicon read depth and detection of challenging variants. **A:** Box plot of read depth of 383 amplicons in each of 191 samples used in validation study. The outliers are indicated as solid dots and the 450× cutoff is indicated as a dashed line. **B:** Scatterplot of median amplicon read depth across 191 samples versus amplicon length (primer trimmed). Sixteen amplicons with median read depth <450× (**dashed line**) in 191 samples are shown as red dots (see amplicon information in Supplemental Table S7). **C:** Reads mapping to the loci of two variants that were not detected on the NCI-MPACT assay were examined by the Integrated Genome Viewer.[54] The deleted or inserted bases are indicated by **red arrows**. **Insets** summarizing the loci base count show both that indel variants reside in the mapped reads and that the TS3.2.1 pipeline failed to call the variants. **D:** Effects of indel gap size and homopolymeric (HP) length on the ability of the NCI-MPACT assay to detect variants. **Top panel:** Thirty detected (red) and two missed (gray) indel type variants (six indels, 15 indels at HP regions, and 11 large indels) plotted versus gap size. **Lower panel:** Twenty-four detected (red) and one missed (gray) HP adjacent variants (one large indel, six single nucleotide variants at HP, and 15 indels at HP) plotted versus their HP length.

validation study, this study utilized both clinical specimens and cell lines. The use of cell lines offered ability to select variants of different types in different genes and pathways for the validation study, as such variants are relatively rare in clinical specimens. At the end of the validation plan, 10 core needle biopsy samples from patients with metastatic cancers were collected and processed per the clinical protocol as fit-for-purpose specimens before study opening. Additionally, the first 50 specimens in the clinical trial were planned to have been verified by Sanger sequencing to further demonstrate assay performance. To assess the overall coverage in the NCI-MPACT assay panel, the read-depth information of 383 amplicons in 191 sample runs was used and are depicted as boxplots (Figure 3A).

Sensitivity Assessment

NCI-MPACT assay sensitivity was determined based on the ability of the assay to detect known variants in each of the five different variant types and in all types together. A total of 33 DNA samples (24 unique FF cell lines, eight unique FFPE xenografts, and one FF CPSC at 10%), containing 105 known variants identified in the COSMICv61, and control plasmids were used for assessing sensitivity in the five variant classes, which consisted of 64 SNVs, nine SNVs at HP, six small indels, 15 indels at HP, and 11 large indels. Variants detected by the NCI-MPACT assay were compared to data from the same cell lines documented in the COSMICv61 or plasmid-borne variants, and each call for these 105 known variants was indicated as either a true-positive call (TP, known variant detected) or a false-negative call (FN, known variant missed). The analytical sensitivity was calculated by dividing the number of TP calls by the sum of the TP and FN calls [TP/(TP + FN)],[51] and the 95% CI was estimated using the Clopper and Pearson method.[52,53]

Specificity Assessment

NCI-MPACT assay specificity was assessed based on FP calls. Three unique, well-characterized HapMap samples, CEPH (NA12878), Yoruban (NA18507), and Chinese Female (NA18526), were chosen. The CEPH NA12878 genome was selected by the National Institute of Standards and Technology as a reference genome, and verified high-confidence sequence is available.[50] Yoruban (NA18507) and Chinese Female (NA18526) represented different ethnic groups with adequate verified data to ensure the accuracy of no-calls by the NCI-MPACT assay. Tumor cell lines were not considered as ideal candidates for specificity assessment because of the absence of well-characterized genomic data, instability in their genomes, and variations in source and culture conditions. The three HapMap cell lines were sequenced independently 96 times, including 84 runs for CEPH FF samples, three runs for each of Yoruban and Chinese female FF samples, and six runs of FFPE samples (twice for each of the three HapMaps). The resulting sequence was examined for aMOIs that were falsely detected (FP) or were true negative (TN) within the 380 aMOI loci (Supplemental Table S3). The analytical specificity was calculated by dividing the number of TN calls by the sum of the TN and FP calls [TN/(TN + FP)] in the entire 96 runs.[51] The 95% CI approximations were estimated for each aMOI using the Clopper and Pearson method.[52,53] To verify specificity-call accuracy, 27 aMOI loci representing three variant types in each of the three drug-target pathways from the three HapMap samples were selected for analysis by Sanger sequencing. To evaluate the effect of the newly released TS4.0 pipeline on specificity, raw data from 75 FF CEPH samples from previous runs and 34 new sequencing runs were analyzed by TS4.0. The call for FP aMOIs, if any, followed the same aMOI verification and review process mentioned above.

Accuracy Assessment

Accuracy was calculated using TP and FN results from the sensitivity experiment and TN and FP results from the specificity experiment, as follows[51]:

$$\text{Accuracy} = [(\text{TP} + \text{TN})/(\text{TP} + \text{TN} + \text{FN} + \text{FP})] \quad (1)$$

The 95% CI approximations were estimated using the Clopper and Pearson method.[52,53] After discussions with the FDA, a subset of variants or loci in the samples used in sensitivity experiments was examined by Sanger sequencing to verify the positive and negative accuracy of the NCI-MPACT assay. Thirty-nine of 105 known variants used in the sensitivity assessment, including all variants that were missed by the NCI-MPACT assay, were selected to test for concordance with Sanger verification. Those variants were selected to represent different mutation types at different variant-allele frequencies. Another 39 genetic loci determined as wild-type sequences by the NCI-MPACT assay in the sensitivity assessment were also verified by Sanger sequencing. The criteria for selecting these 39 wild-type loci were that these variants represent genetic loci in three drug-targeted pathways and different variant types. The concordance between Sanger sequencing and NCI-MPACT assay results was used as a second level of accuracy assessment.

Reproducibility Assessment

To assess the reproducibility within and between operators, three FFPE cell-line xenograft samples and one FF CPSC at 10% were sequenced by the NCI-MPACT assay by two operators, three times, on different dates. Operator 1 sequenced the samples twice, and operator 2 sequenced the samples once. Reproducibility was assessed by calculating positive concordances between pairwise inter- and intra-operator comparisons. *Positive pairwise concordance* was defined as the number of positive calls in agreement divided by the total number of positive calls between the two replicates being compared. Due to the differences in tissue format and number of variants, concordances among all reported aMOIs in the three FFPE cell lines and 27 variants on the spiked-in CPSC at 10% were calculated separately.[53]

The intraoperator (one pair) and the mean interoperator (two pairs) comparison concordances were used for representing the reproducibility.

Proficiency Sample Testing

Ten DNA samples derived from clinical, archived FFPE specimens that had been characterized by a pyrosequencing-based mutation-detection assay in another CLIA laboratory (Center for Cancer Research, NCI, NIH, Bethesda, MD) were analyzed by the NCI-MPACT assay. The operator and data analyst for the NCI-MPACT assay were blinded to the mutation information of these 10 samples (R.D.H. and D.J.S.). NCI-MPACT assay results were sent back to the original CLIA laboratory, whose personnel calculated the concordance in mutation detection between the pyrosequencing assay and the NCI-MPACT assay. This concordance was used for assessing the proficiency of the operator and the ability of the NCI-MPACT assay to correctly identify mutations in previously assayed clinical samples.

Fit-for-Purpose Testing

After analytical validation, the NCI-MPACT assay was used for analyzing unknown clinical specimens to examine the reproducibility in aMOI detection and treatment selection. Ten core needle tumor biopsy samples from 10 different patients with metastatic cancer were collected, shipped, and processed according to the methods specified in the NCI-MPACT clinical protocol. Each sample was analyzed independently four times by two operators (two times per operator). The concordance of aMOI detection and treatment selection was used for assessing the reliability of the NCI-MPACT assay. The aMOIs identified by NCI-MPACT assay were verified by Sanger sequencing. Specimens without detected aMOIs, frequently mutated regions in tumor such as the *KRAS* p.G12/13 codons, were examined by Sanger sequencing to confirm the wild-type sequences.

Sanger Sequencing Verification

Regions containing aMOIs that required confirmation by Sanger sequencing were PCR-amplified using the same primer pairs used in the NCI-MPACT panel with the M13 forward (5′-GTAAAACGACGGCCAGT-3′) and reverse (5′-GGAAACAGCTATGACCATG-3′) sequence added to the 5′ ends of the primer. PCR amplification of one amplicon required 50 ng of genomic DNA. These PCR products were purified with Agencourt Ampure XP Beads, and Sanger sequenced by the 3730XL DNA analyzer (Applied Biosystems, Carlsbad, CA).

Application of NCI-MPACT Assay

The NCI-MPACT clinical trial was opened to accrual in January 2014. Since that time, all core needle tumor biopsies from patients were collected, shipped, and processed according to NCI-MPACT study protocols. Each sample was analyzed once by NCI-MPACT assay. For the first 50 specimens, every sample, whether or not an aMOI was discerned by the NCI-MPACT assay, was planned to be verified by Sanger sequencing if there was a sufficient quantity of DNA remaining from the NCI-MPACT assay. As in the fit-for-purpose testing, specimens without detected aMOIs underwent Sanger sequencing of the *KRAS* p.G12/13 codons and/or *TP53* codons mutated most frequently in the same histological type as the specimen in COSMICv61 database (eg, p.R173, p.R273, p.R248) to confirm the wild-type sequences.

Results

An overview of the NCI-MPACT assay workflow and turnaround time of each step in the process, from patients' biopsy sample collection to clinical report, is illustrated in Figure 1. One goal of the NCI-MPACT assay—development process was to streamline the timeline of NGS results reporting compared to that of the current gold standard method for identifying mutations, Sanger sequencing. The NCI-MPACT assay requires 20 ng of input DNA from FFPE tumor tissue or cell pellets or FF cell pellets, and it takes no more than 10 days to report the aMOIs. The NCI-MPACT assay is performed in a CLIA-accredited laboratory.

Analytical Validation

NGS Data Quality and Read Depth

NGS data from 191 runs of the NCI-MPACT assay were used for analytical validation. The NCI-MPACT assay produced a median of 631,000 (631 K) reads per sample (range, 370 K to 1153 K), a median read length of 152 bp (range, 140 to 161 bp), and a median amplicon failure rate of 4.18% per sample (range, 2.35% to 14.88%) (<80% of amplicons with coverage greater than 450×). Interestingly, but not surprisingly, we observed in the NGS data from the FFPE samples that the median number of reads per sample was relatively less (612 K in FFPE versus 645 K in FF), the median read length was approximately identical (152 bp in FFPE versus 153 bp in FF), and the median amplicon fail rate was slightly greater (5.48% in FFPE versus 3.92% in FF). On analysis of the read depths of all 383 amplicons across 191 runs (Figure 3A), 1499× median of median read depth of all amplicons, indicating that the read depths of most NCI-MPACT coverage regions were much greater than the 450× cutoff. A scatterplot of the median read depths (from 191 assay runs) against the read lengths of each of the amplicons identified 16 amplicons that consistently failed to pass the 450× threshold (Figure 3B), including three amplicons that contained a total of three aMOIs (Supplemental Table S7). Because a 450× read depth was required by the assay standard operating procedures for the verification of a positive call, those aMOIs, if detected in a clinical sample, would require verification by Sanger sequencing during the laboratory review process.

Sensitivity

The NCI-MPACT assay achieved 100% sensitivity for SNVs, SNVs at HP, and large indels; 83.33% for small indels; and 93.33% in indels at HP, resulting in 98.11% overall sensitivity with high 95% CIs (Table 3). The distribution of 105 variants in three sample types included 65 variants in 24 FF cell lines (33 SNVs, three SNVs at HP, six indels, 14 indels at HP, and nine large indels), 13 variants in eight FFPE xenograft samples (10 SNVs, one SNV at HP, one indel at HP, and one large indel), and 27 variants in CPSC at 10% (21 SNVs, five SNVs at HP, and one large indels). For detected variants, the ranges of variant allele frequency in each variant type were: 3% to 100% for SNVs, 5% to 99% for SNVs at HP, 70% to 98% for indels, 25% to 94% for indels at HP, and 16% to 98% for large indels. The source of samples, gene and chromosomal locations of mutations, variant allele frequency, and read depth are listed in Supplemental Table S8. On inspection of the BAM files using the Integrated Genome Viewer[54] of the two variants not detected, an indel and an indel at HP, the expected variants were present in the sequencing reads at approximately the correct variant allele frequency (Figure 3C). This finding strongly suggests that failure to call these variants in the sequencing report resulted from the bioinformatics pipeline in TS3.2.1. On evaluation of the effect of indel gap size and HP length on variant detection, the NCI-MPACT assay was capable of detecting indel variants with a gap size up to 26 bp and variants within or adjacent to homopolymeric stretches up to 7 bp (Figure 3D). On re-analysis of data using an updated bioinformatic pipeline version (TS4.0), the two FN calls in our validation study were rescued, resulting in 100% sensitivity.

Specificity

The NCI-MPACT assay reported no FP aMOIs (380 known or novel) in 96 independent runs and therefore achieved 100% specificity for all five types of variants (Table 3). Twenty-seven aMOI loci representing the three variant types (SNV, indel, and large indel) of aMOI in each of three signaling pathways (DNA repair, *AKT/PI3K/MTOR*, and *RAS/RAF/MEK*) were examined by Sanger sequencing to verify wild-type sequences in each of three HapMap cell lines. Concordance from 27 Sanger sequencing runs was 100%, verifying that the NCI-MPACT assay accurately reported these loci in three cell lines (Supplemental Table S9). On re-analysis of CEPH data from 75 previous assays and 34 fresh CEPH assay runs, no FP aMOIs were identified by the new pipeline, TS4.0, indicating that TS4.0 performance improved sensitivity and did not diminish specificity.

Accuracy

On review of the sensitivity and specificity assessments, the overall NCI-MPACT assay accuracy was 99.99% (Table 3). The accuracy of the NCI-MPACT assay was further assessed by calculating both the positive (variant detected by NCI-MPACT assay) and negative (NCI-MPACT assay detected no variant) accuracy by Sanger sequencing using a subset of samples from the sensitivity study. Thirty-nine of one hundred five known variants in the sensitivity study, including two variants not detected by the NCI-MPACT assay, were selected for Sanger verification, with a 94.87% positive accuracy (Supplemental Table S8). Conversely, another 39 loci in the sensitivity samples called as reference/wild-type sequence by the NCI-MPACT assay were verified as wild-type sequence by Sanger sequencing, for negative accuracy of 100% (Supplemental Table S10).

Reproducibility

The intraoperator and mean interoperator positive concordance were each 100% for five aMOIs reported in three FFPE cell lines. However, for CPSC at 10% samples, one spiked-in SNV at HP variant (*ERCC1*) whose allele frequency was near the threshold was not detected in one replicate performed by operator 1. This finding led to

Table 3 Analytical Performance of the NCI-MPACT Assay

Variant type	Sensitivity, % (*N*, 95% CI)	Specificity, % (*N*, 95% CI)	Accuracy, % (95% CI)	Intraoperator concordance, %	Mean interoperator concordance, %
SNV	100.00 (64, 94.4—100.0)	100.00 (25,152, 96.2—100.0)	100.00 (99.9—100.00)		
SNV at HP	100.00 (9, 66.4—100.0)	100.00 (672, 96.2—100.0)	100.00 (99.5—100.00)		
Indel	83.33 (6, 35.9—99.6)	100.00 (6432, 96.2—100.0)	99.98 (99.9—100.00)		
Indel at HP	93.33 (15, 68.1—99.8)	100.00 (1440, 96.2—100.0)	99.94 (99.6—100.00)		
Large indel	100.00 (11, 71.5—100.0)	100.00 (2784, 96.2—100.0)	100.00 (99.9—100.00)		
Overall	98.11 (105, 93.3—99.8)	100.00 (36,480, 96.2—100.0)	99.99 (99.9—100.00)	100, 96.3	100, 98.1

CPSC, control plasmid spike-in CEPH at 10%; HP, homopolymeric region; *N*, total number of known variants for sensitivity, total number of actionable mutations of interest (aMOI) loci for specificity; SNV, single-nucleotide variant.

Table 4 Proficiency Sample Testing for the NCI-MPACT Assay

Histological diagnosis	Previously identified mutations	Type	NCI-MPACT assay—detected VAF
Thymoma	None		
Non-small cell lung carcinoma	*BRAF*, p.V600E	SNV	0.37
Non-small cell lung carcinoma	*EGFR*, p.711Gdel	indel	0.25
Non-small cell lung carcinoma	*KRAS*, p.G12V	SNV	0.22
	TP53, p.E298*	SNV	0.31
Small cell lung carcinoma	*PIK3CA*, p.H1047L	SNV	0.43
	TP53, p.R342*	SNV	0.85
Thymoma	None		
Thymoma	None		
Melanoma	*NRAS*, p.Q61K	SNV	0.28
Small cell lung carcinoma	*KRAS*, p.G12D	SNV	0.84
	AKT1, p.E17K	SNV	0.51
Non-small cell lung carcinoma	*EGFR*, p.L858R	SNV	0.92
	TP53, p.272-278del	Large indel	0.25

*Point mutation introduced a stop codon.
SNV, single-nucleotide variant; VAF, variant allele frequency.

intraoperator concordance and mean interoperator concordance of 96.3% and 98.1%, respectively, for 27 variants spiked in CPSC at 10% (Table 3 and Supplemental Table S11). The assay reproducibility was further assessed by calculation of the R^2 values of variant allele frequency of 32 detected variants for each pair of replicates. The R^2 values were >0.99 for both the intra- and interoperator comparisons, suggesting that the NCI-MPACT assay was highly reproducible.

Proficiency Sample Testing

Given that the analytical-performance metrics met the criteria established for use in the NCI-MPACT study, proficiency testing was conducted using 10 clinical specimens with known mutations characterized by an orthogonal assay platform in another CLIA laboratory. Seven samples with 11 previously identified mutations (nine SNVs, one indel, and one large indel) and three samples containing no relevant mutations were tested by the NCI-MPACT assay in a blinded fashion. The NCI-MPACT assay achieved 100% concordance with the known results (Table 4). This finding suggests that the laboratory technical staff was proficient in performing the assay by obtaining accurate and expected results.

Fit for Purpose

Although the NCI-MPACT assay performance was acceptable, most of the results were obtained using DNA samples from cell lines. Before completion of the validation study, it was required to test whether the NCI-MPACT assay could successfully analyze unknown clinical specimens after the entire clinical protocol process (from biopsy collection to aMOI report). A total of 10 clinical tumor specimens with completely unknown mutational information were collected and processed following the standard operating procedures intended for the NCI-MPACT study. The hematoxylin and eosin–stained images of the biopsy specimens received are depicted in Supplemental Figure S2. One sample (Supplemental Figure S2I) of the 10 collected specimens required macrodissection to enrich the tumor tissue as it contained <50% tumor content. Each of the extracted DNA samples was analyzed by the NCI-MPACT assay four times by two operators. The NCI-MPACT assay detected four aMOIs in three patient specimens (one specimen contained two aMOIs), and no aMOIs were detected in the seven remaining specimens. The variant allele frequencies of the four detected aMOIs in each of the four replicates were very similar, and treatment selections in all sample replicates were 100% concordant. All detected aMOIs were confirmed by Sanger sequencing. Examination of the frequently mutated regions in *KRAS* p.G12/13 codons by Sanger sequencing confirmed that those loci were wild type in the seven specimens that contained no aMOIs (Table 5).

Application of the NCI-MPACT Assay

The study plan and assay validation results were reviewed by the FDA in an NCI Investigational New Drug Application, and the trial was opened for accrual in January 2014. As of October 2014, 38 core needle biopsy specimens of metastatic tumors from 38 patients with 25 different histological tumor types had been analyzed with the NCI-MPACT assay. Four biopsies (10.5%) could not be sequenced by the NCI-MPACT assay because of insufficient tumor content or insufficient DNA yield. Of 34 specimens sequenced, all samples passed assay QC testing and were moved forward to treatment assignment, with a mean 6.7-day turnaround time. A total of 21 aMOIs were identified in 18 patients (52.9%), whereas no aMOIs were

Table 5 Fit-for-Purpose Sample Testing in the NCI-MPACT Assay

Histological diagnosis (tumor content, %)	aMOI detected by NCI-MPACT	Treatment selection	Replicate 1 VAF, read depth	Replicate 2 VAF, read depth	Replicate 3 VAF, read depth	Replicate 4 VAF, read depth	aMOIs confirmed by Sanger	22 Wild-type *KRAS* loci confirmed by Sanger
Acinic salivary gland tumor (100)	*TP53*, p.R175H	AZD1775 + carboplatin	0.56, 828	0.36, 458	0.42, 704	0.45, 602	Yes	
Renal cell carcinoma (100)	*PIK3CA*, p.N345K	Everolimus	0.47, 151	0.47, 73	0.44, 66	0.3, 149	Yes	
Hepatocellular cancer (50)	None							Yes
Hepatocellular cancer (50)	None							Yes
Hepatocellular cancer (50)	None							Yes
Mesothelioma (50)	None							Yes
Mesothelioma (20)	None							Yes
Mesothelioma (90)	None							Yes
Leiomyosarcoma (75)	None							Yes
Colorectal cancer (70)	*TP53*, p.R306*	AZD1775 + carboplatin	0.7, 1232	0.66, 955	0.71, 1478	0.65, 1577	Yes	
	KRAS, p.G12V		0.46, 2065	0.5, 2262	0.52, 2435	0.46, 2707	Yes	

*Point mutation introduced a stop codon.
aMOI, actionable mutations of interest; VAF, variant allele frequency.

detected in the other 16 patients; three specimens contained more than one aMOI. Consistent with previous reports, the gene with the greatest aMOI frequency was *TP53*, followed by *KRAS* (Table 6). On Sanger sequencing results from these 34 specimens, agreement with the NCI-MPACT results was 100% for both aMOI-positive samples and wild-type *KRAS* and *TP53* loci in aMOI-negative samples.

Discussion

Although reports on the AmpliSeq and PGM technologies have been published by others detailing mutation detection in cancer specimens,[49,57–59] most of those articles have focused on research purposes. Here we report processes of assay development and analytical validation for a custom NGS-based mutation-detection assay that can be used for screening patients for enrollment and for determining treatment within the framework of a clinical research study. It is important to note that the NCI-MPACT trial recruits only patients whose tumors have progressed after standard treatment and that the required biopsy samples are collected only if deemed low risk by the interventional radiologists. These aspects of the intended use helped to guide our expected NCI-MPACT assay—performance metrics and validation plan. The results from the NCI-MPACT assay are intended to serve only as a means of selecting and enrolling patients into a treatment arm in the NCI-MPACT trial, and their use is not intended for other clinical purposes. During development, the analytical specificity of the assay was considered of greater importance than the analytical sensitivity because a FP result might permit the enrollment of a patient incorrectly, whereas a FN assay result would not be considered as risky. This validation effort was informed by discussions with the FDA Center for Diagnostics and Radiological Health before the activation of the clinical trial, and it may serve as a template for others who intend to develop and apply NGS assays to clinical studies.

In the development and validation of assays, it is important to clearly outline the intended use and associated patient risks so that the validated assay is fit for the intended purpose. The validation plan outlined in this article will likely not be optimal for assays intended for uses different from that of the NCI-MPACT clinical trial. The process of selecting the genes and variants and rules for treatment selection are provided in detail. During the preliminary feasibility study, QC thresholds were determined (Table 2), FP loci within the reportable range were identified, and standard operating procedures were finalized and locked. The locked system was then tested for analytical performance before the use of the assay as an integral component of the NCI-MPACT study. The data from the analytical performance of the NCI-MPACT assay suggested that our design, approach, and process used during assay development produced an assay capable of meeting the intended use for this study.

One of the features of the NCI-MPACT study was the use of a rule-based system for gene selection, aMOI data review, and treatment selection. Rule-based systems that incorporate expert knowledge in formulating the rules for accomplishing defined tasks in a systematic manner have been successfully applied to the diagnosis and treatment of diseases[60,61] and microarray data analysis.[62,63] Although many studies that have implemented the NGS assay have relied on a tumor board to review the sequencing results for determining treatment,[64–66] we used a rule-based, online informatics system called GeneMed.[47] The rules applied in GeneMed were generated by a group of experts in clinical and molecular biology before analytical validation was initiated for annotating aMOIs and assigning treatment. The automated application of the rules of interpreting NGS assay

Table 6 NCI-MPACT Assay Results from 34 Patient Biopsies

Histological diagnosis	Detected aMOI	Variant type	VAF	Read depth	Confirmed by Sanger	Select treatment
Alveolar soft part sarcoma	None				Yes	
Bladder; small cell carcinoma	*TP53*, p.V157F	SNV	0.91	1335	Yes	AZD1775 + carboplatin
Invasive ductal carcinoma of breast	*TP53*, FRAMESHIFT	indel at HP	0.51	734	Yes	AZD1775 + carboplatin
Invasive ductal carcinoma of breast	*PIK3CA*, p.H1047R	SNV	0.57	1999	Yes	Everolimus
Chondrosarcoma	None				Yes	
Cholangiocarcinoma	*PTEN*, FRAMESHIFT	indel at HP	0.65	1898	Yes	Everolimus
Colon adenocarcinoma	*TP53*, p.R213*	SNV at HP	0.73	943	Yes	AZD1775 + carboplatin
	KRAS, p.G12C	SNV	0.45	1307	Yes	
Colon adenocarcinoma	*TP53*, p.R248W	SNV	0.19	1089	Yes	Trametinib
	KRAS, p.G13D	SNV	0.66	1661	Yes	
Colon adenocarcinoma	*TP53*, FRAMESHIFT	Large indel	0.8	469	Yes	AZD1775 + carboplatin
Colon adenocarcinoma	None				Yes	
Colon adenocarcinoma	*TP53*, p.R175H	SNV	0.13	1020	Yes	AZD1775 + carboplatin
Colorectal adenocarcinoma	*KRAS*, p.G12V	SNV	0.53	1120	Yes	Trametinib
Embroyonal cell sarcoma	*TP53*, p.R273C	SNV	0.91	1038	Yes	AZD1775 + carboplatin
Ovarian granulosa cell tumor	None				Yes	
Head and neck squamous cell carcinoma	None				Yes	
Hepatocellular carcinoma	None				Yes	
Leiomyosarcoma	None				Yes	
Myxofibrosarcoma	None				Yes	
Neuroendocrine lung carcinoma	None				Yes	
Non-small cell lung carcinoma	*TP53*, p.E336*	SNV	0.73	929	Yes	AZD1775 + carboplatin
Ovarian serous carcinoma	*KRAS*, p.G12V	SNV	0.91	1702	Yes	Trametinib
Ovarian carcinosarcoma	*TP53*, p.R248Q	SNV	0.96	599	Yes	AZD1775 + carboplatin
Pancreatic adenocarcinoma	*TP53*, p.C176F	SNV at HP	0.34	588	Yes	AZD1775 + carboplatin†
	KRAS, p.G12D	SNV	0.38	1669	Yes	
Pancreatic adenocarcinoma	*KRAS*, p.G12D	SNV	0.09	1252	Yes	Trametinib
Papillary ovarian carcinoma	None				Yes	
Papillary ovarian carcinoma	None				Yes	
Parotid gland adenocarcinoma	None				Yes	
Parotid gland; acinic cell carcinoma	None				Yes	
Peritoneal serous carcinoma	*TP53*, p.Y220C	SNV	0.45	1045	Yes	AZD1775 + carboplatin
Prostate adenocarcinoma	None				Yes	
Serous ovarian carcinoma	*TP53*, FRAMESHIFT	indel at HP	0.59	648	Yes	AZD1775 + carboplatin
Small bowel adenocarcinoma	None				Yes	
Spindle cell sarcoma	*TP53*, p.R273C	SNV	0.67	575	Yes	AZD1775 + carboplatin
Urothelial carcinoma	None				Yes	

*Point mutation introduced a stop codon.

†NCI-MPACT trial protocol rules state that pancreatic cancer patients with *KRAS* mutations are not eligible for trametinib treatment arm[55,56]; thus, though the *KRAS* mutation was detected at a similar variant allele frequency in this specimen, *TP53* mutation was selected for targeted treatment.

aMOI, actionable mutation of interest; HP, homopolymeric region; SNV, single-nucleotide variant; VAF, variant allele frequency.

data and selecting treatment may result in a more uniform application of rules compared with tumor board–based review of sequencing results, and may result in less bias, which was important for the objectives of the NCI-MPACT trial. The NCI-MPACT trial objectives were based on the hypothesis that the selection of treatment based on chosen aMOIs would yield a better clinical response rate for targeting regimen than would the selection of treatment options without molecular data. The rules and NCI-MPACT assay system were designed to be adapted (or versioned) and revalidated as needed.

In our read-depth analysis, we identified 16 amplicons (4%) of 383 amplicons with median read depths less than the 450× threshold. Although no correlation was identified between amplicon read depth and length (Figure 3B) or GC content (data not shown), we did notice that extraordinarily long and dense HP regions were located in several of the low read-depth amplicons. Those amplicons will be redesigned in the next version of the assay.

The NCI-MPACT assay achieved 100% sensitivity in SNV, SNV at HP, and large indels, 83.33% for indels, and 93.33% in indels at HP-type variants, resulting in a 98.11% sensitivity overall in a total of 105 known variants (Table 3 and Supplemental Table S8). The two FN variants verified by Sanger sequencing were likely due to mapping and variant call errors in the TS3.2.1 bioinformatics pipeline

(Figure 3C). Similar results were reported previously, in which spurious calls occurred for indels and indels at HP.[49] A systematic study suggested that the main error source was inaccurate flow calls.[67] Re-analysis of data by the new TS4.0 pipeline not only rescued the two FN calls by TS3.2.1 but also retained 100% specificity. These results suggested that the specific data-analysis pipeline has a significant effect on assay performance. To compensate for the known weakness of variant calling at HP regions, we added a verification step in the NCI-MPACT−assay process by which any aMOI found within a HP region must be verified by Sanger sequencing. The high sensitivity of the NCI-MPACT assay provided high confidence that specimens carrying an aMOI in the NCI-MPACT trial will be successfully identified. We recognized that clinical specimens were the preferable specimen for use in analytical-performance testing. Such clinical specimens ideally should be collected and processed as intended for use in the validated assay. In our study, we did not have access to adequate numbers of FFPE clinical specimens with verified mutations representing all variant types contained in the three different treatment-associated pathways. Therefore, to test analytical performance, we chose to utilize well-documented cell lines and xenograft tissues containing the required types of variants. To compensate for the limited number of FFPE clinical specimens used in the validation study, we performed extensive Sanger sequencing verification of the specimens with positive aMOIs and *KRAS* G12/13 loci or *TP53* hotspot mutation loci in specimens negative for any aMOIs during fit-for-purpose testing and will continue to do this for patients' biopsy specimens collected in the NCI-MPACT trial (Tables 5 and 6). To date, 34 biopsy specimens have been tested, with 100% agreement in both aMOI-positive and -negative samples demonstrated by Sanger sequencing. This finding suggests that the NCI-MPACT assay maintains high sensitivity and specificity in testing clinical FFPE specimens.

From 96 replicate sequencing runs of the three HapMap DNA samples, we observed that the NCI-MPACT assay achieved 100% specificity in testing all five types of variants. Beadling et al[57] reported 95.1% specificity using a 46-gene cancer hotspot panel assay with an 8% variant allele frequency cutoff. We would like to emphasize that our specificity was determined within the scope of a predefined reportable range (380 known plus loss-of-function aMOIs in 13 tumor-suppressor and DNA-repair genes) at a 4% variant allele frequency cutoff for SNVs and 15% variant allele frequency for indels. The high specificity of the NCI-MPACT assay ensures that patients without aMOIs will not be erroneously enrolled in the NCI-MPACT trial, thus further mitigating excess risk to the patients. Although increased confidence in the assessment of specificity of the NCI-MPACT assay could be achieved by sequencing additional well-characterized, normal specimens, we believe that the results from the repetitive sequencing of the three HapMap genomes were adequate for our intended application. It should also be noted that, the 16 clinical specimens from the NCI-MPACT study had no aMOIs reported by the assay; all were confirmed to have wild-type sequence for the *KRAS* and *p53* loci as tested by Sanger sequencing. Therefore, we believe that the data generated as a part of specificity estimation using the three HapMap genomes resulted in a good assessment of the specificity of the assay.

The accuracy of the NCI-MPACT assay was 99.99%, as calculated using data from the 129 runs used for sensitivity and specificity studies (Table 3). Sanger verification showed a 94.87% positive accuracy in 39 known variants (Supplemental Table S8) (100% when tested with the TS4.0 pipeline), a 100% negative accuracy in 39 known wild-type loci in the sensitivity study (Supplemental Table S10), a 100% concordance in 27 variants in the specificity study (Supplemental Table S9), and a 100% concordance in 10 fit-for-purpose clinical specimens (Table 5). These data suggest that the NCI-MPACT assay can detect variants with high accuracy, a key criterion of the selection of patients for a targeted treatment. In keeping with the allocation of specimens as outlined in the plan for analytical validation of the assay, the results from proficiency and fit-for-purpose testing were used only for their intended purpose. It should be noted that the results from proficiency and fit-for-purpose testing did increase our confidence in the analytical accuracy of this assay. Although Sanger sequencing is still regarded as the gold standard method of verifying identified mutations, we found Sanger verification to be the main bottleneck in our workflow due to the limitations of detecting variants at a low allele frequency, low sample/analyte throughput, high amount of input DNA required, high cost, and long turnaround time (Figure 1). We expect that in the near future NGS will become a gold standard.

The NCI-MPACT assay demonstrated a high degree of reproducibility in sequencing technical replicates. There was 100% concordance in identified aMOIs and treatment selection in all four replicates performed by two operators in fit-for-purpose testing (Table 5), which established a solid reliability for the investigational use of this assay in the NCI-MPACT clinical trial. Recently, McShane et al[68] developed a 30-point checklist for evaluating whether an omics-based assay is sufficiently reliable and ready for clinical use. Our analytical validation study addressed applicable checkpoints on this list. Preliminary results from the first 34 biopsy specimens in the NCI-MPACT trial indeed showed that the assay identified aMOIs with perfect concordance with Sanger and in the expected turnaround time.

In summary, our findings suggest that the NCI-MPACT NGS selection assay is sufficiently sensitive, specific, accurate, reliable, and well-suited for the intended investigational use in the NCI-MPACT clinical trial.

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Supplemental Data

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References

1. Macconaill LE, Garraway LA: Clinical implications of the cancer genome. J Clin Oncol 2010, 28:5219–5228
2. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr, Kinzler KW: Cancer genome landscapes. Science 2013, 339: 1546–1558
3. Savage DG, Antman KH: Imatinib mesylate—a new oral targeted therapy. N Engl J Med 2002, 346:683–693
4. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L: Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 2001, 344:783–792
5. Sosman JA, Kim KB, Schuchter L, Gonzalez R, Pavlick AC, Weber JS, McArthur GA, Hutson TE, Moschos SJ, Flaherty KT, Hersey P, Kefford R, Lawrence D, Puzanov I, Lewis KD, Amaravadi RK, Chmielowski B, Lawrence HJ, Shyr Y, Ye F, Li J, Nolop KB, Lee RJ, Joe AK, Ribas A: Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. N Engl J Med 2012, 366:707–714
6. Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, Ou SH, Dezube BJ, Janne PA, Costa DB, Varella-Garcia M, Kim WH, Lynch TJ, Fidias P, Stubbs H, Engelman JA, Sequist LV, Tan W, Gandhi L, Mino-Kenudson M, Wei GC, Shreeve SM, Ratain MJ, Settleman J, Christensen JG, Haber DA, Wilner K, Salgia R, Shapiro GI, Clark JW, Iafrate AJ: Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J Med 2010, 363:1693–1703
7. The Cancer Genome Atlas Network: Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 2008, 455:1061–1068
8. The Cancer Genome Atlas Network: Integrated genomic analyses of ovarian carcinoma. Nature 2011, 474:609–615
9. The Cancer Genome Atlas Network: Comprehensive genomic characterization of squamous cell lung cancers. Nature 2012, 489: 519–525
10. The Cancer Genome Atlas Network: Comprehensive molecular portraits of human breast tumours. Nature 2012, 490:61–70
11. Dias-Santagata D, Akhavanfard S, David SS, Vernovsky K, Kuhlmann G, Boisvert SL, Stubbs H, McDermott U, Settleman J, Kwak EL, Clark JW, Isakoff SJ, Sequist LV, Engelman JA, Lynch TJ, Haber DA, Louis DN, Ellisen LW, Borger DR, Iafrate AJ: Rapid targeted mutational analysis of human tumours: a clinical platform to guide personalized cancer medicine. EMBO Mol Med 2010, 2:146–158
12. MacConaill LE, Campbell CD, Kehoe SM, Bass AJ, Hatton C, Niu L, Davis M, Yao K, Hanna M, Mondal C, Luongo L, Emery CM, Baker AC, Philips J, Goff DJ, Fiorentino M, Rubin MA, Polyak K, Chan J, Wang Y, Fletcher JA, Santagata S, Corso G, Roviello F, Shivdasani R, Kieran MW, Ligon KL, Stiles CD, Hahn WC, Meyerson ML, Garraway LA: Profiling critical cancer gene mutations in clinical tumor samples. PLoS One 2009, 4:e7887
13. Kummar S, Williams PM, Lih CJ, Polley EC, Chen AP, Rubinstein LV, Zhao Y, Simon RM, Conley BA, Doroshow JH: Application of molecular profiling in clinical trials for advanced metastatic cancers (commentary). J Natl Cancer Inst 2015, 107: djv003
14. Meshinchi S, Hunger SP, Aplenc R, Adamson PC, Jessup JM: Lessons learned from the investigational device exemption review of Children's Oncology Group trial AAML1031. Clin Cancer Res 2012, 18:1547–1554
15. Schilsky RL, Doroshow JH, Leblanc M, Conley BA: Development and use of integral assays in clinical trials. Clin Cancer Res 2012, 18: 1540–1546
16. Yamaguchi T, Kakefuda R, Tajima N, Sowa Y, Sakai T: Antitumor activities of JTP-74057 (GSK1120212), a novel MEK1/2 inhibitor, on colorectal cancer cell lines in vitro and in vivo. Int J Oncol 2011, 39:23–31
17. Motzer RJ, Escudier B, Oudard S, Hutson TE, Porta C, Bracarda S, Grunwald V, Thompson JA, Figlin RA, Hollaender N, Urbanowitz G, Berg WJ, Kay A, Lebwohl D, Ravaud A: Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial. Lancet 2008, 372:449–456
18. Grunwald V, Karakiewicz PI, Bavbek SE, Miller K, Machiels JP, Lee SH, Larkin J, Bono P, Rha SY, Castellano D, Blank CU, Knox JJ, Hawkins R, Anak O, Rosamilia M, Booth J, Pirotta N, Bodrogi I: An international expanded-access programme of everolimus: addressing safety and efficacy in patients with metastatic renal cell carcinoma who progress after initial vascular endothelial growth factor receptor-tyrosine kinase inhibitor therapy. Eur J Cancer 2012, 48:324–332
19. Palma JP, Wang YC, Rodriguez LE, Montgomery D, Ellis PA, Bukofzer G, Niquette A, Liu X, Shi Y, Lasko L, Zhu GD, Penning TD, Giranda VL, Rosenberg SH, Frost DJ, Donawho CK: ABT-888 confers broad in vivo activity in combination with temozolomide in diverse tumors. Clin Cancer Res 2009, 15:7277–7290
20. Leijen S, Beijnen JH, Schellens JH: Abrogation of the G2 checkpoint by inhibition of Wee-1 kinase results in sensitization of p53-deficient tumor cells to DNA-damaging agents. Curr Clin Pharmacol 2010, 5: 186–191
21. Forbes SA, Bindal N, Bamford S, Cole C, Kok CY, Beare D, Jia M, Shepherd R, Leung K, Menzies A, Teague JW, Campbell PJ, Stratton MR, Futreal PA: COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. Nucleic Acids Res 2011, 39:D945–D950
22. Forbes SA, Beare D, Gunasekaran P, Leung K, Bindal N, Boutselakis H, Ding M, Bamford S, Cole C, Ward S, Kok CY, Jia M, De T, Teague JW, Stratton MR, McDermott U, Campbell PJ:

COSMIC: exploring the world's knowledge of somatic mutations in human cancer. Nucleic Acids Res 2015, 43:D805–D811
23. Gilmartin AG, Bleam MR, Groy A, Moss KG, Minthorn EA, Kulkarni SG, Rominger CM, Erskine S, Fisher KE, Yang J, Zappacosta F, Annan R, Sutton D, Laquerre SG: GSK1120212 (JTP-74057) is an inhibitor of MEK activity and activation with favorable pharmacokinetic properties for sustained in vivo pathway inhibition. Clin Cancer Res 2011, 17:989–1000
24. Solit DB, Garraway LA, Pratilas CA, Sawai A, Getz G, Basso A, Ye Q, Lobo JM, She Y, Osman I, Golub TR, Sebolt-Leopold J, Sellers WR, Rosen N: BRAF mutation predicts sensitivity to MEK inhibition. Nature 2006, 439:358–362
25. Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J, Hamid O, Schuchter L, Cebon J, Ibrahim N, Kudchadkar R, Burris HA 3rd, Falchook G, Algazi A, Lewis K, Long GV, Puzanov I, Lebowitz P, Singh A, Little S, Sun P, Allred A, Ouellet D, Kim KB, Patel K, Weber J: Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. N Engl J Med 2012, 367:1694–1703
26. Mao JH, Kim IJ, Wu D, Climent J, Kang HC, DelRosario R, Balmain A: FBXW7 targets mTOR for degradation and cooperates with PTEN in tumor suppression. Science 2008, 321:1499–1502
27. Bos JL: ras oncogenes in human cancer: a review. Cancer Res 1989, 49:4682–4689
28. Garon EB, Finn RS, Hosmer W, Dering J, Ginther C, Adhami S, Kamranpour N, Pitts S, Desai A, Elashoff D, French T, Smith P, Slamon DJ: Identification of common predictive markers of in vitro response to the Mek inhibitor selumetinib (AZD6244; ARRY-142886) in human breast cancer and non-small cell lung cancer cell lines. Mol Cancer Ther 2010, 9:1985–1994
29. Malumbres M, Barbacid M: RAS oncogenes: the first 30 years. Nat Rev Cancer 2003, 3:459–465
30. Mori R, Ishiguro H, Kimura M, Mitsui A, Sasaki H, Tomoda K, Mori Y, Ogawa R, Katada T, Kawano O, Harada K, Fujii Y, Kuwabara Y: PIK3CA mutation status in Japanese esophageal squamous cell carcinoma. J Surg Res 2008, 145:320–326
31. Di Nicolantonio F, Arena S, Tabernero J, Grosso S, Molinari F, Macarulla T, Russo M, Cancelliere C, Zecchin D, Mazzucchelli L, Sasazuki T, Shirasawa S, Geuna M, Frattini M, Baselga J, Gallicchio M, Biffo S, Bardelli A: Deregulation of the PI3K and KRAS signaling pathways in human cancer cells determines their response to everolimus. J Clin Invest 2010, 120:2858–2866
32. Yoshikawa K, Hamada J, Tada M, Kameyama T, Nakagawa K, Suzuki Y, Ikawa M, Hassan NM, Kitagawa Y, Moriuchi T: Mutant p53 R248Q but not R248W enhances in vitro invasiveness of human lung cancer NCI-H1299 cells. Biomed Res 2010, 31:401–411
33. Dearth LR, Qian H, Wang T, Baroni TE, Zeng J, Chen SW, Yi SY, Brachmann RK: Inactive full-length p53 mutants lacking dominant wild-type p53 inhibition highlight loss of heterozygosity as an important aspect of p53 status in human cancers. Carcinogenesis 2007, 28:289–298
34. Monti P, Campomenosi P, Ciribilli Y, Iannone R, Aprile A, Inga A, Tada M, Menichini P, Abbondandolo A, Fronza G: Characterization of the p53 mutants ability to inhibit p73 beta transactivation using a yeast-based functional assay. Oncogene 2003, 22:5252–5260
35. Shi XB, Nesslinger NJ, Deitch AD, Gumerlock PH, deVere White RW: Complex functions of mutant p53 alleles from human prostate cancer. Prostate 2002, 51:59–72
36. Xue C, Haber M, Flemming C, Marshall GM, Lock RB, MacKenzie KL, Gurova KV, Norris MD, Gudkov AV: p53 determines multidrug sensitivity of childhood neuroblastoma. Cancer Res 2007, 67:10351–10360
37. Aurelio ON, Kong XT, Gupta S, Stanbridge EJ: p53 mutants have selective dominant-negative effects on apoptosis but not growth arrest in human cancer cell lines. Mol Cell Biol 2000, 20:770–778
38. Gaiddon C, Lokshin M, Ahn J, Zhang T, Prives C: A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain. Mol Cell Biol 2001, 21:1874–1887
39. Chen JY, Funk WD, Wright WE, Shay JW, Minna JD: Heterogeneity of transcriptional activity of mutant p53 proteins and p53 DNA target sequences. Oncogene 1993, 8:2159–2166
40. Brachmann RK, Vidal M, Boeke JD: Dominant-negative p53 mutations selected in yeast hit cancer hot spots. Proc Natl Acad Sci U S A 1996, 93:4091–4095
41. Monti P, Campomenosi P, Ciribilli Y, Iannone R, Inga A, Abbondandolo A, Resnick MA, Fronza G: Tumour p53 mutations exhibit promoter selective dominance over wild type p53. Oncogene 2002, 21:1641–1648
42. Forrester K, Lupold SE, Ott VL, Chay CH, Band V, Wang XW, Harris CC: Effects of p53 mutants on wild-type p53-mediated transactivation are cell type dependent. Oncogene 1995, 10:2103–2111
43. Hassan NM, Tada M, Hamada J, Kashiwazaki H, Kameyama T, Akhter R, Yamazaki Y, Yano M, Inoue N, Moriuchi T: Presence of dominant negative mutation of TP53 is a risk of early recurrence in oral cancer. Cancer Lett 2008, 270:108–119
44. Waddell S, Jenkins JR, Proikas-Cezanne T: A "no-hybrids" screen for functional antagonizers of human p53 transactivator function: dominant negativity in fission yeast. Oncogene 2001, 20:6001–6008
45. Epstein CB, Attiyeh EF, Hobson DA, Silver AL, Broach JR, Levine AJ: p53 mutations isolated in yeast based on loss of transcription factor activity: similarities and differences from p53 mutations detected in human tumors. Oncogene 1998, 16:2115–2122
46. Subler MA, Martin DW, Deb S: Activation of the human immunodeficiency virus type 1 long terminal repeat by transforming mutants of human p53. J Virol 1994, 68:103–110
47. Zhao Y, Polley EC, Li MC, Lih CJ, Palmisano A, Sims DJ, Rubinstein LV, Conley BA, Chen AP, Williams PM, Kummar S, Doroshow JH, Simon RM: GeneMed: an informatics hub for the coordination of next-generation sequencing studies that support precision oncology clinical trials. Cancer Inform 2015, 14:45–55
48. Cingolani P, Platts A, Wang le L, Coon M, Nguyen T, Wang L, Land SJ, Lu X, Ruden DM: A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly (Austin) 2012, 6:80–92
49. Singh RR, Patel KP, Routbort MJ, Reddy NG, Barkoh BA, Handal B, Kanagal-Shamanna R, Greaves WO, Medeiros LJ, Aldape KD, Luthra R: Clinical validation of a next-generation sequencing screen for mutational hotspots in 46 cancer-related genes. J Mol Diagn 2013, 15:607–622
50. Zook JM, Chapman B, Wang J, Mittelman D, Hofmann O, Hide W, Salit M: Integrating human sequence data sets provides a resource of benchmark SNP and indel genotype calls. Nat Biotechnol 2014, 32:246–251
51. Pont-Kingdon G, Gedge F, Wooderchak-Donahue W, Schrijver I, Weck KE, Kant JA, Oglesbee D, Bayrak-Toydemir P, Lyon E: Design and analytical validation of clinical DNA sequencing assays. Arch Pathol Lab Med 2012, 136:41–46
52. Clopper CJ, Pearson ES: The use of confidence or fiducial limits illustrated in the case of the binomial. Biometrika 1934, 26:404–413
53. R Development Core Team: R: a language and environment for statistical computing. Vienna, R Foundation for Statistical Computing, 2014
54. Thorvaldsdottir H, Robinson JT, Mesirov JP: Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief Bioinform 2013, 14:178–192
55. Infante JR, Somer BG, Park JO, Li CP, Scheulen ME, Kasubhai SM, Oh DY, Liu Y, Redhu S, Steplewski K, Le N: A randomised, double-blind, placebo-controlled trial of trametinib, an oral MEK inhibitor, in combination with gemcitabine for patients with untreated metastatic adenocarcinoma of the pancreas. Eur J Cancer 2014, 50:2072–2081

56. Bodoky G, Timcheva C, Spigel DR, La Stella PJ, Ciuleanu TE, Pover G, Tebbutt NC: A phase II open-label randomized study to assess the efficacy and safety of selumetinib (AZD6244 [ARRY-142886]) versus capecitabine in patients with advanced or metastatic pancreatic cancer who have failed first-line gemcitabine therapy. Invest New Drugs 2012, 30:1216–1223
57. Beadling C, Neff TL, Heinrich MC, Rhodes K, Thornton M, Leamon J, Andersen M, Corless CL: Combining highly multiplexed PCR with semiconductor-based sequencing for rapid cancer genotyping. J Mol Diagn 2013, 15:171–176
58. Hadd AG, Houghton J, Choudhary A, Sah S, Chen L, Marko AC, Sanford T, Buddavarapu K, Krosting J, Garmire L, Wylie D, Shinde R, Beaudenon S, Alexander EK, Mambo E, Adai AT, Latham GJ: Targeted, high-depth, next-generation sequencing of cancer genes in formalin-fixed, paraffin-embedded and fine-needle aspiration tumor specimens. J Mol Diagn 2013, 15:234–247
59. Kanagal-Shamanna R, Portier BP, Singh RR, Routbort MJ, Aldape KD, Handal BA, Rahimi H, Reddy NG, Barkoh BA, Mishra BM, Paladugu AV, Manekia JH, Kalhor N, Chowdhuri SR, Staerkel GA, Medeiros LJ, Luthra R, Patel KP: Next-generation sequencing-based multi-gene mutation profiling of solid tumors using fine needle aspiration samples: promises and challenges for routine clinical diagnostics. Mod Pathol 2014, 27:314–327
60. Shortliffe EH, Davis R, Axline SG, Buchanan BG, Green CC, Cohen SN: Computer-based consultations in clinical therapeutics: explanation and rule acquisition capabilities of the MYCIN system. Comput Biomed Res 1975, 8:303–320
61. Miller RA, Pople HE Jr, Myers JD: Internist-1, an experimental computer-based diagnostic consultant for general internal medicine. N Engl J Med 1982, 307:468–476
62. Pan KH, Lih CJ, Cohen SN: Analysis of DNA microarrays using algorithms that employ rule-based expert knowledge. Proc Natl Acad Sci U S A 2002, 99:2118–2123
63. Pan KH, Lih CJ, Cohen SN: Effects of threshold choice on biological conclusions reached during analysis of gene expression by DNA microarrays. Proc Natl Acad Sci U S A 2005, 102:8961–8965
64. Von Hoff DD, Stephenson JJ Jr, Rosen P, Loesch DM, Borad MJ, Anthony S, Jameson G, Brown S, Cantafio N, Richards DA, Fitch TR, Wasserman E, Fernandez C, Green S, Sutherland W, Bittner M, Alarcon A, Mallery D, Penny R: Pilot study using molecular profiling of patients' tumors to find potential targets and select treatments for their refractory cancers. J Clin Oncol 2010, 28:4877–4883
65. Roychowdhury S, Iyer MK, Robinson DR, Lonigro RJ, Wu YM, Cao X, Kalyana-Sundaram S, Sam L, Balbin OA, Quist MJ, Barrette T, Everett J, Siddiqui J, Kunju LP, Navone N, Araujo JC, Troncoso P, Logothetis CJ, Innis JW, Smith DC, Lao CD, Kim SY, Roberts JS, Gruber SB, Pienta KJ, Talpaz M, Chinnaiyan AM: Personalized oncology through integrative high-throughput sequencing: a pilot study. Sci Transl Med 2011, 3:111ra21
66. Hollebecque A, Massard C, De Baere T, Auger N, Lacroix L, Koubi-Pick V, Vielh P, Lazar V, Bahleda R, Ngo-camus M, Angevin E, Varga A, Deschamps F, Gazzah A, Mazoyer C, Richon C, Vassal G, Eggermont AM, Andre F, Soria J-C: Molecular screening for cancer treatment optimization (MOSCATO 01): a prospective molecular triage trial—interim results (abstract). 2013 ASCO Annual Meeting. J Clin Oncol 2013, 31(Suppl):2512, Abstract 2512
67. Bragg LM, Stone G, Butler MK, Hugenholtz P, Tyson GW: Shining a light on dark sequencing: characterising errors in Ion Torrent PGM data. PLoS Comput Biol 2013, 9:e1003031
68. McShane LM, Cavenagh MM, Lively TG, Eberhard DA, Bigbee WL, Williams PM, Mesirov JP, Polley MY, Kim KY, Tricoli JV, Taylor JM, Shuman DJ, Simon RM, Doroshow JH, Conley BA: Criteria for the use of omics-based predictors in clinical trials. Nature 2013, 502:317–320