Advances in Molecular Diagnostics

Review Article Open Access

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Received: September 29, 2015; Accepted: October 24, 2015; Published: October 27, 2015

Citation: Hesse A, Chen C, Reddi HV (2015) Clinical Next-Generation Sequencing for Somatic Mutation Detection – Advancements and Commercialization Strategies. Adv Mol Diag 1:101. ' 2 ,

Pathologists can readily score sections from biopsies to identify samples with greater than 10% tumor burden [9], but the emerging field of liquid biopsies will enable far more sensitive detection methods. To lower the limit of mutation detection, researchers have developed ways to simplify extraction of liquid biopsies, improve sequencing technology, enrich for mutant populations, and enhance bioinformatics software (Figure 1). This review will focus on the latest developments within each of these methods and discuss the utilization of combinations of technologies and strategies for commercialization.

Sequencing Methods

Whole exome sequencing will typically identify more than 20,000 variants [10], but most of these are clinically insignificant polymorphisms, non-pathogenic missense mutations or false positive calls resulting from sequencing errors. The simplest way to sift through the noise of NGS data is to perform deeper sequencing. This method of adding coverage to increase base call reliability was verified by Izawa et al. in a 2012 study which demonstrated that a variant with a 1% allele fraction can be detected with statistical confidence at 700x coverage comprised of 350 reads from each strand [11]. The drawback of deep sequencing is the increase in cost. A most practical way to increase coverage is to focus sequencing on a small panel of genes relevant to the disease state rather than whole genome or exome. Accordingly, for Cancer, many companies are beginning to introduce panels tailored to a "broad spectrum" common mutation cancer panel or a type-specific cancer. Another simple way to reduce the noise from NGS is to perform "paired tumor-normal" sequencing. This technique involves orthogonally sequencing (independent, simultaneous runs of paired specimens) somatic and normal tissue sample from whole blood. Common experimental designs produce indepe 7depe
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a larger panel that covers 409 genes and, when coupled with the Ion Proton sequencer, generates more than 10GB of data. The clinical utility of the Ion Proton and AmpliSeq Comprehensive Cancer Panel was demonstrated in a study by Singh et al that utilized these tools to discover somatic variation in multiple cancer types from formalin- xed para n-embedded biopsy samples. Finally, there is a more specialized AmpliSeq Colon and Lung Cancer Research Panel v2. This panel covers hotspots from 24 genes and was validated on 155 unique FFPE samples from the OncoNetwork Consortium.

Ion Torrent recently upgraded their sequencing chemistry for the PGM with the launch of the Hi-Q™ sequencing kit. In developing the Hi-Q kits, mutated polymerases were screened to identify a novel enzyme that reduces the false positives caused by insertion/deletion polymerase errors by 90%. Furthermore, the new chemistry supports 400 base pair read lengths. Ion Torrent technology o ers lower cost equipment and faster turnaround times than Illumina, but more expensive sequencing runs. Comparison of the Illumina and ermo Fisher commercial cancer sequencing kits and technologies is listed in Tables 1 and 2.

Mutation enrichment

One method to reliably sequence rare mutations below the existing limits of detection is to speci cally enrich variants from the wild-type sequence to easily detectable levels before sequencing. ere have been many methods developed for this purpose and can be divided into those that detect speci c known mutations and those that can enrich unknown mutations.

Enriching for known mutations can easily be done by designing PCR primers speci c for the mutation. ere have been a number of methods developed with this basic premise including ampli cation refractory mutation system (ARMS), allele-speci c ampli cation (ASPCR), allele-speci c ampli cation (ASA), PCR ampli cation of speci c alleles (PASA), PCR ampli cation of multiple speci c alleles (PAMSA), competitive oligonucleotide priming (COP), mutant enrichment PCR [enriched or mutant-enriched PCR (EPCR or ME-PCR)], mismatch ampli cation mutation assay (MAMA), mutant allele–speci c ampli cation (MASA), antiprimer quenching-based real-time PCR (aQRT-PCR), restriction endonuclease–mediated selective PCR (REMS-PCR), Scorpion and Pointman. e di erence among these methods is beyond the scope of this review, but they have been compared in detail by Milbury et al. [16].

Enriching unknown mutations introduces a level of complexity. Enzymatic digests using mismatch speci c endonucleases leaves DNA products unavailable for sequencing. To preserve the DNA, more complex methods like high performance liquid chromatography (HPLC) have been utilized. More recently, CO-Ampli cation at Lower Denaturation temperature or COLD-PCR was developed to circumvent the need for HPLC. COLD-PCR is an ampli cation performed at a reduced denaturation temperature, such that heteroduplex DNA containing a mixture of wild-type and mutant DNA are preferentially

Varscan 2

VarScan is a variant detection so ware developed by the Genome Institute at Washington University with validated, high quality results for somatic mutation calling. e major advantage of VarScan 2 is that it directly performs simultaneous paired tumor-normal analysis position by position to maximize detection of low abundant alleles that were under-sampled in normal tissue. Genotype calls are then made independently by germline consensus method and compared using a parametric decision tree algorithm (varscan.sourceforge.net/somatic-calling.html) Koboldt et al validated VarScan 2 in 2012 using 151 ovarian adenocarcinoma samples that underwent exome scale sequencing [12]. e authors noted that VarScan 2 is an e ective tool for the detection of somatic mutations and identi cation of copy number variations (CNV) and loss of heterozygosity. Additionally, VarScan 2 has a notably low false-negative rate of 0.84%, making it a highly dependable analysis tool. It is important to note that variants missed by VarScan 2 in the Koboldt study were also missed by similar so ware [19], suggesting that this is a limitation of the sequencing rather than the so ware itself.

Mutect

e Gen ome Analysis Tool Kit (GATK), developed by the Broad Institute, is a popular so ware for analysis of human germline mutations. With the increased demand for somatic analysis tools, e Broad Institute developed MuTect, which exhibits high sensitivity and reliable detection of low frequency variants [11,19]. In addition, MuTect can be used with an unmatched normal sample or in the absence of a normal sample; however, extensive post so ware analysis would then be required for the attainment of actionable results. Wang et al examined a number of tumor-normal pairs in order to determine the utility of six such variant-calling tools, including MuTect and VarScan 2. ey found that MuTect outperforms other programs in making accurate calls on lower quality reads (those with low allelic fraction or low coverage), while Varscan 2 showed superiority for high quality calls and for SNVs with alternate alleles. erefore, they concluded that running data through both programs with these complementary strengths should maximize the number of correctly identi ed variants [19].

Torrent suite

Ion Torrent o ers an analysis suite optimized for their sequencing technology. is pipeline performs raw data analysis, mapping, alignment and variant calling. Additional plug–ins for added functionality such as coverage analysis and reporting tools are available as well. A distinguishing feature of the Torrent Suite™ is the availability of technical support from ermo Fisher—a luxury that is not typically found with open source tools. Singh et al demonstrated the capabilities of this pipeline using Ion Torrent's AmpliSeq Comprehensive Cancer Panel. Single nucleotide variant, INDEL and copy number variation were 93% concordant with previously validated mutations from Sanger and FISH assay analysis on the 28 tumor samples with allele frequencie as low as 18%. e 7% discordant variants were the result of allelic

Company	Test Name	Panel Size (genes)	LOD	TAT	Key Features
The Jackson Laboratory for Genomic Medicine	JAX-CTP	190		Not reported	•CNV component •300x avg depth of coverage
Mayo Medical Laboratories	CANCP	50		12-20 days	•Therapy driven •Looks for clinically actionable driver mutations
ARUP	Solid Tumor Mutation Panel	48		< 2 weeks	
EdgeBio		46	Not reported	Not reported	Extremely deep coverage of 2000x achieved
University of Pittsburgh Medical Center	ThyroSeq	14		1 week	•Custom capture for thyroid cancer •Includes W K \ U R L G F D Q F H U V S H F L ¿ F J H Q H
Foundation Medicine	FoundationOne®	315		11-14 days	•Large panel also captures actionable intronic regions •Designed for higher performance with lower tumor purity
Washington University in St Louis	Solid Tumor Gene Set	65	Not reported	3 Weeks	Focus on highly actionable genes
Memorial Sloan Kettering	MSK-IMPACT™	410		3 weeks	•Developed to survey common and rare cancer J H Q H V ‡ ([W U H P H O \ K L J K W K U R X J K S can test thousands of patients per year •Currently only offered to MSK patients
Knight Diagnostic Laboratories	GeneTrails® Solid				

dropout producing no coverage for the region encompassing these mutations—indicating issues with the sequencing chemistry, not the so ware. Furthermore, 4 SNVs were picked up by the Torrent Variant Caller that were not detected with the other assays. When the data was run using paired tumor-normal analysis, all but one variant was detected. is SNV was not detected because of ampli cation failure.

MiSeq Reporter (v1.3+)

Illumina developed a somatic variant caller to complement their TruSeq® Amplicon Cancer Panel and conveniently installed it right into the MiSeq Repoter so ware version 1.3 release (also available on BaseSpace™). While the so ware is not designed for sequencing tumor-normal pairs, it does achieve detection of variants with a frequency below 5%. Similarly, Illumina launched the DS somatic variant caller into MiSeq Reporter so ware version 2.2 with the rollout of their TruSight Tumor panel. is variant caller also achieves a LOD below 5% with data obtained from the MiSeq desktop sequencer and is designed for a8(o ac)6(h0.5(oin)12.1(r p)d1(e M)o-6(a)9(in)4(e)-5(d f)2)19(t)- laie rata91T.7er sd for a8(o ac)6(h0.5(oin)1r)13(e)0.512.1(f)

results in 10-14 days. eir lineup includes the 37 gene Solid Tumor Genotyping Panel, the 23 gene Non-small cell Lung Cancer (NSCLC) panel with FISH translocation analysis available and the 23 gene Gastrointestinal Stromal Tumor (GIST) panel.

(http://www.knightdxlabs.com/featured/targeted-diagnostics-with-genetrails)

NeoGenomics specializes in cancer diagnostics and has the most comprehensive menu to date for clinical tumor testing with their NeoTYPE™ Cancer Pro le line of tests. e two primary categories of testing o ered are the Broad Reach Tumor Pro les and the Next-Gen Cancer-Speci c Pro les. e former encompassing 4 options ranging from 43 genes up to whole cancer exome analysis of over 4800 genes. e Next-Gen Cancer-Speci c Pro les comprise a menu of 24 smaller panels that focus on detecting driver mutations of the speci ed tumor with TATs ranging from 1-2 weeks (www.neogenomics.com/neotype-cancer-pro les/).

GenPath launched OnkoMatch™, a 14 gene (68 hotspot SNVs) mutation genotyping test in 2012 based on exclusively licensed technology from Massachusetts General Hospital and has since expanded their oncology menu using next-generation sequencing with the OnkoSight™ line of tests. OnkoSight is an NGS assay that achieves a 5% LOD and reports results in less than 2 weeks. e Solid Tumor Panel captures 31 genes and GenPath o ers 3 additional targeted panels for melanoma, lung and colorectal cancers. Similarly, the 37 gene Myeloid Malignancy Panel accompanies 3 targeted panels for Acute Myeloid Leukemia, Myelodysplastic Syndrome and Myeloproliferative Neoplasms.

(www.genpathdiagnostics.com/oncology/onkosight-ngs/)

Stanford Health Care developed a custom cancer panel, the Solid Tumor Actionable Mutation Panel (STAMP) that captures 198 genes selected for their value as diagnostic, prognostic and therapeutic markers.