Performance Characteristics of the AmpliSeq Cancer Hotspot Panel v2 in combination with the Ion Torrent Next Generation Sequencing Personal Genome Machine

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Abstract

Next-Generation Sequencing is a rapidly advancing technology that has research and clinical applications. For many cancers, it is important to know the precise mutation(s) present, as specific mutations could indicate or contra-indicate certain treatments as well as be indicative of prognosis. Using the Ion Torrent Personal Genome Machine and the AmpliSeq Cancer Hotspot panel v2, we sequenced two pancreatic cancer cell lines, BxPC-3 and HPAF-II, alone or in mixtures, to determine the error rate, sensitivity, and reproducibility of this system. The system resulted in coverage averaging 2000x across the various amplicons and was able to reliably and reproducibly identify mutations present at a rate of 5%. Identification of mutations present at a lower rate was possible by altering the parameters by which calls were made, but with an increase in erroneous, low-level calls. The panel was able to identify known mutations in these cell lines that are present in the COSMIC database. In addition, other, novel mutations were also identified that may prove clinically useful. The system was assessed for systematic errors such as homopolymer effects, end of amplicon effects and patterns in NO CALL sequence. Overall, the system is adequate at identifying the known, targeted mutations in the panel.

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1 **1. Introduction**

2 Next-Generation Sequencing (NGS) is a rapidly advancing, high-throughput parallel sequencing technology. It
3 has the potential to revolutionize disease research, forensics, and clinical medicine. In the research arena, NGS is being
4 used to gain understanding of disease pathogenesis and to identify new targets for therapy development in a wide
5 variety of cancers and other diseases. For example, the AmpliSeq Cancer Panels are currently being used to identify
6 mutations in Pulmonary Langerhans Cell Histiocytosis as a pathway to understanding the disease and to identify
7 potential treatments (Yousem et al. 2013) and in colorectal cancer patients to identify mutations of clinical relevance
8 (Malapelle et al. 2014). NGS technology is also being evaluated as a potential method to examine mitochondrial DNA as
9 a potential forensic tool (Parson et al. 2013).

10 Clinically, this technology is being used as a rapid method to identify potential therapies. One area of particular
11 interest is the use of NGS technology to identify mutations present in the tumors of patients who do not have the most
12 common mutations in their disease and therefore may not respond to the standard treatment. One example of this is
13 the use of NGS in myeloproliferative neoplasms (Tenedini et al. 2014), where the technology is used to identify new
14 treatment targets. Another area of likely use for NGS is in refining patient prognosis. NGS is currently being examined
15 for refining patient prognosis in myeloproliferative neoplasms, gastric cancer and Non-small cell lung cancer (NSCLC)
16 (Fassan et al. 2014; Geurts-Giele et al. 2013; Tenedini et al. 2014). NSCLC can be monoclonal and multifocal or present
17 with multiple primary tumors. The prognosis is different, depending on which type it is, and prognosis can influence
18 treatment options (Geurts-Giele et al. 2013). In hematopoietic cancers, such as Acute Myeloid Leukemia, mutational
19 screening of several genes is part of routine testing and is involved in determining treatment and prognosis. Current
20 testing methods often require examination of several genes individually, which has a high input DNA requirement and
21 high time and resource demands. NGS technology has the potential to streamline the mutation analysis process, reduce
22 the DNA requirement and provide the ability to identify low frequency genetic mutations, as in the investigation of
23 minimal residual disease (Luthra et al. 2014). In addition to treatment and prognosis, clinical research in pancreatic
24 cancer is currently investigating NGS as a method to determine the tumor cellularity versus stromal cellularity, which is a
25 necessary measure for pathological analysis of tumor samples (Song et al. 2012).

As the use of NGS technology continues to grow, there is also an increasing need to understand the strengths, weaknesses and overall performance of the technology and of the different available systems. As an investigation into the behavior of an NGS system, we used the Ion Torrent Personal Genome Machine (PGM) System with the AmpliSeq Cancer Hotspot panel v2 to examine the mutations present in two pancreatic cancer cell lines. Pancreatic cancer was chosen because the majority of these cell lines contain multiple mutations in genes present on the AmpliSeq Cancer Hotspot panel v2, in particular KRAS, TP53, CDKN2A and SMAD4 (Deer et al. 2010). KRAS is mutated in the majority of pancreatic cancer cell lines but it is wild type in the cell line BxPC-3. BxPC-3 also presents a unique case for study as it has large scale homozygous deletions in both CDKN2A and SMAD4 which will allow assessment of the performance of the AmpliSeq Cancer panel v2 and Ion Torrent NGS technology in the case of large deletions of genetic material. The other cell line chosen was HPAF-II as it contains both simple mutations as well as small deletions (Table 1). Mixtures of DNA extracted from these cell lines provide a useful model to examine the overall performance and limits of detection of the AmpliSeq Cancer Hotspot panel v2 used with the Ion Torrent NGS system.

2. Methods

2.1 Cell lines and cell culture

New aliquots of BxPC-3 and HPAF-II were purchased from American Type Culture Collection (ATCC, Manassas, VA; BxPC-3 [CRL-1687], HPAF-II [CRL-1997]) and maintained in culture as recommended by ATCC. RPMI-1640, Eagle's Minimum Essential Medium, fetal bovine serum, DMSO, Phosphate Buffered Saline, Dulbecco's Phosphate Buffered Saline and Trypsin-EDTA solution were purchased from ATCC. Corning® 96 well plates, Corning® cell culture flasks (untreated, 25 cm², vented cap) and NUNC® CryoTubes™ (1 mL, internal thread, polypropylene) were purchased from Fisher Scientific (Hampton, NH).

2.2 DNA isolation and DNA mix preparation

DNA was isolated from freshly collected cells using the DNeasy Blood & Tissue Kit from Qiagen (Valencia, CA). DNA concentration was determined using the Broad range Qubit DNA kit and Qubit 2.0 Fluorometer (Life Technologies, Grand Island, NY). After determining DNA concentration, mixed samples of BxPC-3 and HPAF-II DNA were prepared. Mixtures of DNA were prepared at 90:10, 95:5, 99:1 and 99.1:0.1 ratios of BxPC-3 to HPAF-II DNA. HPAF-II and BxPC-3 DNA was also analyzed unmixed to determine the mutation characteristics of the cell lines.

52 2.3 Library Preparation

53 AmpliSeq libraries were prepared as described in MAN0006735 Revision 5.0 (this and subsequent Life Technology

54 protocols are available at www.lifetechnologies.com) using the Ion AmpliSeq Library Kit 2.0 and the Ion AmpliSeq Cancer

55 Hotspot Panel v2 (Life Technologies). Included in this panel were primers for the amplification of regions of the

56 following fifty genes: ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, EZH2,

57 FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAS, GNAQ, HNF1A, HRAS, IDH1, JAK2, JAK3, IDH2, KDR, KIT, KRAS, MET,

58 MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11,

59 TP53 and VHL. 10ng of each DNA sample was used as the template to prepare the library. Amplified libraries were

60 quantified using the Qubit 2.0 Fluorometer and the High Sensitivity Qubit Assay Kit (Life Technologies). Amplified

61 libraries were assessed for quality (size and concentration) using the Agilent 2100 Bioanalyzer Instrument (Agilent

62 Technologies, Santa Clara, CA) following the Bioanalyzer standard protocol.

63 2.3 Emulsion PCR

64 The AmpliSeq libraries were clonally amplified on to Ion Sphere Particles (ISPs) using emulsion PCR following standard

65 Ion Torrent protocols. ISP preparation was performed using the automated Ion Torrent OneTouch2 system following

66 the manufacturer's protocol (MAN0007220 Revision 4.0). The Qubit Fluorometer was used to assess ISP quality after ISP

67 preparation but before ISP enrichment. Enrichment of ISPs was performed using the Ion Torrent OneTouch ES system

68 and kit following the manufacturer's protocols.

69 2.4 Sequencing using the Ion Torrent Personal Genome Machine

70 Enriched ISPs were loaded onto 316 chips and a single sample was loaded per chip. Ion Torrent sequencing was

71 performed using the Ion PGM Sequencing 200 Kit v2 and following the standard protocol (MAN0007273 Revision 1.0).

72 2.5 Data Analysis

73 Alignment of the sequences to human genome build 19 reference genome and base calling were performed using the

74 Torrent Suite software. Identification of variants was performed by the Ion Torrent Variant Caller plugin software.

75 Coverage maps were generated using the coverageAnalysis plugin.

76 2.6 Sequences

Sequences have been submitted to the SRA of NCBI under the study PRJNA268438 with the following accession numbers: SRP767414, SRP767507, SRP767509, SRP767511, SRP767512, SRP767513.

2.7 COSMIC database

Mutations found in the unmixed BxPC-3 and HPAF-II were compared to validated mutations listed in the Catalogue of Somatic Mutations in Cancer (COSMIC) Cell Lines Project for both cell lines. The COSMIC cell lines project was accessed June 2014.

3. Results

3.1 HPAF-II and BxPC-3 cell line analysis

The AmpliSeq Cancer Hotspot Panel v2 targets hotspots for mutation in 50 genes. The number of mutations targeted varies by the gene. The number of targeted mutations was found by examining the variant caller frequency data for each cell line. The number of variants specifically examined by the variant caller software is presented in Table 2. In addition to the targeted mutations, all of the sequences produced are compared by the variant caller software to the human genome reference sequence and ‘novel’ calls are also made. These ‘novel’ calls are not targeted variants. The number of targeted mutations found and the number of novel mutations found is noted for both the BxPC-3 and HPAF-II cell lines in Table 2. The variant caller software had one major oversight in the analysis of the initial cell line data: the software did not call or flag the large homozygous deletions present in the CDKN2A or SMAD4 genes present in the BxPC-3 DNA. Examination of the full data tables also did not reveal this issue. The missing data became evident only when the genes were specifically searched for. An additional plugin, coverageAnalysis, can be used that generates visual coverage maps. These coverage maps clearly show the large areas of missing data in BxPC-3 compared to HPAF-II (Figure 1A and B).

Figure 1. Coverage maps of HPAF-II, BxPC-3 and mixed DNA samples. Note the lack of coverage (*) in B, indicating complete deletion of the regions.

A. HPAF-II

B. BxPc-3

C. 99% BxPc-3
1% HPAF-II

D. 99.9% BxPc-3
0.1% HPAF-II

The AmpliSeq variant caller data was compared to the known mutation data from TP53, KRAS, CDKN2A and SMAD4 for both BxPC-3 and HPAF-II. In BxPC-3, as expected, no mutation was found in KRAS and the expected TP53 mutation was found. However, in addition to the expected mutation in TP53, a second ‘novel’ mutation was found that has not previously been reported in this cell line. The Ion Torrent coverage map shows the expected homozygous deletion of the SMAD4 and CDKN2A genes in BxPC-3 (Figure 1B). In the HPAF-II cell line no mutations were expected in SMAD4 and none were found. The expected KRAS mutation was found. However, the expected CDKN2A and TP53 mutations were not found. Closer examination of the variant caller data and comparison to the COSMIC allele IDs shows that the mutation in TP53 found in HPAF-II is not a variant that the caller targets as it has never been found in a somatic tumor (COSMIC). Although small deletions in CDKN2A have previously been reported in the literature in HPAF-II, no validated deletion is listed in the COSMIC cell line data base. Although these deletions in CDKN2A were not specified in COSMIC and the TP53 mutation was not specifically looked for by the variant caller, these mutations would show up as

111 'novel' mutations because they would be present within the examined regions of the gene. We did not see these 'novel'
112 mutations and there are two possible reasons for this. It is possible that the aliquots of the cell lines we examined,
113 although fresh from the supplier, did not possess the mutations. This seems likely for the CDKN2A deletions, which are
114 not validated. However, a more likely explanation for the TP53 mutation is that the mutation fell outside the regions
115 that were examined. The AmpliSeq Cancer Hotspot panel v2 is a multiplex series of PCR-amplified targets that cover a
116 limited portion of each gene (Table 3). Examining TP53, which is the most highly covered gene, the variant caller looks
117 for 1141 variants, but this corresponds to only 4.85% of the total gene.

118 *3.2 Mixed BxPC-3 and HPAF-II DNA sample analysis*

119 Initial sequencing results from the unmixed BxPC-3 and HPAF-II samples showed an average coverage of
120 approximately 2000x per base. Given this estimate of coverage, it is possible that mutations present as low as 1% would
121 be visible in the mixed samples. The ability to see mutations in the presence of normal DNA is highly important in actual
122 tumor analysis, as there is often a large normal component to the samples. Mixed DNA samples were created with the
123 ratios of BxPC-3 to HPAF-II of 90:10, 95:5, 99:1 and 99.9:0.1.

124 There was substantial overlap in the variants called in BxPC-3 and in HPAF-II. Using the standard Torrent Suite
125 variant caller, fifteen variants were called in BxPC-3, of which 5 were unique to BxPC-3. Sixteen variants were called in
126 HPAF-II, of which 7 were unique to HPAF-II (Table 4). In the 90:10 BxPC-3 to HPAF-II sample, all five unique to BxPC-3
127 variants and all seven unique to HPAF-II variants were called. In the 95:5 BxPC-3 to HPAF-II sample, all five unique to
128 BxPC-3 variants were called, but only six of the seven unique to HPAF-II variants were called. The variant that was not
129 called was only present in HPAF-II at 50.6%, resulting in a near cutoff level in the 95:5 mixture. In the 99:1 and 99.9:0.01
130 samples, all five unique to BxPC-3 variants were called while no unique to HPAF-II variants were called.

131 The depth of coverage for each base in the mixed samples remained the same as the unmixed samples of
132 approximately 2000x. Given this depth of coverage, the HPAF-II mutations should be visible in the 99:1 mixed sample,
133 however these mutations were not found by the Variant Caller using the standard sampling parameters. Some of the
134 sampling parameters were changed to determine whether that would allow detection of any of the unique to HPAF-II
135 variants in the 99:1 BxPC-3 to HPAF-II sample. The first parameter changed was min_allele_freq, which is the minimum
136 allele frequency that must be reached in order to be called as a variant. The default is 0.02 (2%) for SNPs and 0.05 (5%)

for INDELs. For both SNPs and INDELs, this was changed to 0.01, which would represent the theoretical 1% level. The analysis was performed multiple times with these new parameters, and different results were obtained with each analysis. The results ranged from all seven unique to HPAF-II variants being called to only two unique to HPAF-II variants being called. This likely was due to the parameter `downsample_to_coverage`. This parameter indicates that the software should only look at the specified number of reads (the default is 2000 and the reads are randomly selected), if the coverage is greater than that value. The `downsample_to_coverage` parameter artificially and randomly reduces the coverage to increase speed of analysis.

Reanalysis was performed with other parameters changed: `gen_min_alt_allele_freq` (default 0.035) and `gen_min_indel_alt_allele_freq` (default 0.1). These two parameters generate a candidate list, to which the `min_allele_freq` parameter is applied and filters the candidate list to generate NOCALLs. The two `gen_..._freq` parameters were lowered to 0.01, the 1% theoretical level, in conjunction with the lowered `min_allele_freq` parameter. With these parameter settings, only two of the seven unique to HPAF-II variants were called in five reanalyses.

Since 1% is the theoretical level for the unique to HPAF-II variants if they are homozygous, the parameters were also all lowered to 0.005, or 0.5%, which is below the theoretical 1% level for homozygous variants and at the theoretical level for heterozygous variants. With these parameter settings, three of the seven unique to HPAF-II variants were called in five reanalyses. The downsampling parameter was then changed in conjunction with the lowered frequency parameters. The `downsample_to_coverage` was increased to 4000 and the other parameters were set to 0.05. The addition of changing the downsampling level did not alter the results obtained. The same three of seven unique to HPAF-II variants were called in five reanalyses with the altered downsampling as were called without altering the downsampling parameter.

3.3 Reproducibility of data

In many of the planned uses for NGS technology, particularly those which anticipate high use, the default software will be employed. To assess reproducibility, we examined the 95:5 sample, as it was the most diluted sample that identified the majority of variants using the default software. Three independent replicates of the 95:5 BxPC-3 to HPAF-II mixture were performed. A large pool of the samples at 95:5 was initially mixed, followed by three separate library preparation and sequencing runs. This ratio was chosen because some of the variants seen were heterozygous,

163 which would result in a final frequency of about 2.5%, which is near the default cutoff (2%) for calling variants. All three
164 sequencing runs found the same variants.

165 One sequencing run found an additional variant not seen in the others. This new variant (chr4: T55972974G)
166 was found at only 5% and at the same location as another variant (chr4: T55972974A). chr4: T55972974A was present in
167 both BxPC-3 and HPAF-II, but it is homozygous in BxPC-3 and heterozygous (51%) in HPAF-II. In the two 95:5 replicates
168 without the additional variant at this site, chr4: T55972974A was present at nearly 98%. In the one 95:5 replicate with
169 the chr4: T55972974G variant, the T55972974A variant was present at 92%, while the T55972974G variant was present
170 at 5%. When the variant file for the sequencing run with the alternate variant was viewed in Integrative Genomics
171 Viewer (IGV) (Robinson et al. 2011; Thorvaldsdóttir et al. 2012) each G variant was preceded by the insertion of an A.
172 This is likely indicative of a software issue in aligning and reading these sequences.

173 Besides the reanalyses described above for the 99:1 BxPC-3 to HPAF-II sample, reanalyses with altered
174 parameters were performed for the three 95:5 BxPC-3 to HPAF-II samples. The downsampling parameter was not
175 changed, but the three frequency (..._freq) parameters were changed to 0.05 (unless the default setting was lower, then
176 it was left as default), which is the theoretical 5% level for a homozygous variant in HPAF-II. This change did not alter
177 the results: the same six of seven unique to HPAF-II variants were called as were called with the default settings. The
178 frequency parameters were then changed to 0.01 (1%), which is below the theoretical 5% level and should identify the
179 one missing unique to HPAF-II variant (present in the 100% HPAF-II sample at 50.6%). With these parameter settings, all
180 of the unique to HPAF-II variants were called.

181 *3.4 NO CALL analysis, Effects of Homopolymer regions and end of amplicon effects*

182 In addition to the behavior of the AmpliSeq Cancer Hotspot panel v2 and Ion Torrent on mutation identification
183 in mixed samples, the overall performance of the system is important. In each sample there were some hotspot
184 mutations that were considered NO CALL due to failure to pass an internal quality requirement of the software. We
185 analyzed the NO CALLs for increases in the percentage of NO CALLs by mutation type and by gene. Analysis was done by
186 pooling the data from all samples, however, data from the 0.1% and 1% mixed samples on the SMAD4 and CDKN2A
187 genes were excluded as these genes were deleted from the BxPc-3 cell line which provided the majority of the genetic
188 material. In the BxPc-3 cell line alone, no data is present in the variant caller data sheet. The addition of even 0.1% of

the HPAF-II leads to limited amplification and recognition by the variant caller that data is present in these two genes, however, almost all hotspots in these genes were NO CALLs, which would inflate the rate of NO CALLs in our analysis.

Examination of the NO CALLs by mutation type found the most NO CALLs in the deletion mutation type where 6.9% of all deletion hotspots targeted by the panel were considered NO CALLs. The next highest rate was with a multi-nucleotide polymorphism (2.4%), followed by single nucleotide polymorphisms (1.1%), and insertions (0.5%).

Examination of NO CALL data for the individual genes found the rates to be very low in the majority of genes (Table 5).

However, there are several genes in which the rate was quite high. Only five genes had NO CALL rates above 5%. These genes were CDH1 (14.3%), MPL (12.5%), NRAS (6.8%), PDGFRA (9.1%) and STK11 (5.4%).

Misidentification of homopolymer regions as insertions or deletions has been seen in the literature with NGS technology and specifically with 454 and Ion Torrent platforms (Bolotin et al. 2012). Additionally, the position of the observed 'mutation' in the amplicon may affect the likelihood that these calls are actually present. To determine if homopolymer regions or positional effects were present in these samples, the novel calls were visually examined in IGV to determine if these calls fell in either a homopolymer region or near the end of a read. Examination of the novel calls showed no positional effects or homopolymer effects.

4. Discussion

BxPc-3 and HPAF-II pancreatic cancer cell lines as well as mixed samples of these two cell lines were assessed using the AmpliSeq Cancer Hotspot panel v2 and the Ion Torrent PGM as a vehicle for assessing performance characteristics. The use of cell lines for analyses like these has the advantage of providing large quantities of high-quality DNA for repeated analysis and these cell lines have been previously examined using other technologies. Some of the mutations are known, which allows selection of cell lines to assess the performance characteristics in specific situations. Although both cell lines have previously been examined for mutations, the analysis of BxPc-3 and HPAF-II using the AmpliSeq Cancer Hotspot panel v2 provided new data on the mutations present within each cell line. Of these new mutations, some of them were targeted mutations previously identified as important in somatic cancer. For example, BxPc-3 has mutations in targeted genes such as HRAS, KIT, PDGFRA and SMARCB1 that had not been previously identified. In addition to these targeted mutations, likely with clinical implications, other novel mutations were identified that add to the body of knowledge of mutations in these cell lines. Further experimentation using a secondary

technology will be required to confirm both the targeted and the novel mutations. However, the examination of the cell lines revealed some issues with the use of this technology for studying cell lines and these issues could also apply to the use in human tissue. Although 50 genes are examined, only a limited region of each gene is covered; these regions are selected based on the known important somatic mutations found in human cancers. Thus, mutations expected in the cell lines may not be detected if they fall outside the amplified region and new mutation discovery is limited to those targeted regions. However, the tradeoff is rapid and concurrent detection of mutations in multiple cancer-associated genes in a system that can be multiplexed to conserve materials.

One area of interest was the sensitivity to mutation of the AmpliSeq Cancer Hotspot panel v2 and the Ion Torrent PGM system. To assess this, we examined mixed samples of HPAF-II on a background of BxPc-3. Using the standard on board variant caller, we were able to see the presence of a 5% mutation. A 5% mutation detection rate in a normal background is consistent with previously reported MiSeq cell line dilution study in AML (Luthra et al. 2014) and with Ion Torrent PGM sensitivity using a different AmpliSeq Cancer panel (Singh et al. 2013). However, this is in contrast to the results from two other groups who reported 1% sensitivity using the Illumina GAIIx analysis and 2% detection using custom primers sets (Hadd et al. 2013) and 1% AmpliSeq Colon and Lung cancer v1 panel sensitivities (Scarpa et al. 2013). We were able to detect 1% mutation by modifying the parameters for analysis; however, modification made the data variable from analysis to analysis and would require multiple analyses of each data set. Performing multiple analyses is very time consuming and unlikely to be a viable option in most commercial settings. Overall, our results show that the AmpliSeq Cancer Hotspot panel v2 in combination with the Ion Torrent PGM using the basic onboard variant caller is as sensitive as pyrosequencing, such as the Roche 454 platform, which commonly detects a 5% mutation rate; is more sensitive than Sanger sequencing, which can only detect a 20% mutation rate (Harrington et al. 2013); and is comparable to other NGS technology. Using the threshold of 5% mutation, the Ion Torrent PGM and the AmpliSeq Cancer Hotspot panel v2 showed reliability in repeated testing of the 5% mixed cell line sample.

The error rates of the Ion Torrent as well as other NGS technologies have previously been examined. Experimentation using the T-cell receptor of the human immune system demonstrated that the error rate in Ion Torrent and 454 was lower than Illumina due to the bridge amplification in Illumina (Bolotin et al. 2012). However, previous use of the Ion Torrent, as well as other sequencing technologies, has found homopolymer regions to be an issue.

241 Homopolymer sequencing errors are often identified as deletions or insertions (Seo et al. 2013). As the homopolymer
242 region increases in length, the likelihood of a sequencing error increases (Loman et al. 2012; Quail et al. 2012).
243 Homopolymers are an issue specific to 454 and Ion Torrent platforms, not present in Illumina technology (Bolotin et al.
244 2012) due to its removable fluorophore. Previous experimentation has shown the rate of homopolymer sequencing
245 error in Ion Torrent sequencing was as high as 1.5 indel errors per 100 bases sequenced (Loman et al. 2012).
246 Examination of our data using the Ion Torrent in combination with the AmpliSeq Cancer Hotspot panel v2 did not reveal
247 any homopolymer-based errors or any errors caused by mutation position in the amplicon. This may be due to the
248 design of the primers used in the AmpliSeq Cancer Hotspot panel v2 or the location of the mutations. The location of
249 the targeted mutations could fall outside homopolymer regions, thus reducing the possibility of homopolymer
250 sequencing errors. Careful design of primers will reduce the potential for positional effect in amplification for the
251 targeted mutations, although the novel mutations cannot be anticipated.

252 In addition to the potential for incorrect calls due to homopolymers, there is also the potential to miss a
253 mutation due to sequencing failure. Analysis of the data collected from the BxPc-3, HPAF-II and mixed cell line samples
254 has shown that there is a variable failure rate by gene in the AmpliSeq Cancer Hotspot panel v2. Additionally, there was
255 an increased failure rate in targeted deletion mutations in comparison to the other types of mutations. Previous
256 experimentation on the Ion Torrent PGM in the context of standard sequencing has provided specific characterization of
257 the errors in Ion Torrent PGM data (Bragg et al. 2013). This work showed the majority of errors were in insertions or
258 deletions at a rate of 2.84% (1.38% after quality clipping) caused by over-calling short homopolymers and under-calling
259 long homopolymers. Using the AmpliSeq Cancer Hotspot panel v2, no homopolymer errors were found, which is likely
260 due to the difference in application as the amplified regions showed very few homopolymers that could contribute to
261 these types of errors. This same group also found a high frequency of insertion and deletion errors, approximately every
262 1000 bases in reference sequence, which occurred more often with the 314 chips and less often with the 316 chips
263 (Bragg et al. 2013). We did find an increase in inability for the variant caller to determine the presence of deletions, as
264 evidenced by the 6.9% NO CALL rate in deletion variants. However, we did not see any increase in insertions and had
265 fairly low error rates overall. This may be due to the depth of coverage in using the AmpliSeq Cancer Hotspot panel v2 in
266 comparison to whole exome or genome sequencing. Previous data has found a difference in the necessary depth of

coverage between insertions or deletions and SNPs. With the Ion Torrent PGM, a minimum of 100-300x coverage is required to identify insertions or deletions, while a minimum of 20-30X coverage is required to identify SNPs. The actual required depth for insertions and deletions varies by context, and is higher in the presence of homopolymers (Tarabeux et al. 2014).

Next Generation Sequencing technology provides some advantages over other standard methods of sequence assessment, such as Sanger sequencing or mutant-specific PCR technologies. In particular, the multiplexed capacity allows the ability to identify mutations in genes other than the primary target, which could either affect the response to treatment or suggest an alternative treatment. For example, response to EGFR-based treatment depends on KRAS mutational status so the ability to assess both genes is necessary. As the understanding of cancer continues to expand, more such combinations are likely to be discovered so multiplexed assays provide an economy of scale with regards to both materials and time.

NGS technologies have also been found to be comparable in sensitivity to other methods. For example, Illumina MiSeq technology has a limit of detection comparable to Taqman and high-resolution melting curve analysis and superior detection to Sanger sequencing while still allowing high sample throughput (Nguyen-Dumont et al. 2013). The Ion Torrent AmpliSeq cancer panel has been compared to mutant specific PNA PCR and found to have comparable sensitivity. The AmpliSeq cancer panel had the advantage of covering more sequence than many mutant-specific PCRs and can detect mutations not found by mutation-specific PCR (Kim et al. 2013). Sanger style sequencing is often considered the gold standard in sequence identification; however there is a growing body of evidence that the NGS technologies provide very good concordance in sequence while also having the advantage of multiplexing samples. In mitochondrial DNA sequencing, very good concordance in sequence was found between Sanger-type sequencing and Ion Torrent, only 0.02% of reads showed a difference and many of these occurred at homopolymer regions (Parson et al. 2013). The concordance is not limited only to standard sequencing; the Ion Torrent AmpliSeq cancer panel has been compared to traditional sequencing in several genes and gene regions and good sequence concordance was found (Geurts-Giele et al. 2013). Illumina and Ion Torrent technologies have been directly compared with both tumor and cell line DNA and showed good sequence concordance (Hadd et al. 2013; Luthra et al. 2014). One major difference is that Ion Torrent had a higher rate of detecting SNPs than Illumina, but Ion Torrent also had a higher rate of error and more

false positive calls than Illumina (Quail et al. 2012). The incorrect calling of SNPs is likely coverage-based, with at least 30-40x coverage important for correct SNP calling (Quail et al. 2012), showing the importance of ensuring sufficient coverage for clinical and research samples.

While sequencing technology provides a wealth of information about gene mutations, this technology is not applicable in all situations. One of the most obvious areas for concern is mutations that fall within homopolymer regions of genes. While the standard software may be unable to accurately call mutations in these regions, mutations in homopolymer regions can be accurately detected but require different analysis than the standard used in many academic and clinical labs (Tarabeux et al. 2014). In addition to the concern presented with homopolymer regions, another area of concern is large rearrangements, such as those found in BRCA genes. Similar to homopolymer regions, large rearrangements can be detected using bioinformatics, however, this method requires comparison between samples and a secondary confirmation method in addition to NGS sequencing is recommended (Tarabeux et al. 2014). However, the most important concern is that sequencing is not able to detect expression levels, which is important for identifying gene fusion products such as the YWHAE-NUTM2A/B fusion sometimes found in Endometrial stromal sarcomas. These tumors can show a high level of KIT expression by immunohistochemistry, which is not detectable by Sanger Sequencing of KIT or using the AmpliSeq Cancer Hotspot panel. Increases in expression can be important for prognosis and treatment options (Lee et al. 2014).

The data presented here focused on the Ion Torrent PGM and the AmpliSeq Cancer Hotspot panel v2. However, there are multiple panels available and new panels are being developed. Future work is necessary to determine whether the performance characteristics seen with this panel are shared with other panels. In addition to premade panels, custom panels are being made for diseases like breast cancer and identifying disease susceptibility in blood²¹. Future work should also characterize the performance of custom panels to determine how universal the observed performance characteristics are to a particular NGS technology and to determine how strong the effect of primer design and individual gene region characteristics are to error rate, sensitivity and reproducibility.

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Author Contributions

KSB and MYLY performed the experiments and wrote the manuscript. ZL, MYLY, and KSB performed data analysis. SCW and RKE provided guidance and intellectual input throughout the project. KSB and SCW secured funding for this work. All authors reviewed the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

Tables

Table 1. Selected mutations present in BxPC-3 and HPAF-II.

Cell Line	KRAS	TP53	CDKN2A	SMAD4
BxPC-3	WT	220 Cys	HD	HD
HPAF-II	12 Asp	151 Ser	Δ^*	WT

WT – wild type, HD – homozygous deletion, Δ – deletion

* deletions in CDKN2A have been reported independently 3 times, however the exact bases deleted were different in each study.

Table 2. The number of mutations targeted in each gene and how many targeted and novel mutations were found in each gene in BxPC-3 and HPAF-II.

Gene	# of targeted mutations	BxPC-3		HPAF-II	
		targeted mutations	novel mutations	targeted mutations	novel mutations
ABL1	19				
AKT1	6				
ALK	8				
APC	135	0	1	0	1
ATM	24				
BRAF	76				
CDH1	7				
CDKN2A	107		*		
CSF1R	8				
CTNNB1	73				
EGFR	108	0	1	0	1
ERBB2	19				
ERBB4	13			0	1
EZH2	11				
FBXW7	25				
FGFR1	2				
FGFR2	8				
FGFR3	16	0	1	0	1
FLT3	30	0	1	0	1
GNA11	5				
GNAS	12			0	1
GNAQ	6				
HNF1A	10				
HRAS	27	1	0		
IDH1	12				
IDH2	12				
JAK2	5				
JAK3	6				
KDR	11	0	3	0	2
KIT	105	1	0		
KRAS	61			1	0
MET	18				
MLH1	1				
MPL	10				
NOTCH1	20				
NPM1	28				
NRAS	35				
PDGFRA	22	1	1	0	1

PIK3CA	97			0	1
PTEN	148				
PTPN11	28				
RB1	18				
RET	17			0	2
SMAD4	31		*		
SMARCB1	11	1	0	1	0
SMO	5				
SRC	1				
STK11	21				
TP53	1141	1	1	0	2
VHL	121				

* Region unflagged by variant calling software, however there is no data present for these regions. These regions are known homozygous deletions.

Table 3. Percentage of each gene covered by targeted amplification of the AmpliSeq panel.

Gene	total length (bp)	covered length (bp)	percent covered
ABL1	173795	408	0.23%
AKT1	26394	224	0.85%
ALK	728838	230	0.03%
APC	138735	826	0.60%
ATM	146268	1533	1.05%
BRAF	190752	243	0.13%
CDH1	98250	325	0.33%
CDKN2A	26740	255	0.95%
CSF1R	60082	181	0.30%
CTNNB1	44445	118	0.27%
EGFR	188307	892	0.47%
ERBB2	40523	365	0.90%
ERBB4	1162911	843	0.07%
EZH2	76978	85	0.11%
FBXW7	213984	492	0.23%
FGFR1	57697	238	0.41%
FGFR2	120129	440	0.37%
FGFR3	15561	518	3.33%
FLT3	97319	400	0.41%
GNA11	27047	92	0.34%
GNAQ	311029	123	0.04%
GNAS	71456	218	0.31%
HNF1A	23766	177	0.74%
HRAS	3309	206	6.23%
IDH1	18854	103	0.55%
IDH2	18497	130	0.70%
JAK2	143098	128	0.09%
JAK3	23251	297	1.28%

KDR	47337	924	1.95%
KIT	82787	854	1.03%
KRAS	45675	331	0.72%
MET	125982	640	0.51%
MLH1	72113	126	0.17%
MPL	16661	118	0.71%
NOTCH1	51343	348	0.68%
NPM1	23181	115	0.50%
NRAS	12431	249	2.00%
PDGFRA	920593	461	0.05%
PIK3CA	86187	1101	1.28%
PTEN	105338	843	0.80%
PTPN11	91182	236	0.26%
RB1	178144	992	0.56%
RET	53281	611	1.15%
SMAD4	90228	1028	1.14%
SMARCB1	47556	475	1.00%
SMO	24673	520	2.11%
SRC	60734	103	0.17%
STK11	22637	616	2.72%
TP53	19149	928	4.85%
VHL	12036	318	2.64%

Table 4. Variant calls in unmixed and mixed HPAF-II and BxPc-3 samples.

HPAF-II:BxPC-3 (%HPAF)	ERBB4	GNAS	HRAS	KDR	KIT	PDGFRA
Undiluted (100%)	65.3%	50.6%	0.0%	ND	0.0%	0.0%
10:90 (10%)	9.8%	5.0%	87.7%	96.6%	45.1%	91.5%
5:95 (5%)	4.5%	ND	94.0%	97.7%	46.0%	95.5%
1:99 (1%)	ND	ND	97.5%	99.0%	49.9%	98.1%
0.1:99.9 (0.1%)	ND	ND	97.8%	100.0%	52.5%	98.9%
call type	novel	novel	targeted	novel	targeted	targeted
percent in undiluted BxPC-3	ND	ND	98.0%	100.0%	48.2%	100.0%
HPAF-II:BxPC-3 (%HPAF)	KRAS	PIK3CA	RET (1)	RET (2)	TP53 (1)	TP53 (2)
Undiluted (100%)	75.9%	100.0%	100.0%	100.0%	0.0%	98.1%
10:90 (10%)	15.4%	15.9%	8.7%	9.1%	90.8%	8.5%
5:95 (5%)	8.3%	9.2%	5.7%	5.6%	95.1%	5.1%
1:99 (1%)	0.0%	ND	ND	ND	98.8%	ND
0.1:99.9 (0.1%)	0.0%	ND	ND	ND	100.0%	ND
call type	targeted	novel	novel	novel	targeted	novel
percent in undiluted BxPC-3	0.0%	ND	ND	ND	100.0%	ND

ND – not detected

Table 5. Percentage of reads in each gene that were flagged NO CALL.

Gene	Percentage no call
ABL1	0.0%
AKT1	0.0%
ALK	0.0%
APC	2.5%
ATM	0.0%
BRAF	0.0%
CDH1	14.3%
CDKN2A	0.0%
CSF1R	0.0%
CTNNB1	0.9%
EGFR	0.0%
ERBB2	0.0%
ERBB4	0.0%
EZH2	0.0%
FBXW7	2.0%
FGFR1	0.0%
FGFR2	0.0%
FGFR3	0.0%
FLT3	1.3%
GNA11	0.0%
GNAS	2.1%
GNAQ	0.0%
HNF1A	0.0%
HRAS	0.0%
IDH1	0.0%
IDH2	0.0%
JAK2	0.0%
JAK3	0.0%
KDR	0.0%
KIT	0.2%
KRAS	2.9%
MET	0.0%
MLH1	0.0%
MPL	12.5%
NOTCH1	5.0%
NPM1	0.0%
NRAS	6.8%
PDGFRA	9.1%
PIK3CA	0.1%
PTEN	3.4%
PTPN11	0.0%
RB1	0.0%
RET	0.0%
SMAD4	0.0%
SMARCB1	0.0%
SMO	0.0%
SRC	0.0%
STK11	5.4%
TP53	1.7%
VHL	2.7%

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