

Clinical Next-Generation Sequencing for Somatic Mutation Detection-Advancements and Commercialization Strategies

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Abstract

Advancements in next-generation sequencing, variant-calling software, and mutation enrichment are facilitating the detection of rare genetic variants. In doing so, these methods have enabled identification of somatic disease, and more specifically cancer, representing as little as 0.01% of bulk genetic material. This improved sensitivity reduces false negative results, and in cancer, allows earlier detection. Such early detection will greatly improve monitoring and treatment of these diseases, especially in the context of liquid biopsies. This review aims to encapsulate the growing spectrum of technology and software currently being utilized to improve the sensitivity of somatic variant detection. Furthermore, industry adoption of these techniques will be summarized.

Keywords: Somatic mutation; Sequencing methods; Liquid biopsy

Background

Unlike germline mutations, which are present in all cells of the body, somatic mutations are mosaic [1] and therefore can be present in very low number relative to healthy cells [2,3]. This makes detection of these rare variants problematic by increasing the diagnostic cost and complexity, as well as presenting new technological challenges. Much of the analytical difficulty is caused by the innate error rate of DNA sequencing methods. These errors behave as sequencing noise, making it difficult to discriminate between genuine rare mutations and mistakes made during in vitro DNA amplification. Next-generation sequencing (NGS) methods, like those run on Illumina and Ion Torrent platforms, are a significant improvement over 1st generation technology, but still cannot easily detect mutations with allelic fractions below 10% [4]. Similarly, false negative results of variants with low allelic fractions pose a problem for clinical laboratories by limiting diagnostic efficacy. In this regard, current sequencing methods show an improvement over the previous gold standard, Sanger sequencing, which has a limit of detection (LOD) of 20% [5] for non-confirmatory testing. The other major problem with current sequencing methods, perhaps the most concerning to the patient, is that solid tissue biopsies are needed to genotype cancer-specific tissue. However, increasing the sensitivity of these technologies and implementing newer, non-invasive enrichment techniques will open doors in clinical molecular diagnostics to safe and early disease screening and monitoring.

An emerging solution to combat both the sensitivity limitations of NGS and the invasiveness of acquiring solid tumor samples is enriching liquid biopsies. Liquid biopsies are blood samples from which either circulating tumor cells (CTC) or circulating cell-free DNA (cfDNA) from tumors can be isolated. Respectively, these are either cancerous cells themselves or DNA from cancerous cells that have been shed into circulation [6]. Currently, the most viable sample type for use with liquid biopsies is blood plasma. It is important to have the plasma isolated as early as possible after the blood draw to mitigate further dilution of

mutant DNA by wild-type DNA from continued lysis and breakdown of healthy blood cells [7]. The ability to readily obtain minimally invasive samples without needing to know tumor location makes liquid biopsies attractive for early disease detection. After detection, these methods can be used to track disease regression or recurrence following treatment [8]. Existing sequencing methods have limits of detection above 10% tumor cells, which in the context of liquid biopsies corresponds to severe disease [6]. Reducing the limit of disease detection would allow clinicians to identify and treat earlier disease states.

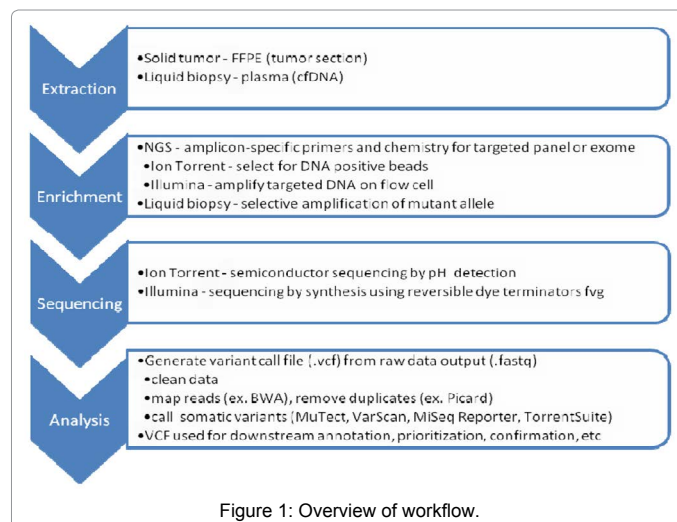


Figure 1: Overview of workflow.

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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 independent sequence files that are imported into analysis software that compare the germline and somatic data to eliminate non-specific variants [12]. Further refinement is accomplished with customized bioinformatics pipelines and confirmation of suspect mutations on an alternate technology. Illumina and Ion Torrent are the current market leaders in NGS platforms, having been largely adopted by industry due to lower error rates compared to Pacific Biosciences and Oxford Nanopore, making them more suitable for somatic mutation sequencing.

Illumina

Currently, Illumina is the most widely used NGS technology with 74% market share (www.marketsandmarkets.com/Market-Reports/next-generation-sequencing-ngs-technologies-market-546.html). Compared to other available sequencing technology, Illumina offers the largest data output, the lowest cost per-base and relatively fast turn-around time. Illumina products amplify fragments by clonal bridge amplification and sequence by synthesis using reversible dye terminators. Three “off the shelf” somatic cancer panels are available: the TruSight Tumor Panel™, the TruSight Myeloid Panel™, and the TruSeq Amplicon Cancer Panel™. The TruSight Tumor Panel consists of 26 genes spanning 21kb of sequence and achieves a minimum coverage of 1,000x per amplicon at 7,000X mean coverage (illumina.com/datasheet.pdf). The TruSeq Amplicon Cancer Panel is composed of 48 genes spanning more than 35kb of sequence with an average coverage of 1,000x per run [13]. The TruSight Myeloid Panel™ covers approximately 141kb from 15 full genes (exons only) and hotspots from 39 additional genes. In this panel, sequencing depth is 500X for 95% of amplicons with an LOD as low as 3% (illumina.com/trusight_tumor.pdf).

Illumina recently released the NextSeq 500 v2. This kit is compatible with the TruSight Myeloid and TruSeq Amplicon Cancer Panels run on NextSeq sequencers. It improves upon the previous sequencing reagents and clustering chemistry with error rates in line with those seen on MiSeq or HiSeq.

Thermo fisher

Ion Torrent products amplify DNA fragments by emulsion PCR, and sequencing is performed directly on a silicon chip that detects changes in pH from the release of a proton during DNA polymerization. The Personal Genome Machine (PGM) and the Ion Proton exhibit the quickest run time, the former in little as 3 hours [14] and yield roughly 1-2 GB and 10-15 GB of data per run, respectively. Ion Torrent has recently released several cancer panels including the 50-gene Ampliseq Hotspot Cancer Panel v2 which is an update of the previous Ampliseq panel adding an additional 4 genes and about 2000 COSMIC mutations (2,800 total). This assay has been validated using various carcinomas, gastrointestinal stromal tumors, melanoma, and brain tumors [13,15]. The panel is composed of a relatively small panel (less than 13.5 kb of sequence), allowing for scalable runs generating as much as 5,000x coverage using Ion Torrent’s 316 chip (www.edgebio.com/ampliseq-cancer-panel). Using a smaller “hotspot” panel on the Ion Torrent further increases the speed of sequencing and allows for faster reporting. The AmpliSeq™ Comprehensive Cancer Panel is

	AmpliSeq Comprehensive Cancer Panel	AmpliSeq Hotspot Cancer Panel V2	TruSeq Amplicon Cancer Panel	TruSight Tumor Panel
Genes	409	50**	48**	26
Amplicons	16,000	207	212	174
COSMIC mutations	15,749	2,800 (3,012 v.61)	(3,156 v.61)	Not reported
AVG Depth	1,000X (PI Chip)	1,400X (8 sample 316 Chip)	> 1,000X (20 sample MiSeq)	7,000X
Sample Type	WB, FFPE, FNA	WB, FFPE, FNA	FFPE	FFPE
Runtime	< 16 hours:3.5 hours library prep One Touch – 8 hours Run time 2-4 hours with PI chip	<15 hours:3.5 hours library prep 5 hours One Touch 3-5 hours run time on 316 chip	< 40 hours:24 hours run time 7.5 hours library prep	<30 hours:<7 hours library prep 22 hour run time
Primary platform	Ion Proton/PGM	Ion PGM	MiSeq	MiSeq
Multiplexing	Up to 4 samples	Up to 16 Samples	Up to 96 Samples*	Up to 4 Samples

*Up to 96 samples pooled using v3 kits (mean coverage remains 1000X)

**AmpliSeq CP contains the following additional genes EZH2 and IDH2 compared to TruSeq CP

Table 1: Commercial sequencing Kit comparison.

	AmpliSeq Colon and Lung Cancer Panel	TruSight Myeloid Panel
Company	Thermo Fisher	Illumina
Genes	22 (+ hotspots from 2 additional genes)	15 (+ hotspots from 39 additional genes)
Amplicons	92	568
Depth of Coverage	> 95% at 500x	95% > 500x
Sample Type	FFPE	WB, FNA, bone marrow
Runtime	24-48 hours (final annotated report)	<72 hours (final annotated report)
Primary platform	Ion Proton/PGM	MiSeq/NextSeq 500
Multiplexing	Up to 16 samples (318™ chip)	Up to 8 samples (MiSeq)

Table 2: Commercial targeted tumor-specific Kits.

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-fixed paraffin-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 offers lower cost equipment and faster turnaround times than Illumina, but more expensive sequencing runs. Comparison of the Illumina and Thermo 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 specifically enrich variants from the wild-type sequence to easily detectable levels before sequencing. There have been many methods developed for this purpose and can be divided into those that detect specific known mutations and those that can enrich unknown mutations.

Enriching for known mutations can easily be done by designing PCR primers specific for the mutation. There have been a number of methods developed with this basic premise including amplification refractory mutation system (ARMS), allele-specific amplification (ASPCR), allele-specific amplification (ASA), PCR amplification of specific alleles (PASA), PCR amplification of multiple specific alleles (PAMSA), competitive oligonucleotide priming (COP), mutant enrichment PCR [enriched or mutant-enriched PCR (EPCR or ME-PCR)], mismatch amplification mutation assay (MAMA), mutant allele-specific amplification (MASA), antiprimer quenching-based real-time PCR (aQRT-PCR), restriction endonuclease-mediated selective PCR (REMS-PCR), Scorpion and Pointman. The difference 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 specific 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-Amplification at Lower Denaturation temperature or COLD-PCR was developed to circumvent the need for HPLC. COLD-PCR is an amplification performed at a reduced denaturation temperature, such that heteroduplex DNA containing a mixture of wild-type and mutant DNA are preferentially amplified over wild-type homoduplexes. The development of Improved and Complete Enrichment CO-amplification at Lower Denaturation temperature or ICE-COLD-PCR (IC-PCR) goes one step further by also including synthetic reference DNA (RS) molecules that compete to bind with wild-type (WT) DNA strands. The synthetic reference sequence is also chemically modified to prevent primer binding and is phosphorylated on the 3' end to prevent polymerization. Spiking the amplification solution with WT RS establishes dynamically favorable binding of polymerase with mutant DNA strands, thereby preferentially

amplifying the mutant strand in high number [3]. Interestingly, IC-PCR exhibits an inverse relationship between amount of enrichment and initial mutation abundance. Milbury et al, substantiated this inverse trend using IC-PCR to enrich mutations for subsequent pyrosequencing. The researchers observed a 5.5-fold increase in sensitivity with 10% pre-enrichment abundance of the mutant allele, a 35-fold increase for a 1% mutant allele and a 75-fold increase when starting with 0.1% mutant allele.

A potential drawback of mutational enrichment using PCR is that it is difficult to extrapolate back to determine the initial ratio of wild-type to mutant DNA. Quantitation is extremely valuable in liquid biopsies because it can be used for disease monitoring before and after treatment. Approximate quantitation can be achieved by comparing results to those obtained using wild-type DNA spiked with known mutant DNA as standards. With the recent development of digital PCR systems that compartmentalize individual template DNAs during PCR, it should be possible to obtain absolute quantitation of these rare mutations in the future.

Bioinformatics and Analysis Software

Calling mosaic variants can be challenging due to low allelic fraction and variability in depth of coverage. Additionally, sequencer error rates may exceed the natural mutation rate with low frequency variants, which results in an increased number of false negative calls [12]. There are an increasing number of software platforms available to aid in overcoming these issues and facilitate the process of variant calling. For applications without the need for de novo assembly, such as re-sequencing testing performed in clinical labs, software can be optimized for low divergence and thus increase the detection of low abundant mutations. In combination with species-specific mutation rate and known error rates of the sequencing platform, statistical assumptions can be made that decrease the demand for computational resources and increase accuracy [9]. The most popular software programs, such as VarScan 2 and MuTect, utilize a method of paired tumor-normal samples that compare normal tissue with somatic tissue for the purpose of eliminating polymorphisms [17]. This review will cover the more widely used software tools. A comprehensive evaluation of over 200 genome software tools has been reviewed by researchers at the Innsbruck Medical University [18]. Somatic variant calling software covered in this review are summarized in Table 3.

Developer	Tool	Primary output files	Support	key features
Genome Institute-Washington University	VarScan 2	VCF	open source	Highest SNV detection rate
The Broad Institute	MuTect	VCF, Call-stats, Wiggle	open source	•Highest performance with lower quality reads • Does not require tumor-normal pairing
Thermo Fisher	TorrentSuite	VCF	tech support	• Comes installed on Torrent Server
Illumina	MiSeq Reporter (v1.3+)	VCF, gVCF	tech support	Software installed on instrument for realtime analysis

Table 3: Somatic software tools.

Varscan 2

VarScan is a variant detection software developed by the Genome Institute at Washington University with validated, high quality results for somatic mutation calling. The 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]. The authors noted that VarScan 2 is an effective tool for the detection of somatic mutations and identification 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 software [19], suggesting that this is a limitation of the sequencing rather than the software itself.

Mutect

The Genome Analysis Tool Kit (GATK), developed by the Broad Institute, is a popular software for analysis of human germline mutations. With the increased demand for somatic analysis tools, The 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 software 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. They 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. Therefore, they concluded that running data through both programs with these complementary strengths should maximize the number of correctly identified variants [19].

Torrent suite

Ion Torrent offers an analysis suite optimized for their sequencing technology. This 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 Thermo 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 frequencies as low as 18%. The 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	10%	Not reported	•CNV component •300x avg depth of coverage
Mayo Medical Laboratories	CANCP	50	5-10%	12-20 days	•Therapy driven •Looks for clinically actionable driver mutations
ARUP	Solid Tumor Mutation Panel	48	5%	< 2 weeks	
EdgeBio		46	Not reported	Not reported	Extremely deep coverage of 2000x achieved
University of Pittsburgh Medical Center	ThyroSeq	14	3%	1 week	•Custom capture for thyroid cancer •Includes thyroid cancer specific gene fusion analysis
Foundation Medicine	FoundationOne®	315	5%	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	2-5%	3 weeks	•Developed to survey common and rare cancer genes •Extremely high throughput workflow that can test thousands of patients per year •Currently only offered to MSK patients
Knight Diagnostic Laboratories	GeneTrails® Solid Tumor Genotyping Panel	37	5-15%	10-14 days	Panel covers rare mutations involved in breast carcinoma and gliomas
	GeneTrails® NSCLC	23	<10%	10-14 days (+ 3 to 7 for FISH)	FISH translocation analysis available
	GeneTrails® GIST	23	<10%	10-14 days	
NeoGenomics	NeoTYPE Broad Reach Cancer Profile	43-4813	Not reported	3 weeks	•Includes FISH analysis of 9 chromosomal loci (for discovery profile) •4 test options
	NeoTYPE™ Next-Gen Cancer-Specific Profile	varies	Not reported	1-2 weeks	•Highly focused cancer-specific driver mutation analysis •24 test options
GenPath	OnkoMatch™	14	Not reported	1 week	Genotyping test that targets 68 known mutations
	OnkoSight™ Solid Tumor Panel	31	5%	< 2 weeks	Deep sequencing with avg coverage depth of 3,000x and minimum depth of 250x
	OnkoSight™ Myeloid Malignancy Panel	37	5%	< 2 weeks	Deep sequencing with avg coverage depth of 3,000x and minimum depth of 250x
Stanford Health Care	STAMP	198	5%	Not reported	Optimized for Ultra-deep sequencing
University of Washington	UW-OncoPlex™	234	6-12%	6 weeks	•Comprehensive panel designed for treatment therapy, prognosis and diagnosis •Detect INDELs, gene amplifications, a number of fusions

Table 4: Solid tumor panels.

Company	Test Name	Panel Size (genes)	LOD	TAT	Key features
Guardant Health	Guardant360™	68	0.10%	< 2 weeks	•Quick turnaround time •CNV, rearrangements and INDEL detection
Transgenomic	MX-ICP	1	0.01%	7-10 days	High sensitivity with detection limit of 0.01% allelic fraction
Biodesix	GeneStrat	3	Not Reported	3 days	•Currently for advanced NSCLC lung cancer patients •Turnaround time of 3 days
Personal Genome Diagnostics	PlasmaSelect-R	63	0.1%	4 weeks	•Company offers a bioinformatics pipeline for use with their kits •CNV, rearrangements and INDEL detection
Pathway Genomics	•CancerIntercept™ Monitor •CancerIntercept™ Detect	9	0.01%	2-3 weeks	•96 known somatic mutations analyzed •Subscription plans for serial testing

Table 5: Sample enrichment panels.

dropout producing no coverage for the region encompassing these mutations—indicating issues with the sequencing chemistry, not the software. 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. This SNV was not detected because of amplification 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 Reporter software version 1.3 release (also available on BaseSpace™). While the software 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 software version 2.2 with the rollout of their TruSight Tumor panel. This variant caller also achieves a LOD below 5% with data obtained from the MiSeq desktop sequencer and is designed for a parallelized, multi-sample workflow (Illumina_somatic_variant_caller.pdf, illumina_amplicon_ds_somatic_variant_caller.pdf)

Development and Commercialization Strategies

Despite the growing availability of somatic genetic testing protocols and commercially available assays, many clinical laboratories continue to offer only hereditary cancer panels. Wide-scale industry adoption of somatic testing will not only require extensive validation of high-throughput repeatability in wet lab, bioinformatics, and reporting procedures, but cost effective workflows that can meet the demand for expected turnaround times. Table 4 lists the labs that have entered the somatic testing space by either utilizing commercially available kits or implementing proprietary assays. Commercial NGS-based tests are listed in Table 4 and liquid biopsy sample enrichment tests are collated in Table 5.

The Jackson Laboratory of Genomic Medicine has recently developed a somatic cancer panel, the Cancer Treatment Panel (JAX-CTP™). This test sequences 190 clinically actionable genes with an average coverage depth of 300X and is designed to detect mutation fractions as low as 10%. JAX validated their protocols and pipeline using HapMap and FFPE samples to evaluate: (1) repeatability, (2) reproducibility, (3) specificity, (4) sensitivity and (5) accuracy. Precision, measured as a composite of repeatability and reproducibility, met the 98% concordance requirement for validation. The JAX-CTP also includes copy number variation (CNV) analysis utilizing NanoString nCounter®, which distinguishes this panel from many other solid tumor NGS tests. However, there are two significant limitations to this component that must be considered when interpreting the data: (1)

the design only allows for reliable detection with copy numbers six or greater—requiring at least 50% tumor purity—and (2) the validation did not quantify detection limits for deletions due to sample availability [20].

Mayo Medical Laboratories designed the Solid Tumor Targeted Cancer Gene Panel by Next-Generation Sequencing (CANCP) that consists of 50 genes sequenced by NGS at a 5-10% allelic fraction detection limit. CANCP is utilized with the goal of discovering mutations known to confer resistance or desirable responses to treatment therapies

(www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/35594).

ARUP offers the Solid Tumor Mutation Panel, a 48-gene NGS-based hotspot panel for solid tumor samples. ARUP has validated a process to sequence somatic tissue with a tumor percentage as low as 10% (not to be confused with mosaic detection limit) and can report results in less than 2 weeks (www.aruplab.com/files/resources/oncology/SolidTumor.pdf).

Washington University in St Louis through their Genomics and Pathology Services laboratory (GPS) provides somatic cancer testing with the Solid Tumor Gene Test: a 65 gene panel for profiling tumors for diagnosis and treatment guidance (gps.wustl.edu/cancer#solid%20tumor).

Foundation Medicine offers FoundationOne™, a solid tumor panel consisting of 315 genes and intronic regions from an additional 28 genes with a median depth of 500x (foundationOne_technicalspecs.pdf). The panel is designed to detect SNVs, INDELs, CNVs as well as selected gene rearrangements with high sensitivity and detection limit as low as 5% [21]. The turnaround time for test results is between 11 and 14 days from receipt of sample. Foundation Medicine also has ongoing clinical trials to evaluate the utility and performance of a liquid biopsy test, which is expect to launch in 2016 (foundationmedicine.com/releasedetail).

Memorial Sloan Kettering Cancer Center developed the Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT™) test to target both common and rare cancer variants using Illumina's HiSeq 2500 as the sequencing workhorse. The panel covers 410 genes with selected intronic regions and analyses SNVs, INDELs, CNVs as well as some structural rearrangements. The test methods achieve low detection limits of 2% and 5% for hotspot and nonhotspot mutations respectively. Currently, this test is only offered to MSK patients [22].

Knight Diagnostic Laboratories specializes in cancer diagnostics and has a line of somatic tumor panels, the GeneTrails® Cancer Panels. Using Ion Torrent PGMs, KnightDx has created tumor panels that yield a minimum of 100x coverage, an LOD of 5-15% and produce final

results in 10-14 days. Their 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 Profile line of tests. The two primary categories of testing offered are the Broad Reach Tumor Profiles and the Next-Gen Cancer-Specific Profiles. The former encompassing 4 options ranging from 43 genes up to whole cancer exome analysis of over 4800 genes. The Next-Gen Cancer-Specific Profiles comprise a menu of 24 smaller panels that focus on detecting driver mutations of the specified tumor with TATs ranging from 1-2 weeks (www.neogenomics.com/neotype-cancer-profiles/).

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. The Solid Tumor Panel captures 31 genes and GenPath offers 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.

www.stanfordlab.com/esoteric/test-stanford-solid-tumor-actionable-mutation-panel.html

University of Washington department of lab medicine uses Illumina instruments to sequence the 234 genes of their solid tumor test, UW-OncoPlex. The test is validated to detect SNVs INDELs, gene amplifications, a limited number of gene fusions and performs microsatellite instability analysis for colorectal cancer samples. The assay achieves an average depth of coverage of 500x with an LOD of 6-10% [23].

EdgeBio utilizes Ion Torrent's 46-gene AmpliSeq Cancer Panel in an effort to devise a more efficient and time-sensitive strategy for providing sequencing data to treating oncologists, according to the Director of Genomic Sciences at Scripps Institute. Levy et al compared colon tumor cells with blood cells from a single patient and were able to generate 5,000x coverage using this technology and the Ion Torrent 316 chip, with an average coverage of 2000x and no regions with less than 10x (www.edgebio.com/ampliseq-cancer-panel-detection-somatic-mutations).

University of Pittsburgh Medical Center designed a custom thyroid cancer panel, ThyroSeq® that covers 12 genes totaling 284 mutation hotspots. The test is run on an Ion Torrent 318 chip achieving analytical accuracy of 100% and a mutation detection limit as low as 3%. This is accomplished by using the Torrent Suite pipeline from Ion Torrent. Additional analysis and annotation is performed by a custom in-house

design developed by UPMC [24]. A second version of ThyroSeq, ThyroSeq v.2 has been released that now includes 14 genes covering over 1000 mutation hotspots and 42 thyroid cancer specific gene fusions. Additionally, a 60 gene version of the ThyroSeq v.2 test is offered through a partnership with CBLPath. www.cblpath.com/

Guardant Health developed a liquid biopsy test for commercial use in 2014, the Guardant360™, which tests for 68 clinically actionable cancer genes across more than 150kb of DNA. The test has a reported specificity greater than 99.99% and, detects SNVs, CNVs, INDELs and genomic rearrangements with a LOD of 0.1 and a turnaround time of 2 weeks.

www.guardanthealth.com/guardant360/

Transgenomic, Inc developed MX-ICP-multiplexed ICE COLD-PCR™. MX-ICP technology produces as much as a 500-fold increase in mutation detection sensitivity, allowing a detection limit as low as 0.01%. Testing is currently offered for EGFR mutations to determine NSCLC and CRC treatment resistance and has a turnaround time of 7-10 days.

www.transgenomic.com/clinical-applications/mx-icp-overview/

Biodesix launched GeneStrat, a commercial liquid biopsy test, in May of 2015. The test targets 3 cancer genes: EGFR, KRAS and BRAF for mutations that provide guidance for treatment decisions of advanced NSCLC lung cancer patients with a turnaround time of 72 hours. Post enrichment sequencing is performed by droplet digital PCR (ddPCR). www.biodesix.com/genestrat/

Pathway Genomics released 2 new liquid biopsy tests to market in 2015, CancerIntercept™ Detect and CancerIntercept™ Monitor. The former intended for early discovery and the latter for serial monitoring of tumor and treatment progress. The tests require 10ml of blood in 2 specialized tubes for a total of 20ml and can achieve a detection limit as low as 0.01% with 300ng of DNA and 0.25% with as little as 10ng of DNA. Furthermore, by enriching the sample for 9 well known driver mutation genes affecting multiple cancer types in combination with the ultra-sensitive design of CancerIntercept, the assay is able to function as an early screening test for common tumors. Post enrichment sequencing is performed on Illumina instruments.

www.pathway.com/cancerintercept-liquid-biopsy/

Personal Genome Diagnostics launched the first liquid biopsy panel for tumor profiling called PlasmaSelect-R. They sequence libraries from this panel using Illumina sequencing. The current version of the test is designed for research use with a clinical (CLIA certified) test scheduled for release later this year.

main.personalgenome.com/wp-content/uploads/2015/04/PGDx_PlasmaSelect-R_Product_Information.pdf

Challenges

The presence of genome heterogeneity (genetic mosaicism) and heterogeneous tissue provoke some of the larger biological problems for clinical laboratories. Genetic mosaicism is an unavoidable characteristic of tumor biology; however, improvements to bioinformatics processes can greatly reduce the burden this phenomenon imposes on clinical interpretation. Indeed, as population genetics data become more plentiful, mutation rate algorithms can be further optimized and accuracy subsequently improved. Heterogeneous tissue—the various normal cells mixed in with a tumor mass—impose difficulty on wet lab procedures resulting in reduced efficiency and accuracy of sample sequences. To combat this challenge, significant progress is being made

in the field of single cell genomics, which will substantially reduce neighboring cell contamination [25]. As it stands, there are a number of problems that must be resolved before single molecule sequencing can be used as a comprehensive diagnostic tool [26]. For example, Pacific Biosciences' RS II sequencer produces a relatively low output of 1GB per run with a relatively high instrument cost of roughly \$700,000 dollars and a considerably high error rate of 14% (compared to 0.1-1.0% for current leading technologies). Detection of CNV is another persistent issue for Next-generation sequencing. Although read depth-based methods for CNV detection are utilized for clinical testing, the methods are limited to high amplification (>6 copies), homozygous deletions and are sensitive to sample purity [20,21]. Despite these challenges, advancements in mutation enrichment of liquid biopsies are enabling genetic testing labs to march forward not only with increased diagnostic sensitivity, but a viable method of cancer treatment monitoring.

While the scope of this review aims to detail the technologies and software used for somatic mutation detection and their adoption by the diagnostic industry, the difficulties of reimbursement and coverage of such tests cannot be ignored. Diagnostic testing for medical treatment and management needs to go hand in hand with reimbursement to address the market need thereby driving further advancements. Two major complications for reimbursement according to Genome.gov are insufficient data regarding the economics of such testing and evaluating the costs of technologies used for testing. While this data will become available with time as more and more genetics laboratories continue providing information, other, more intricate problems will persist. Insurance plans differ considerably on what qualifies for reimbursement regarding genetic tests and many even use "evidence-based coverage" plans that attempt to justify the accuracy of tests and the availability of treatments. Dissimilarity not only between insurance providers but also between laboratory protocols, the technology used and the purpose of testing determined by the patient's physicians as well as clinical utility of the test makes this an incredibly complicated issue.

Conclusion

In the past, cancer and other somatic diseases with mosaic presentations were, for the most part, limited to germline risk assessment. Today, sequencing and computing technology permit targeting and identification of complex and low abundant variation, which is forever changing the diagnostic landscape in medicine. Clinicians now have rapid access to accurate, low-cost genetic information and can therefore develop thorough, highly personalized treatment plans and track the progress through non-invasive serial testing.

There is still a great deal of work to be done, however. While clinicians make use of what is currently available, laboratory and bioinformatics scientists need to design scalable, high throughput processes that can handle commercial volume with high reproducibility

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