Cancer Genetics 228–229 (2018) 55–63

Cancer Genetics

ORIGINAL ARTICLE

Validation of a next-generation sequencing oncology panel optimized for low input DNA

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Abstract

One caveat of next-generation sequencing (NGS)-based clinical oncology testing is the high amount of input DNA required. We sought to develop a focused NGS panel that could capture hotspot regions in relevant genes requiring 0.5–10 ng input DNA. The resulting Penn Precision Panel (PPP) targeted 20 genes containing clinically significant variants relevant to many cancers. One hundred twenty-three samples were analyzed, including 83 solid tumor specimens derived from FFPE. Various input quantities of DNA (0.5–10 ng) were amplified with content-specific PCR primer pools, then sequenced on a MiSeq instrument (Illumina, Inc.) via paired-end, 2×186 base pair reads to an average read depth of greater than 6500x. Variants were detected using an in-house analysis pipeline. Clinical sensitivity and specificity were assessed using results from our previously validated solid tumor NGS panel; sensitivity of the PPP is 96.75% (387/400 variants) and specificity is 99.9% (8427/8428 base pairs). Variant allele frequencies (VAFs) are highly concordant across both assays ($r = 0.98$ $p < 0.0001$). The PPP is a robust, clinically validated test optimized for low-yield solid tumor specimens, capturing a high percentage of clinically relevant variants found by larger commercially available NGS panels while using only 0.5–10 ng of input DNA.

Keywords Solid tumor, Low-yield DNA specimen, Clinical NGS testing, Multi-gene panel.

Introduction

New insights into the molecular biology of cancer provide a means to improve patient management by defining tumor subsets, stratifying risk, and guiding therapy for a variety of tumors [1]. This "personalized" or "precision" approach of identifying and ultimately targeting tumor- and patient-specific molecular abnormalities has required genetic characterization at time of diagnostic evaluation. These analyses can be valuable in making a diagnosis, as in thyroid nodule fine needle aspiration [2]. More frequently, the somatic variant status of a variety of solid tumors, such as non-small cell lung cancer (NSCLC), melanoma and colorectal cancer, is predictive of therapeutic consequences [3–5]. For example, certain acquired somatic activating variants in the epidermal growth factor receptor (*EGFR*) gene predict clinical response to tyrosine kinase inhibitor (TKI) therapy in NSCLC [6].

Traditionally, tumor genetic evaluation includes cytogenetic analysis, fluorescence in situ hybridization (FISH), and single gene/variant studies. As some genetic alterations may be mutually exclusive, and the cost of performing multiple

Received April 19, 2018; received in revised form July 24, 2018; accepted August 19, 2018

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https://doi.org/10.1016/j.cancergen.2018.08.004

single-gene assays can become prohibitive, tiered, algorithmic approaches to molecular testing are often adopted to contain expense and conserve diagnostic tissue [7,8]. However, even an optimal testing algorithm may be ineffective when confronted with limited diagnostic tissue and increasing numbers of clinically actionable genomic alterations.

With the advent of next-generation sequencing (NGS), also known as massively parallel sequencing, researchers continue to elucidate the genomic landscape of a variety of tumors [9–11]. Implementation of NGS in the clinical laboratory is attractive, as it can detect a multitude of genomic alterations at once. A variety of factors, including increasing access to the technology, decreasing costs, robust methodologies to deal with fresh-frozen and formalin-fixed paraffin-embedded (FFPE) specimens, and development of rigorous validation approaches, have rapidly brought NGS-based testing into clinical practice [12].

Most NGS-based clinical testing has focused on sequencing all or most of the exons of clinically-relevant and actionable genes [13,14]. However, these assays often have high DNA input requirements, which present a challenge for testing of minimally invasive diagnostic specimens, such as those collected by fine needle aspiration (FNA). FNA is a standard diagnostic technique for a variety of tumors, providing an alternative to open biopsy procedures, especially in lung cancer [15,16], but diagnostic FNAs commonly yield DNA quantities below the required input of large NGS panels. Although many FNAs have a concurrent or follow-up surgical biopsy, for a variety of reasons, the cytological specimen may be the only diagnostic tissue available. Despite the fact that specimens collected by FNA may be adequate for morphological assessment, there is often insufficient DNA for extensive molecular analyses [17,18]. Furthermore, large panels require a long wet-bench work flow and result in the detection of a large number of DNA variants, all of which require review prior to being reported. Small amplicon-based hotspot panels enable laboratories to have a much shorter turn-around time, to satisfy requests for rapid testing.

NGS is highly suited for multi-target interrogation, so it is not surprising that several groups have developed NGS-based panels optimized for FNA specimens [18–20], most of which still require at least 50 ng of input DNA. NGS multi-gene variant profiling has been successfully performed on specimens with as little as 10 ng of DNA; however, at this input, only half of the cytological samples tested by Kanagal-Shamanna et al. (31 out of 61) had sufficient DNA for testing. To address this pressing clinical need, many laboratories have adopted the use of targeted hotspot panels on the Ion Torrent platform, using either commercially available panels [21–24] or custom designed panels [25]. For laboratories that have invested in Illumina technology, this represents a difficult burden both in the purchase and additional laboratory workflows required to use multiple sequencing technologies.

We developed and validated an NGS panel for clinical specimens with low DNA yield, unable to be evaluated with our more comprehensive NGS assay based on the TruSeq Custom Amplicon® (TSCA, Illumina, Inc.). The TSCA panel provides pre-designed, optimized oligonucleotide probes that generate 204 amplicons for sequencing variant hotspots in 47 genes known to be associated with cancer and tumor development. Although it is possible to successfully run this assay with as little as 10 ng of input DNA, approximately 5% of submitted specimens fail to meet the minimum DNA quality control requirements of this assay.

Reviewing our laboratory data from a 14-month period, these lower quality specimens ($n=44$) had an average DNA concentration of 1.72 ng/μL. The vast majority of these lower-quality samples ($n=42$, 96%) were cytology and/or endobronchial ultrasound (EBUS) specimens, most of which were from lung tumors ($n=23$). The remainder were largely glioblastoma ($n=8$) and melanoma ($n=5$). All insufficient melanoma specimens were either small biopsies (cytology, core biopsies) or older than 2 years, and all of the insufficient glioblastoma specimens were resection specimens (one noted as scant by the pathologist and, another, >10 years old). With these specimens in mind, we selected genes and exons that had well-established clinical utility, and created a streamlined panel that attempted to balance the number of targeted regions with a low DNA input that could be run on Illumina MiSeq instruments. Here, we describe the validation and performance characteristics of this assay.

Materials and methods

Assay design

The Penn Precision Panel (PPP) targets hotspot regions in 19 genes (*AKT1, ALK, BRAF, CSF1R, EGFR, ERBB2, HRAS, IDH1, IDH2, KIT, KRAS, MAP2K1, MET, NOTCH1, NRAS, PDGFRA, PIK3CA, PTEN*, and *RET)* and the entire coding region of *TP53* (Table 1), to detect clinically relevant variants involved in prognostication and/or therapy selection for solid tumors. For each gene, representative hotspots, as well as the exons and amino acids captured by the panel, are shown in Table 1. Amplicons range in length from 67 to 134 base pairs, mean amplicon length = 112 base pairs. The primers for each amplicon are included in Table S6.

DNA extraction

FFPE tissues were macro-dissected from between 5 and 15 slides with 5 μm thick sections or 1–3 10 μm thick rolls. Scrapings were dewaxed with Deparaffinization Solution (Qiagen, Hilden Germany). The tissue was then lysed using ATL Buffer (Qiagen) and Proteinase K (Qiagen) overnight in a Thermomixer (Eppendorf, Mt Laurel, NJ) at 56 °C with periodic shaking every ten minutes. If tissue was still present in the tube(s) after overnight incubation, more Proteinase K was added. The lower solution phase containing the lysed cellular material was then removed and put into individual fresh tubes. To remove any crosslinking, specimens were then put on a heat block at 90 °C for an hour. After allowing tubes to cool to room temperature, RNaseA (Qiagen) was added for five minutes. Proteins were then separated using Protein Precipitation solution (Qiagen) and centrifugation; samples were kept chilled over this process. The resulting supernatant was then added to isopropanol with Glycogen (Qiagen) to precipitate genomic DNA. This DNA was washed once with 70% ethanol then eluted in variable amounts of Hydration Buffer (Qiagen).