Next Generation MUT-MAP, a High-Sensitivity High-Throughput Microfluidics Chip-Based Mutation Analysis Panel

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Abstract

Molecular profiling of tumor tissue to detect alterations, such as oncogenic mutations, plays a vital role in determining treatment options in oncology. Hence, there is an increasing need for a robust and high-throughput technology to detect oncogenic hotspot mutations. Although commercial assays are available to detect genetic alterations in single genes, only a limited amount of tissue is often available from patients, requiring multiplexing to allow for simultaneous detection of mutations in many genes using low DNA input. Even though next-generation sequencing (NGS) platforms provide powerful tools for this purpose, they face challenges such as high cost, large DNA input requirement, complex data analysis, and long turnaround times, limiting their use in clinical settings. We report the development of the next generation mutation multi-analyte panel (MUT-MAP), a high-throughput microfluidic, panel for detecting 120 somatic mutations across eleven genes of therapeutic interest (AKT1, BRAF, EGFR, FGFR3, FLT3, HRAS, KIT, KRAS, MET, NRAS, and PIK3CA) using allele-specific PCR (AS-PCR) and Taqman technology. This mutation panel requires as little as 2 ng of high quality DNA from fresh frozen or 100 ng of DNA from formalin-fixed paraffin-embedded (FFPE) tissues. Mutation calls, including an automated data analysis process, have been implemented to run 88 samples per day. Validation of this platform using plasmids showed robust signal and low cross-reactivity in all of the newly added assays and mutation calls in cell line samples were found to be consistent with the Catalogue of Somatic Mutations in Cancer (COSMIC) database allowing for direct comparison of our platform to Sanger sequencing. High correlation with NGS when compared to the SuraSeq500 panel run on the Ion Torrent platform in a FFPE dilution experiment showed assay sensitivity down to 0.45%. This multiplexed mutation panel is a valuable tool for high-throughput biomarker discovery in personalized medicine and cancer drug development.

Introduction

Biological markers, or biomarkers, have been defined as “any substance, structure or process that can be measured in bi-specimen and which may be associated with health-related outcomes” [1]. Currently biomarkers are being used for prognostic, diagnostic, and predictive purposes in the field of oncology and as such play a vital role in personalized medicine. Biomarkers can be used to determine subsets of a population that may or may not respond to drug treatment/therapy and can even be used to prescreen patients in clinical trials. The reliable detection and validation of these markers is therefore essential.

In the last ten years, developments in genome-wide analytic methods have made the profiling of gene expression and genetic alternations of the cancer genome possible. By determining the molecular profile of a tumor (both mutational status and gene expression), a patient’s disease can be characterized. This information can then be used to determine which course of treatment a patient should follow. A recent example of such targeted therapy is the development of ZELBORAF for treatment of patients whose unresectable or metastatic melanoma harbors a BRAF V600E mutation [2]. A companion diagnostic assay was developed with this drug to screen patients, allowing only those patients whose tumors were biomarker positive to receive the treatment. Somatic mutations, therefore, can serve as tumor specific biomarkers, allowing for the use of targeted therapies.

One of the biggest challenges in using clinical samples for biomarker detection is the fact that most tumor biopsies are formalin-fixed and paraffin-embedded (FFPE) for long term storage of the tissue [3]. This treatment leads to lower yield and quality of isolated genomic DNA (gDNA) from the samples due to cross-linking and fragmentation.
### Table 1. Mutation Coverage Breakdown by Gene.

| Gene | Mutation Count | Exon | Mutation ID | cDNA Mutation Position | Amino Acid Mutation Position |
|------|----------------|------|-------------|------------------------|-----------------------------|
| PIK3CA | 17 | 1 | 746 | 263 G>A | R88Q |
| | | 4 | 754 | 1034 A>T | N345K |
| | | 7 | 757 | 1258 T>C | C420R |
| | | 9 | 760 | 1624 G>A | E542K |
| | 12458 | 1634 A>C | E543A |
| | | 764 | 1634 A>G | E545G |
| | | 765 | 1635 G>T | E545D |
| | | 763 | 1633 G>A | E545K |
| | | 147 | 1636 C>G | Q546E |
| | | 766 | 1636 C>A | Q546K |
| | 12459 | 1637 A>G | Q546R |
| | | 25041 | 1637 A>T | Q546L |
| | 20 | 773 | 3129 G>T | M1043I |
| | | 776 | 3140 A>T | H1047L |
| | | 775 | 3140 A>G | H1047R |
| | | 774 | 3139 C>T | H1047Y |
| | 12597 | 3145 G>C | G1049R |
| HRAS | 11 | 2 | 480 | 34 G>A | G12S |
| | | 481 | 34 G>T | G12C |
| | | 483 | 35 G>T | G12V |
| | | 484 | 35 G>A | G12D |
| | | 487 | 37 G>A | G13S |
| | | 486 | 37 G>C | G13R |
| | 3 | 496 | 681 C>A | Q61K |
| | | 498 | 342 A>G | Q61R |
| | | 499 | 181 A>T | Q61L |
| | | 502 | 183 G>C | Q61Hc |
| FGFR3 | 9 | 6 | 714 | 742 C>T | R248C |
| | | 715 | 746 C>G | S249C |
| | | 8 | 718 | 1118 A>G | Y373C |
| | | 716 | 1108 G>T | G370C |
| | | 17461 | 1111 A>T | S371C |
| | | 24842 | 1138 G>A | G380R |
| | 13 | 719 | 1948 A>G | K650E |
| | | 720 | 1949 A>T | K650M |
| | 15 | 24802 | 2089 G>T | G697C |
| FLT3 | 4 | 20 | 785 | 2503 G>C | D835H |
| | | 783 | 2503 G>T | D835Y |
| | | 784 | 2504 A>T | D835V |
| | | 787 | 2505 T>A | D835E |
| MET | 4 | 2 | 710 | 1124 A>G | N375S |
| | | 14 | 707 | 3029 C>T | T1010I |
| | | 19 | 699 | 3743 A>G | Y1248C |
| | | 700 | 3757 T>G | Y1253D |
| KIT | 8 | 11 | 1219 | 1669 T>C | W577R |
| | | 1221 | 1669 T>G | W577G |
| | | 1290 | 1727 T>C | L576P |
Characterization of the cancer genome by next generation sequencing (NGS) methods have emerged, ignited by the increased understanding of somatic alternations in cancer and their value in the development of personalized therapeutics. However, NGS lacks the analytical sensitivity and quantitative performance required for mutation detection in FFPE tissues. Furthermore, currently NGS requires larger DNA quantities for analysis, has complex and time consuming data analysis pipelines, and involves high costs, all of which makes NGS impractical for routine clinical use.

We previously developed a mutation multi-analyte panel (MUT-MAP) that allowed for the detection of 71 mutations across six oncogenes. This panel utilized the Fluidigm microfluidics technology which allowed for simultaneous detection of these mutations in a single sample. We report here the development and validation of the next generation MUT-MAP, a high-throughput platform that can now detect 120 hotspot mutations in eleven genes across six oncogenes. This panel utilized the Fluidigm microfluidics technology which allowed for simultaneous detection of mutations in FFPE tissues. Furthermore, these mutations in a single sample. We report here the

Materials and Methods

Microfluidics

The updated MUT-MAP panel was run on the BioMark platform (Fluidigm Corp.) using a 96.96 dynamic array as described previously [11] with a few alterations. Preamplified DNA combined with qPCR reagents and 10× assays mixed with the Fluidigm 20× sample loading reagent (Fluidigm Corp.) were loaded onto the chip as per the manufacturer’s protocol. All newly added assays were allele-specific PCR (AS-PCR) assays which utilized an engineered Thermus specie Z05 DNA polymerase (AS1) and primers to allow for allelic discrimination between the wild-type and mutant sequence. [12,13] An exon specific probe was used in all assays.

DNA Preamplification

DNA preamplification procedures were performed as described previously [11]. Briefly, DNA was preamplified in a 10 μl reaction for 20 cycles in the presence of a preamplification primer cocktail mix (Table S1 shows sequences of newly added primers) and 1× ABI Preamp Master Mix (Applied Biosystems; Foster City, CA). All samples were exonuclease treated after PCR amplification to remove the remaining primers before being loaded onto the chip. Exonuclease I (16 U) (New England Biolabs; Ipswitch, MA) in exonuclease reaction buffer and nuclease-free water were added to each 10 μl PCR amplification and incubated at 37°C for 30 min followed by a 15 min incubation at 80°C for enzyme inactivation. Samples were then diluted four-fold in nuclease-free water and stored at 4°C or −20°C until needed.

A positive control was prepared in bulk by amplification of a cocktail of relevant mutant plasmids for all eleven genes in the presence of a wild-type human genomic DNA background; this positive control was run in triplicate on every chip for quality control purposes.

Preparation of Reagents

All assays from the previous MUT-MAP were prepared as described previously [11]. Final primer and probe concentrations of 200 and 100 nM were used respectively for the newly designed custom AS-PCR assays which were added to the panel. These assays are currently under development at Roche Molecular Systems, Inc. (Pleasanton, CA).

A commercially available COBAS PIK3CA Mutation Test (Roche Molecular Systems) was modified to achieve compatibility with the two-color BioMark readout (FAM and VIC) for mutation detections in the PIK3CA gene.

All assays were prepared by diluting assays with the 20× sample loading buffer (Fluidigm Corp.). Diluted samples were mixed with AS1 qPCR master mix and run in duplicate by loading 5 μl into

Table 1. Cont.

| Eleven-Gene Mutation Coverage by AS-PCR Assays |
|------------------------------------------------|
| Gene | Mutation Count | Exon | Mutation ID | cDNA Mutation Position | Amino Acid Mutation Position |
|------|----------------|------|-------------|------------------------|-----------------------------|
| 13   | 1304           | 1924 A>G | K642E       |
| 17   | 1311           | 2446 G>C | D816H       |
| 1310 | 2446 G>T       | D816Y       |
| 1314 | 2447 A>T       | D816V       |

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each well of a primed 96.96 Fluidigm Chip. The 96.96 dynamic array was loaded and then analyzed with the BioMark reader as previously described [11].

Data was analyzed and cycle threshold (C_T) values were determined using the BioMark real-time PCR analysis software (Fluidigm Corp.) and automated mutation calls were determined using an algorithm based on the difference in C_T (ΔC_T) values between wild-type and mutant assays for all AS-PCR assays.

Eleven-Gene Mutation Panel

This MUT-MAP panel can screen 120 hotspot mutations across the AKT1, BRAF, EGFR, FGFR3, FLT3, HRAS, KIT, KRAS, MET, NRAS, and PIK3CA genes. The mutation coverage of additional content on this panel is presented in Table 1.

Assay Specificity and Sensitivity

Individual plasmids, each containing a single mutation correlating to each newly added assay on the 11-gene panel were used as samples to determine assay specificity and determine potential cross-reactivity between different hotspots.

Five linearized mutant plasmids were mixed to a final concentration of 4 ng/μL. The resulting mixes were diluted in either nuclease-free water or wild-type genomic DNA (Taqman Control Human Genomic DNA, Life Technologies, Cat# 4312660) where the genomic DNA concentration was kept constant at 10 ng. All of the samples were analyzed by the 11-gene panel along with a standard curve of wild-type human gDNA alone. Percentage of each mutation detected was calculated and the lower limit of detection (LLOD) of the assays in a genomic DNA background was determined for each assay evaluated. The samples diluted in nuclease-free water allowed for the assessment of assay linearity.

Platform Validation

Mutation calls were validated using cell lines as well as FFPE tissues. Cell lines with known mutations reported in the literature were used to confirm the sensitivity and specificity of the assays. Further, a total of nine FFPE samples with known mutation status were mixed together with varying DNA inputs into seven Latin square mixes. The final DNA concentration of each mix was 40 ng/μl. These seven mixes were analyzed on MUT-MAP as well as by the SuraSeq500 panel on the Ion Torrent platform [14] in order to compare mutation calls and sensitivity levels of both platforms. The resulting data has been uploaded to the European Nucleotide Archive, http://www.ebi.ac.uk/ena/data/view/PRJEB5209.

Results

Panel Contents

To increase the coverage of our MUT-MAP platform, AS-PCR assays for HRAS, FGFR3, FLT3, KIT, MET, and PIK3CA were added (Table 1). The updated panel can now detect 120 somatic mutations across eleven genes of therapeutic interest for a single sample. By multiplexing assays and using two detection channels (FAM and VIC), we were able to consolidate all the assays onto a single Fluidigm microfluidics chip allowing for the simultaneous detection of 120 mutations in 44 samples.

Mutant Control Formulation

A single control sample was formulated to be used as a positive control for every assay on MUT-MAP using the process described in Figure 1A. The positive control was generated by mixing mutant plasmids in the presence of a wild-type human genomic DNA background. The positive control was further preamplified and diluted to a concentration that resulted in C_T ranges from 9–16 across all wild-type and mutant assays.

Figure 1. (A) Schematic diagram for the process of generating the positive control for MUT-MAP. (B) The positive control is a mixture of mutant plasmids and wild-type human genomic DNA. The positive control was created such that the resulting C_T range from 9–16 across all wild-type and mutant assays. Pk_H1047X covers multiple hotspot mutations resulting in a lower overall C_T as it is detecting more than one plasmid in the positive control. doi:10.1371/journal.pone.0090761.g001
Assay Validation
A series of experiments were performed to validate the new assays added to the panel to ensure specificity and reproducibility. As described previously [11], a complete cross-reactivity analysis was conducted by screening a set of plasmids containing the mutant sequences against every assay on the panel. The CT values generated from these experiments are shown in Tables 2 and 3. A CT value of 30.0 indicates no amplification and that the specific mutation was not detected in that sample. Any CT value lower than 30.0 indicate amplification and those values generated by mutation-specific assays on their corresponding mutant plasmid are indicated in bold (Tables 2 and 3).

By utilizing the new AS-PCR assays, we were able to prevent the cross-reactivity found in certain instances on our previous panel (Tables 2 and 3). This highlights the specificity of our assays as some of the mutations are in the exact same position but have a single altered base, as in the case of Hr_G12S (position 34 G>A) and Hr_G12C (position 34 G>T) in Table 2.

Platform Reproducibility
The reproducibility of the mutation detection assays were evaluated by the comparison of duplicate experiments. The inter- and intra-chip variability in assay CT values was examined as shown in Figure 2. Inter-chip reproducibility was accessed by
directly comparing the CT values of the mutant control between two chips and the Pearson correlation coefficient \( r^2 \) was calculated to be 0.995. A total of 5290 duplicate pairs were mapped on a scatter plot to determine the intra-chip reproducibility and the \( r^2 \) value was found to be 0.990.

To insure that no variability was introduced by different operator analysis, data from a single MUT-MAP experiment was analyzed by three independent operators. The CTs for the mutant control were found to have an \( r^2 \) value of 0.993 after multiple regression analysis (data not shown).

**Assay Sensitivity and Linearity**

When sensitivity of assays were assessed by diluting plasmids serially either in nuclease-free water or a constant wild-type genomic DNA background (10 ng), most assays showed a lower limit of detection (LLOD) of 0.1–0.2% with a few exceptions. A few examples of such sensitivity analysis are shown in Figure 3 and the remaining data is shown in Figure S1. The wild-type and mutant CTs for these samples are graphed in blue, clearly showing that in the constant wild-type genomic DNA background the indicated mutation can be detected down to LLOD of 0.1–0.2% with a few exceptions as marked in Figure 3. The plasmid diluted in nuclease-free water (red squares) illustrates excellent linearity of the assays.

**Validation of Cell Line Samples**

For cell line samples, gene-specific custom algorithms were written, taking into account the control CT and the mutant CT values. Samples showing \( \Delta C_{\text{T}} < 6 \) were determined as positive for the specific mutation.

Over 600 cell lines have been analyzed by the MUT-MAP to detect mutations across the eleven genes. Table 4 highlights some of the cell lines that were found to have mutations that were detected by the newly added assays. These mutation calls were compared with the published characteristics of these cell lines annotated in the COSMIC database [15].

**Benchmarking Sensitivity of MUT-MAP with NGS**

To assess the accuracy and sensitivity of the MUT-MAP, we compared it with a commonly used NGS platform. Seven Latin Square mixes were formulated by mixing nine different FFPE samples containing twelve hotspot mutations (\( \text{AKT1 E17K, BRAF V600E, EGFR deletion and L858R, HRAS Q61R, KRAS G12A, D, S and G13D, MET T1010I, and PIK3CA E545K and H1047L} \)). When possible, the percentage of each mutation in the parental samples was determined by the SuraSeq500 panel (Figure 4A). Based on these percentages, the amount of each mutation in the seven Latin Square mixes were calculated and ranged from 0.14–32\% (Figure 4B). By analyzing these samples on both platforms we were able to directly compare the sensitivity of twelve of our assays with the SuraSeq500 panel (Figure 4C).

MUT-MAP was able to detect down to a 1.87% mutation for \( \text{PIK3CA H1047X} \) while NGS detected down to 0.94\%. For \( \text{BRAF V600E} \), MUT-MAP utilizes a TaqMan assay which was found to be less sensitive than the SuraSeq500 panel (9.05\% and 0.28\% respectively). Both platforms showed similar sensitivity to the \( \text{AKT1 E17K} \) mutation, as well as, the \( \text{KRAS G12A and D, and G13D} \) mutations. For the \( \text{PIK3CA E545X} \) and \( \text{KRAS G12S} \) mutations, both platforms were able to detect the lowest concentration present in our Latin Square mixes. The MUT-MAP panel also was able to detect \( \text{HRAS Q61R} \) down to a frequency of 0.39\% while the SuraSeq500 panel did not detect the mutation at all in the Latin Square mixes or in the parental sample.

**Disease-Specific Prevalence Study Analyses**

We have performed oncogene mutation profiling on over 1000 individual tumor samples, including FFPE samples, from various cancer types. As an example, using the data generated with MUT-MAP we were able to determine the prevalence of specific mutations in breast and colon cancer (Figure 5A and B, respectively). For a collection of over 500 breast cancer samples we found 29.1\% \( \text{PIK3CA} \) mutations, which is consistent with the COSMIC database [15,16,17,18]. We observed many \( \text{KRAS} \) (52.9\%), \( \text{PIK3CA} \) (12.4\%), and \( \text{NRAS} \) (7.4\%) mutations in a colon cancer tissue collection (N = 121). The prevalence of these mutations also correlate well with those listed in the COSMIC database and other literature [19,20,21,22,23,24,25,26,27,28,29,30]. These results show that MUT-MAP is a sensitive and accurate platform to determine the
Figure 3. Evaluation of assay sensitivity. Linearized plasmids containing the mutant sequence were mixed and diluted into a background of wild-type genomic DNA from 50-0.1% mutant (blue diamonds). A sample containing 5% of the corresponding mutant plasmid with a wild-type genomic DNA background was diluted in nuclease-free water (red squares). Samples were run on the panel and assay sensitivity was determined. The CT of wild-type genomic DNA alone is indicated by the green triangles.

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Table 4. Correlation Between Mutation Calls in Cell Lines and Those Reported in the Literature.

| Cosmic ID | Samples | AKT1 | BRAF | PIK3CA | NRAS | KRAS | EGFR | FGFR3 | FLT3 | HRSAS | KIT | MET |
|-----------|---------|------|------|--------|------|------|------|-------|------|-------|-----|------|
| 687505    | C-33 A  | MND  | MND  | R88Q   | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 909757    | SW 948  | MND  | MND  | E542K  | MND  | Q61L | MND  | MND   | MND  | MND   | MND | MND |
| 906824    | Ca Ski  | MND  | MND  | E545K  | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 908138    | MXN-1   | MND  | MND  | E545X  | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 924239    | L-363   | MND  | MND  | E545X  | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 910698    | BFTC-909| MND  | MND  | E545X  | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 924100    | 22Rv1   | MND  | MND  | Q546X  | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 1752763   | Detroit | MND  | MND  | H1047X | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 177555    | MCAS    | MND  | MND  | H1047R | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 1707557   | HEC-1-A | MND  | MND  | G12D   | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 1576458   | HEC-1-B | MND  | MND  | G12D   | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 1740213   | KMS-11  | MND  | MND  | Y373C  | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 909249    | OPM-2   | MND  | MND  | K650E  | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 1339921   | KYSE-30 | MND  | MND  | Q61L   | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 1752766   | SCC-25  | MND  | MND  | N375S  | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 668093    | Caov-4  | MND  | MND  | MND    | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 1436036   | OVCAR-8 | MND  | MND  | MND    | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 909777    | U-698-M | MND  | MND  | MND    | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 1086323   | BJAB    | MND  | MND  | MND    | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |
| 1295511   | SU-DHL-8| MND  | MND  | MND    | MND  | MND  | MND  | MND   | MND  | MND   | MND | MND |

MND, mutation not detected.
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mutational status in FFPE tissues and may be utilized to classify patients in clinical trials who may derive greater benefit with a targeted therapy.

Discussion

Targeted therapies based on the mutational profiles of the tumor have become increasingly important in cancer diagnostics. We report here an updated MUT-MAP with expanded mutational coverage that includes 120 hotspot mutations in eleven cancer related genes. This panel requires as little as 2 ng of high quality gDNA from fresh frozen tissues or 100 ng of gDNA from FFPE tissues and validation using mutant plasmids showed robust assay signal and low cross-reactivity with all of the newly added assays. Mutation calls in cell lines were found to be consistent with the COSMIC database and MUT-MAP showed a 0.45% sensitivity in FFPE samples.

A

| Parental Samples |
|------------------|
| Mutation         | Percent       |
| Eg_Del           | ND            |
| 2 Kr_G12A/G13D/Me_T1010l | 1.8/20.68/ND |
| 3 Ak_E17K/Kr_G12D | 32.31/31.19   |
| 4 Br_V600E       | 18.1          |
| 5 Hr_Q61R        | MND           |
| 6 Eg_L858R       | 13.14         |
| 7 Pk_H1047L      | 59.9          |
| 8 Kr_G12D        | 21.96         |
| 9 Pk_E545K/Kr_G12S | 31.99/34.28   |

ND= Not detectable by this version of SuraSeq panel
MND= Mutation Not Detected

B

| Latin Square Formulation |
|--------------------------|
| Eg_Del* Me_T1010* Kr_G13D Kr_G12A Kr_G12D Ak_E17K Br_V600E Hr_Q61R Eg_L858R Pk_H1047X Pk_E545X Kr_G12S |
| LS1 3.30 12.60 5.17 0.45 7.50 4.04 1.13 1.67 0.21 0.47 3.76 4.29 |
| LS2 0.39 25.00 10.34 0.50 15.00 8.08 2.26 3.15 0.41 0.94 3.76 4.29 |
| LS3 0.78 0.39 0.16 0.01 32.75 16.16 4.53 6.30 0.82 1.87 0.00 0.00 |
| LS4 1.56 0.78 0.32 0.03 3.22 0.25 9.05 12.60 1.64 3.74 0.00 0.00 |
| LS5 3.13 1.56 0.65 0.06 2.31 0.50 0.14 25.20 3.29 7.49 0.00 0.00 |
| LS6 6.25 3.13 1.29 0.11 3.25 1.01 0.28 0.39 6.57 14.98 0.00 0.00 |
| LS7 12.50 6.25 2.59 0.23 4.44 2.02 0.57 0.79 0.10 29.95 0.00 0.00 |

* Assumed to be 50% mutant in the FFPE parental sample
Rest of the % mutations are based on NGS data

C

| Lowest Percent Mutation Detected |
|----------------------------------|
| Gene   | Mutation | MUT-MAP | SuraSeq |
| AKT1   | E17K     | 1.01    | 1.01    |
| BRAF   | V600E    | 9.05    | 0.28    |
| EGFR   | L858R    | 1.64    | 0.41    |
|        | Deletion | 0.78    | ND      |
| HRAS   | Q61R     | 0.39    | MND     |
| KRAS   | G12A     | 0.45    | 0.45    |
|        | G12D     | 2.31    | 2.31    |
|        | G12S*    | 4.29    | 4.29    |
|        | G13D     | 0.65    | 0.65    |
| MET    | T1010I   | 0.78    | ND      |
| PIK3CA | E545X*   | 3.76    | 3.76    |
|        | H1047X   | 1.87    | 0.94    |

*These mutations were not present in a lower concentration
ND= Not detectable by this version of SuraSeq panel
MND= Mutation Not Detected

Figure 4. Comparison of the sensitivity of MUT-MAP and a next generation sequencing platform. (A and B) Nine FFPE samples with known mutation status were mixed together in varying concentrations following a Latin Square design to generate a seven-member Latin Square panel. The percentage of the mutant allele in each mix was calculated based on the mutant fraction of the parental samples as determined by analysis with the SuraSeq500 panel. For those mutations not detected by the NGS panel, 50% mutation in the parental sample was assumed. (C) The seven Latin Square samples were analyzed on MUT-MAP as well as by the SuraSeq500 panel on Ion Torrent in order to compare mutation calls and sensitivity levels of both platforms.

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mutational status in FFPE tissues and may be utilized to classify patients in clinical trials who may derive greater benefit with a targeted therapy.
In comparison to the SuraSeq500 panel we have demonstrated that MUT-MAP is more sensitive in detecting the HRAS Q61R mutation in FFPE samples and has a similar sensitivity for detecting AKT1 E17K, KRAS G12A, D, and G13D mutations. SuraSeq500 was more sensitive in detecting BRAF V600E and EGFR L858R. Furthermore, MUT-MAP was able to detect these mutations with a much shorter turnaround time from start to finish, including data analysis, than the NGS platform used. While MUT-MAP lacks the breadth of coverage and flexibility of NGS, the platform can accurately and reliably detect hotspot mutations down to 0.45% (KRAS G12A) with very little FFPE DNA input. To date, we have utilized the platform to support multiple clinical programs and to study the prevalence of mutations in various disease settings to assist decision-making in drug development.

In conclusion, we describe here the development and validation of MUT-MAP, a high-sensitivity microfluidics chip-based mutation analysis panel to assay 120 hotspots across eleven oncogenes. This panel can rapidly and accurately determine the mutation status of cancer patient samples in a cost-effective and high-throughput manner. The mutation profiling data generated by MUT-MAP can be used to guide clinical decision-making and inform future clinical trial designs that could aid in the development of personalized health care.

Supporting Information

Figure S1 Evaluation of assay sensitivity and linearity. (TIF)

Table S1 The preamplification primer sequences for the new MUT-MAP content: oncogenes PIK3CA, HRAS, FGFR3, FLT3, KIT and MET. (XLSX)

Table S2 Cross-reactivity matrix for the newly added assays in FGFR3, FLT3, KIT, and MET. (XLSX)
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