Mutation Scanning Using MUT-MAP, a High-Throughput, Microfluidic Chip-Based, Multi-Analyte Panel

Rajesh Patel1*, Alison Tsan2, Rachel Tam1, Rupal Desai1, Nancy Schoenbrunner2, Thomas W. Myers3, Keith Bauer3, Edward Smith3, Rajiv Raja1

1 Oncology Biomarker Development, Genentech Inc., South San Francisco, California, United States of America, 2 Chemistry and Innovation Technology, Pleasanton, California, United States of America, 3 Program in Core Research Roche Molecular Systems Inc., Pleasanton, California, United States of America

Abstract

Targeted anticancer therapies rely on the identification of patient subgroups most likely to respond to treatment. Predictive biomarkers play a key role in patient selection, while diagnostic and prognostic biomarkers expand our understanding of tumor biology, suggest treatment combinations, and facilitate discovery of novel drug targets. We have developed a high-throughput microfluidics method for mutation detection (MUT-MAP, mutation multi-analyte panel) based on TaqMan or allele-specific PCR (AS-PCR) assays. We analyzed a set of 71 mutations across six genes of therapeutic interest. The six-gene mutation panel was designed to detect the most common mutations in the EGFR, KRAS, PIK3CA, NRAS, BRAF, and AKTI oncogenes. The DNA was preamplified using custom-designed primer sets before the TaqMan/AS-PCR assays were carried out using the Biomark microfluidics system (Fluidigm; South San Francisco, CA). A cross-reactivity analysis enabled the generation of a robust automated mutation-calling algorithm which was then validated in a series of 51 cell lines and 33 FFPE clinical samples. All detected mutations were confirmed by other means. Sample input titrations confirmed the assay sensitivity with as little as 2 ng gDNA, and demonstrated excellent inter- and intra-chip reproducibility. Parallel analysis of 92 clinical trial samples was carried out using 2–100 ng genomic DNA (gDNA), allowing the simultaneous detection of multiple mutations. DNA prepared from both fresh frozen and formalin-fixed, paraffin-embedded (FFPE) samples were used, and the analysis was routinely completed in 2–3 days: traditional assays require 0.5–1 μg high-quality DNA, and take significantly longer to analyze. This assay can detect a wide range of mutations in therapeutically relevant genes from very small amounts of sample DNA. As such, the mutation assay developed is a valuable tool for high-throughput biomarker discovery and validation in personalized medicine and cancer drug development.

Introduction

Biomarkers have assumed a central role in oncology, enabling the detection, characterization, and targeted treatment of a range of cancer types [1]. The successful application of targeted anticancer therapies depends on the detection of disease subtypes that are most likely to respond to treatment. As such, the detection and validation of tumor biomarkers is critical for the ongoing development of personalized healthcare, both through the support of effective and robust drug trials, and the effective employment of targeted therapies in the clinic [2].

Biomarkers are classified according to their utility: diagnostic biomarkers are indicators of biological status that allow classification of tumors according to their genetic and/or phenotypic characteristics. Predictive biomarkers allow the response to a particular line of treatment to be anticipated, based on the known mode of action of the chosen therapy and an understanding of the underlying tumor biology. Prognostic biomarkers enable the prediction of disease progression in the absence of treatment, and have been used to identify signaling pathways that are potential drivers of disease, and putative drug targets [3].

Although techniques such as tissue microarray immunohistochemistry (IHC) and reverse-transcription polymerase chain reaction (RT-PCR) allow high-throughput screening of protein and mRNA biomarkers in clinical samples [4], significant challenges remain. Biomarker levels vary across human populations, and significant heterogeneity may be observed within single cancer types, even within samples from a single tumor [5,6]. This is exacerbated by the possibility that first-line chemotherapy may induce DNA damage in tumor cells, leading to changes in biomarker status; as biopsy samples are often obtained before first-line treatment, this may be an obstacle to the correct selection of subsequent targeted therapies, although the extent of this effect remains unclear [6].

While some anticancer therapeutics are entering the clinic with companion diagnostic tests, a wider characterization of tumor gene expression and mutation status will enable targeted therapies...
Table 1. Mutation Coverage Breakdown by Gene.

| Gene     | Mutation Count | Exon | Mutation ID | cDNA Mutation Position | Amino Acid Mutation Position |
|----------|----------------|------|-------------|------------------------|-----------------------------|
| EGFR     | 43             | 18   | 6252        | 2155 G>A               | G719S                       |
|          |                |      | 6253        | 2155 G>T               | G719C                       |
|          |                |      | 6239        | 2156 G>C               | G719A                       |
|          |                |      |             |                        |                             |
|          | 19             |      |             |                        |                             |
|          | 20             |      | 6241        | 2303 G>T               | S768I                       |
|          |                |      | 12376       | 2307_2308 ins 9(gccagctg) | V769_D770insASV             |
|          |                |      | 13558       | 2309_2310 complex(ac:ccagctggaat) | V769_D770insASV             |
|          |                |      | 12378       | 2310_2311 ins GGT      | D770_N771insG              |
|          |                |      | 13428       | 2311_2312 ins 9(gctgta) | D770_N771insSVD            |
|          |                |      | 12377       | 2319_2320 ins CAC      | H773_V774insH              |
|          |                |      | 6240        | 2369 C>T               | T790M                       |
|          |                |      |             |                        |                             |
|          | 21             |      | 6224        | 2573 T>G               | L858R                       |
|          |                |      | 12429       | 2573–2574 TG>G         | L858R                       |
|          |                |      | 6213        | 2582 T>A               | L861Q                       |
| PIK3CA   | 4              | 9    | 760         | 1624 G>A               | E542K                       |
|          |                |      | 763         | 1633 G>A               | E545K                       |
|          |                |      |             |                        |                             |
|          | 20             |      | 775         | 3140 A>G               | H1047L                      |
|          |                |      | 776         | 3140 A>T               | H1047R                      |
| KRAS     | 18             | 2    | 522         | 35 G>C                 | G12A                        |
to be combined for specific patient groups without multiple biopsy procedures. A deeper understanding of different tumor subtypes will help explain mechanisms of drug resistance and open up new channels of therapy and research. For this reason, “biomarker pipelines” play an important role in the development of molecular targeted therapies [7].

There are additional challenges associated with biomarker identification using clinical samples containing poor-quality or degraded DNA in limited quantities. Most clinical samples are formalin fixed and paraffin embedded (FFPE) for preservation and storage. While enabling samples to be archived for subsequent biomarker identification and comparison with patient outcomes, this method of preservation leads to nucleic acid fragmentation and cross-linking, so only a small proportion of sample DNA can be probed successfully [8]. Traditional methods of biomarker detection require 0.5–1 μg high-quality DNA and results may take a significant amount of time to analyze, particularly if samples are to be screened for multiple mutations.

We have developed a high-throughput method for mutation detection (MUT-MAP, mutation multi-analyte panel) based on TaqMan and allele-specific PCR (AS-PCR) assays using a microfluidic chip-based technology. This approach allows the rapid analysis of 71 mutations across a panel of six genes of therapeutic interest. Parallel analysis of 92 clinical trial samples can be carried out using miniscule amounts of DNA (2–100 ng, based on the quality of genomic DNA [gDNA] isolated), allowing the simultaneous detection of multiple mutations in a single sample. DNA can be isolated from both fresh frozen and FFPE samples, and the analysis is routinely completed in 2–3 days.

The six-gene panel mutation assay was designed to detect the most common mutations found in EGFR, KRAS, PIK3CA, NRAS, BRAF, and AKT1. Activating mutations in these genes cause aberrant cell signaling and are found in various types of cancer; their encoded proteins are therefore targets for therapeutic inhibition. For example, mutations in EGFR are linked with increased activation of the epidermal growth factor receptor (EGFR) signaling pathway, which drives tumor growth and promotes survival in several types of cancer [9]. The EGFR and KRAS mutation status is predictive of response to anti-EGFR-targeted therapies such as erlotinib, gefitinib [10], and cetuximab [11]. Additionally, the BRAF inhibitor vemurafenib is only effective in patients with V600 mutation-positive melanoma [12,13], and the phosphoinositide-3-kinase (PI3K) inhibitor GDC-0941 is most effective in preclinical tumor models with PIK3CA mutations [14].

Although next-generation parallel sequencing holds great promise for mutation detection across the whole genome, these technologies are not yet mature enough for routine, high-throughput analysis of precious clinical samples. Parallel sequencing generally requires larger quantities of DNA for analysis and

| Table 1. Cont. |
|----------------|

| Six-Gene Mutation Coverage by TaqMan and Prototype EGFR and KRAS AS-PCR Assays |
|----------------|
| **Gene** | **Mutation Count** | **Exon** | **Mutation ID** | **cDNA Mutation Position** | **Amino Acid Mutation Position** |
|----------------|
| 516 | 34 | G>T | G12C |
| 521 | 35 | G>A | G12D |
| 517 | 34 | G>A | G12S |
| 518 | 34 | G>C | G12R |
| 520 | 35 | G>T | G12V |
| 512 | 34, 35 | GG>T | G13D |
| 532 | 38 | G>A | G13F |
| 533 | 38 | G>C | G13A |
| 527 | 37 | G>T | G13C |
| 529 | 37 | G>C | G13R |
| 528 | 37 | G>A | G13S |
| 534 | 38 | G>T | G13V |
| 3 | 554 | 183 | A>C | Q61H |
| 555 | 183 | A>T | Q61H |
| 549 | 181 | C>A | Q61K |
| 553 | 182 | A>T | Q61L |
| 552 | 182 | A>G | Q61R |
| BRAF | 1 | 15 | 476 | 1799 T>A | p.V600E |
| NRAS | 4 | 2 | 564 | 38 | G>A | p.G13D |
| 580 | 181 | C>A | p.G13K |
| 3 | 584 | 182 | A>G | p.Q61R |
| 583 | 182 | A>T | p.Q61L |
| AKT1 | 1 | 4 | 33765 | 49 | G>A | p.E17K |

[doi:10.1371/journal.pone.0051153.t001]
takes longer to generate data in comparison with our approach. The MUT-MAP microfluidics system provides a readily available platform for the exploratory detection of predictive and prognostic biomarkers in support of current and future personalized healthcare.

Materials and Methods

Overview of the MUT-MAP Microfluidics System

Mutation screening with the MUT-MAP microfluidics system is a multi-stage process. First, DNA is preamplified using custom-designed primer sets for the exons/genes of interest. The BioMark platform (Fluidigm Corp.) is then used to conduct a combination of quantitative PCR (qPCR) mutation detection assays. We employ two assay formats for mutation detection: both formats utilize TaqMan detection of the amplified product [15]. In one format, which we refer to as TaqMan genotyping or, simply, TaqMan, the discrimination between mutant and wild-type is driven by a differentially-labeled mutant- and wild-type-specific probe [16]. In the other assay format, the discrimination is driven by a mutant-specific primer, or allele-specific PCR (AS-PCR [17,18]). The AS-PCR assays incorporate the use of an engineered Thermus species Z05 DNA polymerase (AS1) and, in some cases, covalently modified primers to enhance the specificity of allele-specific qPCR [19,20].

The AS-PCR assays were used for KRAS and EGFR mutation analysis, and have broader coverage of the predominant mutations in these two genes compared with some commercially available assays. An overview of the protocol and process flow is presented in figure 1.

The BioMark protocol involves the introduction of premixed qPCR reagents and preamplified DNA onto the MUT-MAP assay chip via the sample inlets. Assay-specific TaqMan primer/probe mixes are normally added via assay ports. This protocol was modified due to the presence of primers and probes in the qPCR reagents for some reactions (EGFR Mutation Test; Roche Molecular Systems, Inc. [RMS]; Pleasanton, CA). To ensure compatibility with the BioMark platform, these samples were

| Mutation Count | Mutation ID | cDNA Mutation Position | Amino Acid Mutation Position |
|----------------|-------------|------------------------|-----------------------------|
| 30             | 26038       | 2233_2247del15         | K745_E749del                |
|                | 13550       | 2235_2248>AAATTC       | E746_A750>IP                |
|                | 6223        | 2235_2249del15         | E746_A750del                |
|                | 13552       | 2235_2251>AAATTC       | E746_T751>IP                |
|                | 13551       | 2235_2252>AAAT         | E746_T751>I                 |
|                | 12385       | 2235_2255>AAAT         | E746_S752>1                 |
|                | 12413       | 2236_2248>AGAC         | E746_A750>RP                |
|                | 6225        | 2236_2250del15         | E746_A750del                |
|                | 12728       | 2236_2253del18         | E746_T751del                |
|                | 12678       | 2237_2251del15         | E746_T751>A                 |
|                | 12386       | 2237_2252>T            | E746_T751>V                 |
|                | 12416       | 2237_2253>TTGCT        | E746_T751>VA                |
|                | 12367       | 2237_2254del18         | E746_S752>A                 |
|                | 12384       | 2237_2255>T            | E746_S752>V                 |
|                | 18427       | 2237_2257>TCT          | E746_P753>V5                |
|                | 12422       | 2238_2248>GC           | L747_A750>P                 |
|                | 23571       | 2238_2252del15         | L747_T751del                |
|                | 12419       | 2238_2252>GCA          | L747_T751>Q                 |
|                | 6220        | 2238_2255del18         | E746_S752>D                 |
|                | 6218        | 2239_2247del19         | L747_E749del                |
|                | 12382       | 2239_2248TTAAGAGAGAAG> | L747_A750>P                 |
|                | 12383       | 2239_2251>C            | L747_T751>P                 |
|                | 6254        | 2239_2253del15         | L747_T751del                |
|                | 6255        | 2239_2256del18         | L747_S752del                |
|                | 12403       | 2239_2256>CAA          | L747_S752>Q                 |
|                | 12387       | 2239_2258>CA           | L747_P753>Q                 |
|                | 6210        | 2240_2251del12         | L747_T751>S                 |
|                | 12369       | 2240_2254del15         | L747_T751del                |
|                | 12370       | 2240_2257del18         | L747_P753>S                 |
|                | 13556       | 2253_2276del24         | S752_I759del                |

Table 2. Mutation Coverage for EGFR Exon 19 Deletions.

doi:10.1371/journal.pone.0051153.t002
introduced via the assay inlets, and both TaqMan and AS-PCR assay reagents were added via the sample inlets on the microfluidic chip. Data analysis was also modified to accommodate these changes.

DNA Preamplification

DNA was preamplified in 10 µl reactions on a 96-well plate using a preamplification primer cocktail (Table S1) in the presence of 1x ABI PreAmp Master Mix (Applied Biosystems; Foster City, CA). gDNA (2–10 ng) was isolated from cell lines and fresh frozen samples. However, due to the poor quality of DNA obtained from FFPE clinical samples, 50–100 ng was used for preamplification from this source. Primer concentrations were 100 nM during the amplification reaction. Each preamplification sample set included a gDNA control to determine the presence of 1x ABI PreAmp Master Mix (Applied Biosystems; Foster City, CA). gDNA (2–10 ng) was isolated from cell lines and fresh frozen samples. However, due to the poor quality of DNA obtained from FFPE clinical samples, 50–100 ng was used for preamplification from this source. Primer concentrations were 100 nM during the amplification reaction. Each preamplification sample set included a gDNA control to determine preamplification performance as well as a no-template control. An additional positive control was made in bulk by preamplification of a cocktail of relevant mutant plasmids for all six genes; this control was run on every chip.

Samples were preamplified using a Tetrad Thermal Cycler (BioRad; Hercules, CA) according to the following protocol: 95°C for 10 minutes, then thermal cycling (20 cycles, each of 15 seconds at 95°C followed by 2 minutes at 60°C). Samples were diluted fourfold, mixed, centrifuged at 3500 rpm (5810 R; Eppendorf; Hauppauge, NY), and stored at 4°C or –20°C until further processing. Following preamplification, rigorous procedures were followed to prevent sample contamination, including the use of dedicated workspaces and pipettes for pre- and post-PCR reaction set-up, laminar flow hoods, and personal protective equipment.

Preparation of Reagents

 Primer/probe concentrations of 900/200 nM were used in the TaqMan reactions to detect mutations in the PIK3CA, BRAF, NRAS, and AKT genes. Custom AS-PCR assays (Roche Molecular Systems) were used to detect mutations in KRAS and EGFR genes along with custom wild-type assays for both genes. A complete description of primers and probes for the TaqMan reactions is presented in table S2.

A commercially available EGFR Mutation Test (Roche Molecular Systems) was modified to achieve compatibility with the two-color BioMark readout (FAM and VIC) for detection of mutations in EGFR. Hexachlorofluorescein (HEX)-labeled probes were spiked into kit mastermixes to detect S768I and T790M in the VIC channel. Additionally, a custom fourth tube was designed to separately detect exon 20 insertion mutations using MMX3 from the RMS EGFR Mutation Test. The KRAS allele-specific assays utilized a research kit from Roche Molecular Systems.

Both TaqMan and AS-PCR assays were carried out using the AS1 qPCR master mix. Rox dye (final concentration 55 nM) for signal normalization and 20x gel electrophoresis sample loading buffer (Fluidigm Corp.) were added to the qPCR reactions. Assays along with AS1 qPCR master mix were run in duplicate by loading 5 µl into each well of the primed 96.96 Fluidigm Chip. The diluted pre amplified DNA samples were mixed with equal volumes of 2x DNA assay loading buffer (Fluidigm Corp.). The samples were run by loading 5 µl into each well on the chip. The

Table 3. Cross-Reactivity of AKT1, BRAF, PIK3CA, and NRAS Mutants.

| Assays | Plasmid controls AKT1, BRAF, PIK3CA, and NRAS | Controls |
|--------|-----------------------------------------------|----------|
| RNaseP | 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 11.5 30.0 |          |
| AKT1 WT| 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 15.8 30.0 |          |
| AKT1 E17K| 20.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 |          |
| Br. WT | 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 13.1 30.0 |          |
| Br. V600E| 30.0 22.4 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 |          |
| Pk_E542 WT| 30.0 30.0 30.0 20.6 30.0 30.0 30.0 30.0 30.0 15.5 30.0 |          |
| Pk_E542K| 30.0 30.0 16.6 30.0 30.0 30.0 30.0 30.0 30.0 30.0 |          |
| Pk_E545 WT| 30.0 30.0 15.3° 30.0 30.0 30.0 30.0 30.0 30.0 30.0 |          |
| Pk_E545K| 30.0 30.0 30.0 17.2 30.0 30.0 30.0 30.0 30.0 12.0 30.0 |          |
| Pk_H1047 WT| 30.0 30.0 30.0 30.0 20.0 30.0 30.0 30.0 30.0 |          |
| Pk_H1047K| 30.0 30.0 30.0 30.0 15.7 19.1° 30.0 30.0 30.0 |          |
| Pk_H1047 WT| 30.0 30.0 30.0 30.0 26.5 30.0 30.0 30.0 30.0 |          |
| Pk_H1047L| 30.0 30.0 30.0 30.0 16.9 30.0 30.0 30.0 30.0 |          |
| Nr. Q61 WT| 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 |          |
| Nr. Q61K| 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 10.5 30.0 |          |
| Nr. Q61 WT| 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 |          |
| Nr. Q61K| 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 10.6 30.0 |          |
| Nr. G12 WT| 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 16.7 30.0 |          |
| Nr. G12D| 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 30.0 |          |

**Proposed**
**Table 4. Cross-Reactivity of KRAS Mutants.**

| Assays     | Plasmid Controls |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|------------|------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kr controls| 12.1             | 12.2| 12.1| 12.0| 12.1| 12.4| 13.1| 12.5| 12.6| 12.0| 13.3| 11.6| 12.3| 12.9| 12.4| 12.6| 13.0| 12.9| 10.4| 30.0 |
| Kr G12S    | 14.3             | 24.8| 28.0| 28.3| 26.6| 25.7| 30.0| 25.8| 27.7| 26.1| 26.4| 25.7| 25.8| 24.2| 24.6| 24.7| 26.7| 27.3| 23.9| 30.0 |
| Kr G12C    | 24.9             | 14.6| 24.2| 29.4| 27.9| 29.1| 17.6a| 26.9| 28.9| 27.9| 30.0| 29.6| 26.4| 28.7| 28.9| 28.5| 30.0| 25.6| 30.0 |
| Kr G12R    | 28.7             | 24.2| 14.2| 29.8| 30.0| 28.2| 30.0| 28.9| 26.0| 30.0| 28.2| 30.0| 28.4| 30.0| 29.4| 29.0| 28.3| 28.5| 30.0| 28.5 |
| Kr G12D    | 27.6             | 28.5| 23.8| 13.6| 24.8| 28.1| 30.0| 25.2| 25.2| 24.5| 25.3| 24.9| 25.7| 25.0| 24.8| 25.0| 25.9| 27.4| 22.9| 30.0 |
| Kr G12V    | 18.2             | 23.4| 25.8| 13.7a| 13.8| 22.7| 20.3| 24.2| 24.3| 24.8| 23.5| 23.9| 22.9| 22.6| 23.3| 23.1| 23.8| 23.4| 21.0| 30.0 |
| Kr G12A    | 27.4             | 26.2| 21.8| 25.0| 22.8| 14.1| 30.0| 28.6| 30.0| 24.8| 27.1| 28.7| 26.9| 28.6| 28.4| 27.6| 28.1| 29.6| 25.3| 30.0 |
| Kr G12F    | 30.0             | 23.4| 30.0| 30.0| 20.0| 29.3| 13.1| 30.0| 30.0| 30.0| 30.0| 28.3| 30.0| 29.3| 30.0| 30.0| 29.8| 30.0| 29.8| 30.0 |
| Kr G13S    | 15.8a            | 22.9| 22.1| 24.5| 22.1| 25.0| 26.4| 13.3| 23.8| 24.7| 240| 22.7| 22.9| 23.3| 23.0| 22.8| 24.4| 24.2| 21.4| 30.0 |
| Kr G13C    | 17.3             | 24.8| 24.7| 22.4| 13.0a| 22.0| 24.1| 23.8| 13.3| 22.4| 23.9| 23.7| 23.7| 22.7| 22.4| 23.0| 23.1| 23.1| 20.5| 30.0 |
| Kr G13R    | 24.9             | 26.6| 29.9| 27.7| 23.3| 28.0| 29.8| 25.9| 22.6| 13.3| 28.0| 16.9| 26.5| 30.0| 28.6| 30.0| 28.5| 30.0| 26.5| 30.0 |
| Kr G13D    | 29.4             | 29.0| 28.2| 19.9| 23.7| 23.9| 30.0| 30.0| 29.1| 29.9| 15.0| 25.4| 28.1| 22.8| 22.8| 23.3| 23.7| 23.6| 21.4| 30.0 |
| Kr G13V    | 25.5             | 20.5| 26.0| 18.5| 25.8| 26.8| 28.0| 29.3| 24.8| 30.0| 243| 12.9| 20.2| 26.2| 25.9| 25.0| 26.7| 26.1| 23.9| 30.0 |
| Kr G13A    | 23.6             | 22.1| 23.4| 17.0| 20.2| 20.5| 24.7| 21.6| 13.9a| 20.0| 26.7| 21.7| 12.9| 21.5| 21.3| 21.4| 22.0| 21.9| 19.5| 30.0 |
| Kr Q61K    | 26.7             | 27.8| 27.4| 26.6| 27.3| 26.5| 27.5| 25.5| 27.5| 25.0| 29.1| 25.2| 25.8| 15.9| 30.0| 30.0| 30.0| 30.0| 25.3| 30.0 |
| Kr Q61L    | 28.2             | 28.9| 27.3| 26.3| 28.0| 28.4| 28.3| 26.8| 27.9| 27.1| 29.9| 26.4| 27.4| 28.7| 16.5| 28.6| 30.0| 29.8| 25.4| 30.0 |
| Kr Q61R    | 26.4             | 26.8| 26.8| 27.0| 27.2| 27.4| 26.4| 25.3| 27.3| 25.1| 28.3| 24.6| 25.3| 28.5| 23.6| 15.4| 27.8| 27.0| 24.2| 30.0 |
| Kr Q61Hc   | 30.0             | 30.0| 29.5| 29.6| 30.0| 30.0| 29.6| 29.7| 30.0| 29.3| 30.0| 29.3| 30.0| 30.0| 26.6| 17.1| 26.6| 27.5| 30.0 |
| Kr Q61Ht   | 28.0             | 27.4| 29.2| 27.1| 27.0| 29.5| 28.0| 26.2| 28.4| 25.4| 29.6| 25.6| 25.9| 26.8| 28.4| 26.5| 28.8| 16.0| 24.8| 30.0 |

*aCross-reactions between the assays are unidirectional and hence do not interfere with accurate mutation calls.*

doi:10.1371/journal.pone.0051153.t004
Table 5. Cross-Reactivity of EGFR Mutants.

| Assays   | Plasmid Controls | EGFR |
|----------|------------------|------|
| Eg_ex20_Cntrl | 30.0              | 30.0 |
| Eg_19del   | 13.0              | 30.0 |
| Eg_S768I   | 30.0              | 30.0 |
| Eg_L858R   | 30.0              | 30.0 |
| Eg_T790M   | 30.0              | 30.0 |
| Eg_L861Q   | 30.0              | 30.0 |
| Eg_G719X   | 30.0              | 30.0 |
| Eg_ins     | 30.0              | 30.0 |
| gDNA      | 11.8              | 30.0 |
| NTC       | 10.1              | 30.0 |
|                | 24.0              | 30.0 |
|                | 26.4              | 30.0 |
|                | 27.3              | 30.0 |
|                | 23.0              | 30.0 |
|                | 23.2              | 30.0 |
|                | 20.5              | 30.0 |
|                | 23.0              | 30.0 |

The C_T values and cross-reactivities obtained from the plasmid data were instrumental in generating an automated mutation-calling algorithm to detect the presence or absence of mutations in clinical samples for each of the six genes in the panel. Samples were re-run on multiple chips to validate both intra- and inter-chip reproducibility.

In general, all samples were correctly identified with high reproducibility and no confounding cross-reactivity. Where cross-reactivity did occur, it was generally an easily discriminated partial reaction. For example, in the TaqMan assays, the cross-reactivity observed between alleles such as PIK3CA E545 wild-type and E542K can be attributed to cross-reactivity of probes with highly similar sequences. In the KRAS AS-PCR assays, cross-reactivity is likely due to sequence content at the 3’ end of the primer sequences. The unidirectional nature of these cross-reactions made it easy to build an algorithm to classify mutation status.

Results

Plasmid Validation

A series of validation experiments was carried out to confirm the reproducibility and accuracy of the microfluidic assay panel. In order to validate the discrimination of closely related sequences by the mutation screening panel, a complete cross-reactivity analysis was conducted by screening every mutant plasmid target against every mutant-specific assay. The C_T values were generated by the BioMark real-time PCR analysis software (Fluidigm Corp.), and plotted as shown in tables 3, 4, and 5. A C_T value of 30.0 represents no reactivity, and is indicative of the absence of that allele from the sample. Deviations from this baseline represent assay reactivity, with a lower C_T value indicative of increased reactivity. The C_T values generated by mutant-specific assays on their corresponding mutant plasmid targets are highlighted in boxed cells (Tables 3, 4, and 5).

Six-Gene Mutation Panel

The use of MUT-MAP in this study allowed the screening of 71 mutations across the EGFR, KRAS, PIK3CA, NRAS, BRAF, and AKT1 genes. The mutation coverage of this panel is presented in tables 1 and 2. Validation of mutations detected in clinical samples was performed using commercial mutation detection assays (Qiagen DxS assays for PIK3CA, KRAS, and EGFR mutations), and in-house developed and validated TaqMan assays (for BRAF, NRAS, and AKT1).

Validation with FFPE Samples

The assay was further validated using clinical FFPE samples harboring known mutations in the genes of interest. A series of 33 FFPE tumor biopsy samples were analyzed by the six-gene mutation panel. Results were compared with data from traditional micro-well plate qPCR assays: mutations in EGFR, KRAS, and PIK3CA were confirmed using Qiagen DxS assays whereas mutations in BRAF, NRAS, and AKT1 were validated with custom in-house-developed TaqMan assays. Execution of the experiments was notably faster with the multiplex assay than with the traditional methods. The MUT-MAP system also required only 10 minutes for each sample, whereas the traditional methods required 10 minutes for each sample.

A good correlation was observed between the experimental results and the traditional mutation detection assays (Table 7). Where samples were available, all outputs were in agreement. The
### Table 6. Correlation Between Mutation Calls in Cell Lines and Those Reported in the Literature.

| Cosmic ID | Samples   | AKT1 | BRAF | PIK3CA | NRAS | KRAS | EGFR |
|-----------|-----------|------|------|--------|------|------|------|
| 1286013   | MGH-U3    | E17K | MND  | MND    | MND  | MND  | MND  |
| 905954    | SK-MEL-28 | MND  | V600E| MND    | MND  | MND  | MND  |
| 909747    | SW1417    | MND  | V600E| MND    | MND  | MND  | MND  |
| 905988    | MDA-MB-435| MND  | V600E| MND    | MND  | MND  | MND  |
| 906844    | DU4475    | MND  | V600E| MND    | MND  | MND  | MND  |
| 908125    | MEL-IJSQ  | MND  | MND  | MND    | Q61L | MND  | MND  |
| 910926    | BFTC      | MND  | MND  | MND    | Q61L | MND  | MND  |
| 724831    | H1299     | MND  | MND  | MND    | Q61K | MND  | MND  |
| 905955    | SKMEL-2   | MND  | MND  | MND    | Q61R | MND  | MND  |
| 909771    | THP-1     | MND  | MND  | MND    | G12D | MND  | MND  |
| 1018466   | BT483     | MND  | MND  | MND    | E542K| MND  | MND  |
| 905946    | MCF-7     | MND  | MND  | MND    | E545K| MND  | MND  |
| 908121    | MDA-MB-361| MND  | MND  | MND    | E545K| MND  | MND  |
| 906851    | EFM19     | MND  | MND  | MND    | H1047L| MND  | MND  |
| 905945    | T-47D     | MND  | MND  | MND    | H1047R| MND  | MND  |
| 905945    | T-47D     | MND  | MND  | MND    | H1047R| MND  | MND  |
| 910948    | MFM-223   | MND  | MND  | MND    | H1047R| MND  | MND  |
| 908122    | MDA-MB-453| MND  | MND  | MND    | H1047R| MND  | MND  |
| 909778    | UACC-893  | MND  | MND  | MND    | H1047R| MND  | MND  |
| 1479574   | LS180     | MND  | MND  | MND    | H1047R| MND  | G12D |
| 905949    | A549      | MND  | MND  | MND    | MND  | G12S |
| 905942    | NCI-H23   | MND  | MND  | MND    | G12C |
| 910546    | PSN-1     | MND  | MND  | MND    | G12R |
| 910702    | AsPC-1    | MND  | MND  | MND    | G12D |
| 908122    | SW403     | MND  | MND  | MND    | G12V |
| 753624    | CAPAN-1   | MND  | MND  | MND    | G12V |
| 724873    | NCI-H2009 | MND  | MND  | MND    | G12A |
| 907790    | LoVo      | MND  | MND  | MND    | G13D |
| 905960    | MDA-MB-231| MND  | MND  | MND    | G13D |
| 907790    | LOVO      | MND  | MND  | MND    | G13D |
| 687800    | NCI-H1650 | MND  | MND  | MND    | MND  | 19del|
| 1028938   | HCC4006   | MND  | MND  | MND    | MND  | 19del|
| 1028936   | HCC827    | MND  | MND  | MND    | MND  | 19del|
| 1336875   | pC9       | MND  | MND  | MND    | MND  | 19del|
| 924244    | NCI-H1975 | MND  | MND  | MND    | L858R/T790M|
| 909751    | SW48      | MND  | MND  | MND    | G719X|
| 905934    | PC-3      | MND  | MND  | MND    | MND  | MND  |
| 910781    | AN3 CA    | MND  | MND  | MND    | MND  | MND  |
| 687804    | NCI-H1770 | MND  | MND  | MND    | MND  | MND  |
| 905947    | 786-O     | MND  | MND  | MND    | MND  | MND  |
| 908471    | NCI-H1581 | MND  | MND  | MND    | MND  | MND  |
| 908481    | NCI-H2196 | MND  | MND  | MND    | MND  | MND  |
| 909907    | ZR-75-30  | MND  | MND  | MND    | MND  | MND  |
| 688015    | NCI-H2171 | MND  | MND  | MND    | MND  | MND  |
| 905986    | SF-268    | MND  | MND  | MND    | MND  | MND  |
| 749712    | HCC1395   | MND  | MND  | MND    | MND  | MND  |
| 749714    | HCC1937   | MND  | MND  | MND    | MND  | MND  |

MND, mutation not detected.

doi:10.1371/journal.pone.0051153.t006
discrepant sample HP-45416 (lung) was not tested for the EGFR T790M mutation as the Qiagen DxS assays did not carry the T790M assay at the time of the study, and retesting is not possible due to lack of additional sample material.

Sample Input Titrations

In order to confirm the reproducibility and consistency of the methodology, sample input titrations were carried out. To define the effective DNA input concentration over which the assay could be considered accurate, and identify the wild-type and mutant CT values for each gene, DNA input was varied for plasmids, cell lines, and FFPE samples, with sample preamplification (Table 8). The C_T values for both the mutant and wild-type show the expected response to input concentration over the titration range.

Platform Reproducibility Validation

The reproducibility of data from mutation detection assays was also evaluated by the comparison of duplicate experiments. The inter- and intra-chip variability in assay C_T values was assessed as shown in figure 2. A total of 5664 duplicate pairs were mapped on a scatter plot, and the Pearson correlation coefficient (R^2) was calculated. The R^2 values were found to be over 0.99 for FAM as well as VIC channels, indicating excellent inter- and intra-chip reproducibility of data generated by the assay.

### Table 7. Correlation Between Mutation Calls in FFPE Samples and Those Determined by TaqMan/Qiagen DxS Assays.

| Samples | Tissues | Six-Gene Mutation Panel | TaqMan/Qiagen DxS |
|---------|---------|-------------------------|-------------------|
|         |         | AKT1 BRAF PIK3CA NRAS KRAS EGFR AKT1 | BRAF PIK3CA NRAS KRAS EGFR |
| HP-40263 | CO | MND | V600E H1047R | MND MND MND MND |
| HP-41765 | CO | E17K | V600E | MND MND MND MND |
| HP-32864 | NOS | MND | E17K V600E | MND MND MND MND |
| HP-3002 | CO | MND | V600E | MND MND MND MND |
| HP-30760 | NOS | MND | V600E | MND MND MND MND |
| HP-4024 | CO | MND | V600E | MND MND MND MND |
| HP-4175 | CO | MND | Q61R | MND MND MND MND |
| HP-40253 | CO | MND | G12A | MND MND MND MND |
| HP-4264 | CO | MND | E545K | MND MND MND MND |
| HP-40122 | CO | MND | G12S | MND MND MND MND |
| HP-32375 | NOS | MND | G12S | MND MND MND MND |
| HP-45416 | LU | MND | L858R/ T790M | MND MND MND MND |
| HP-4563 | NOS | MND | L858R | MND MND MND MND |
| HP-46155 | LU | MND | 19del | MND MND MND MND |
| HP-44217 | NOS | MND | 19del | MND MND MND MND |
| HP-46155 | LU | MND | 19del | MND MND MND MND |
| HP-29847 | CO | MND | E545K | MND MND MND MND |
| HP-30384 | CO | MND | G12A | MND MND MND MND |

MND, mutation not detected.
CO, Adenocarcinoma of Colon.
LU, Adenocarcinoma of Lung.
NOS, Not otherwise specified.
_\text{a}, Insufficient DNA to complete analysis.
doi:10.1371/journal.pone.0051153.t007
Discussion

The future of oncology biomarker detection can be delivered by many promising technologies, including multiplexed protein assays, and parallel next-generation genome sequencing [22,23]. The limited maturity of many of these techniques, combined with their timescale and infrastructure demands, means that there is an unmet need for robust high-throughput biomarker detection methods in the clinical drug development setting.

Our validation has demonstrated that MUT-MAP offers a means of detecting a wide range of mutations in a panel of therapeutically relevant genes, enabling the detection of predictive and prognostic biomarkers from very small amounts of sample DNA. A cross-reactivity analysis showed that this platform has the ability to reliably discriminate between closely related mutations. In addition, the ability of the assay to provide robust reproducible data has been validated in both cancer cell lines and FFPE biopsy samples using considerably smaller amounts of sample DNA than traditional assays. Such an approach enables the study of a wide range of oncogenic mutations in precious clinical samples with very little tissue available for analysis.

As mutations previously thought to be unique to particular tumor types have been shown to be present across a range of cancers (Sanger COSMIC database [24]), the six-gene sample panel used here could be applied to multiple clinical and preclinical studies. The parallel detection of multiple mutations in a single sample also supports biomarker development for combination treatment regimens, where previous analyses would have taken place independently. Parallel analysis also removes the need for sample tracking over multiple assays, which arises with traditional screening methods. The process is further optimized for clinical research and clinical trials by the availability of commercial kit components, facilitating adaptation of this technique to select patients for experimental therapeutic regimens based on gene mutation biomarker combinations which are identified using the multiplex approach.

In addition to biomarker mapping in the clinical setting, MUT-MAP will enable the retrospective analysis of stored FFPE samples, allowing additional data to be obtained from previous studies and possibly identifying previously unknown biomarker associations. The AS-PCR component of the assay uses proprietary primer modifications and an enzyme screened for improved mismatch discrimination. This enables the high level of sensitivity demonstrated in our study and allows us to multiplex allele-specific assays. This sensitivity enables the accurate and reliable identification of mutation status in multiple genes, from poor-quality, low-mass, preserved clinical samples, thereby allowing the maximum amount of data to be obtained from each sample, and repeat experiments to be conducted from the same biopsy. This capability has exciting potential for the future study of low-yield exploratory biomarkers such as circulating tumor DNA [25]. This highly flexible platform can be used to detect mutations beyond the six genes included in this study; in addition, the precise quantification of

| Table 8. Sample Input Titrations: Effect on Assay Performance. |
|-----------------|-----------------|-----------------|
|             | Mutation Status | Fg Plasmid | Wild-type C<sub>T</sub> | Mutant C<sub>T</sub> |
| Plasmid #1    | Pk_E542K         | 100       | 30           | 12.28          |
|               |                  | 10        | 30           | 15.71          |
|               |                  | 1         | 30           | 18.55          |
| Plasmid #2    | Pk_E545K         | 100       | 30           | 13.23          |
|               |                  | 10        | 30           | 16.23          |
|               |                  | 1         | 30           | 19.98          |
| Plasmid #3    | Pk_H1047R        | 100       | 30           | 11.02          |
|               |                  | 10        | 30           | 15.33          |
|               |                  | 1         | 30           | 19.12          |
| Plasmid #4    | Pk_H1047L        | 100       | 30           | 13.63          |
|               |                  | 10        | 30           | 17.50          |
|               |                  | 1         | 30           | 21.37          |
| FFPE DNA      | Mutation Status  | DNA (ng)  | Wild-type C<sub>T</sub> | Mutant C<sub>T</sub> | ΔC<sub>T</sub> |
| HP-30770      | Kr_G12R          | 160       | 10.66        | 15.87          | 5.21        |
|               |                  | 40        | 12.66        | 17.88          | 5.23        |
|               |                  | 10        | 14.48        | 19.99          | 5.51        |
| HP-30630      | Pk_E542K         | 160       | 14.81        | 15.21          | 0.40        |
|               |                  | 40        | 16.64        | 16.68          | 0.04        |
|               |                  | 10        | 18.60        | 18.93          | 0.33        |
| Cell Line DNA | Mutation Status  | DNA (ng)  | Wild-type C<sub>T</sub> | Mutant C<sub>T</sub> | ΔC<sub>T</sub> |
| MGH-U3        | Ak_E17K          | 120       | 12.01        | 11.44          | −0.57       |
|               |                  | 15        | 15.23        | 15.17          | −0.06       |

Figure 2. Inter- and Intra-Chip Reproducibility Titrations. The MUT-MAP panel qPCR assays were run in duplicate and C<sub>T</sub> outputs were plotted to determine both inter- and intra-chip reproducibility. Data for a typical mutation panel run are shown, with R² correlations of 0.9939 and 0.9909 for inter- and intra-chip reproducibility, respectively. doi:10.1371/journal.pone.0051153.g002
each amplicon opens up the possibility of being able to detect copy number variations. Most significantly, however, the MUT-MAP assay can form the basis for the development of a platform to support efficient biomarker discovery and validation in support of detection and personalized healthcare.

**Supporting Information**

**Table S1 Preamplification Primer Sequences.**

**References**

1. Wistuba II, Gelovani JG, Jacoby JJ, Davis SE, Herbst RS (2011) Methodological and practical challenges for personalized cancer therapies. Nat Rev Clin Oncol 8: 135–141.
2. Savays CL (2008) The cancer biomarker problem. Nature 452: 548–552.
3. ODwyer D, Rahou LD, O'Shea A, Murray GI (2011) The proteomics of colorectal cancer: identification of a protein signature associated with prognosis. PLoS ONE 6: e27718.
4. Denkert C, Sinn BV, Issa Y, Maria MB, Maich A, et al. (2011) Prediction of response to neoadjuvant chemotherapy: New biomarker approaches and concepts. Breast Care (Basel) 6: 265–272.
5. Prat A, Ellis MJ, Perou CM (2011) Practical implications of gene-expression-based assays for breast oncologists. Nat Rev Clin Oncol 9: 48–57.
6. Jakobsen JN, Sorensen JB (2011) Intratumor heterogeneity and chemotherapy-induced changes in EGFR status in non-small cell lung cancer. Cancer Chemother Pharmacol 68: 1–15.
7. Weberpalis JF, Koti M, Squire JA (2011) Targeting genetic and epigenetic alterations in the treatment of serous ovarian cancer. Cancer Genet 204: 325–335.
8. Mittempergher L, de Ronde JJ, Nieuwland M, Kerkhoven RM, Simon I, et al. (2011) Gene expression profiles from formalin fixed paraffin embedded breast cancer tissue are largely comparable to fresh frozen matched tissue. PLoS ONE 6: e17163.
9. Kuan CT, Wikstrand CJ, Bigner DD (2001) EGF mutant receptor vIII as a molecular target in cancer therapy. Endocr Relat Cancer 8: 83–96.
10. Hatzivassiliou G, Song K, Ven I, Brandhuber BJ, Anderson DJ, et al. (2010) RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. Nature 464: 431–435.
11. Bollag G, Hirth P, Tsai J, Zhang J, Ibrahim PN, et al. (2010) Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. Nature 467: 596–599.
12. Forbes SA, Bhamra G, Bamford S, Dawson E, Kok CY, et al. (2008) The Catalogue of Somatic Mutations in Cancer (COSMIC). Curr Protoc Hum Genet Chapter 10: Unit 11.
13. Shi MI, Hirth P, Zhang J, Ibrahim PN, et al. (2010) Training the Rapidity and Efficiency of Oncogenes Detection (TREAD) system (ARMS). Nucleic Acids Res 17: 2503–2516.
14. Okayama H, Curie DT, Brandy ML, Holmes MD, Crystal RG (1989) Rapid, nonradioactive detection of mutations in the human genome by allele-specific amplification. J Lab Clin Med 114: 105–113.
15. Will SG, Tsan A, Newton N (2011) “Allele-Specific Amplification.” U.S. Patent 2010/0099110 A1. Available: http://patft.uspto.gov/. Accessed: 2012 Jul 7.
16. Reichert F, Bauer K, Myers TW, Schoenbrunner NJ, San Filippo J (2012) “DNA polymerases with increased 3’-mismatch discrimination,” U.S. Patent 2011/0312041 A1; Reichert F, Bauer K, Myers TW, “DNA polymerases with increased 3’-mismatch discrimination,” U.S. Patents US 2011–0318785 A1, US-2012–0015405 A1, US-2012–0009628 A1, US 2011–0312037 A1, US 2011–0312039 A1, US 2011–0312038 A1, US 2011–0318768 A1. Available: http://patft.uspto.gov/. Accessed: 2012 Jul 7.
17. Forbes SA, Bhamra G, Bamford S, Dawson E, Kok C, et al. (2006) The Catalogue of Somatic Mutations in Cancer (COSMIC). Curr Protoc Hum Genet Chapter 10: Unit 11.
18. Bollag G, Hirth P, Tsai J, Zhang J, Ibrahim PN, et al. (2010) Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. Nature 467: 596–599.
19. Forbes SA, Bhamra G, Bamford S, Dawson E, Kok C, et al. (2006) The Catalogue of Somatic Mutations in Cancer (COSMIC). Curr Protoc Hum Genet Chapter 10: Unit 11.
20. Vakoc B, Rushing JG, Song K, Ven I, Brandhuber BJ, Anderson DJ, et al. (2010) RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. Nature 464: 431–435.
21. Bollag G, Hirth P, Tsai J, Zhang J, Ibrahim PN, et al. (2010) Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. Nature 467: 596–599.
22. O'Brien C, Wallin JJ, Sampath D, GuhaThakurta D, Savage H, et al. (2010) Predictive biomarkers of sensitivity to the phosphatidylinositol 3’ kinase inhibitor GDC-0941 in breast cancer preclinical models. Clin Cancer Res 16: 3670–3683.

**Table S2 TaqMan and Mutation Detection Assays.**

**Author Contributions**

Conceived and designed the experiments: RP. Performed the experiments: RP RD RT. Analyzed the data: RP. Contributed reagents/materials/analysis tools: RP AT RT RD NS TWM KB ES RR. Wrote the paper: RP AT RT RD NS TWM KB ES RR.