Video Article

Next Generation Sequencing for the Detection of Actionable Mutations in Solid and Liquid Tumors

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Abstract

As our understanding of the driver mutations necessary for initiation and progression of cancers improves, we gain critical information on how specific molecular profiles of a tumor may predict responsiveness to therapeutic agents or provide knowledge about prognosis. At our institution a tumor genotyping program was established as part of routine clinical care, screening both hematologic and solid tumors for a wide spectrum of mutations using two next-generation sequencing (NGS) panels: a custom, 33 gene hematological malignancies panel for use with peripheral blood and bone marrow, and a commercially produced solid tumor panel for use with formalin-fixed paraffin-embedded tissue that targets 47 genes commonly mutated in cancer. Our workflow includes a pathologist review of the biopsy to ensure there is adequate amount of tumor for the assay followed by customized DNA extraction is performed on the specimen. Quality control of the specimen includes steps for quantity, quality and integrity and only after the extracted DNA passes these metrics an amplicon library is generated and sequenced. The resulting data is analyzed through an in-house bioinformatics pipeline and the variants are reviewed and interpreted for pathogenicity. Here we provide a snapshot of the utility of each panel using two clinical cases to provide insight into how a well-designed NGS workflow can contribute to optimizing clinical outcomes.

Video Link

The video component of this article can be found at https://www.jove.com/video/52758/

Introduction

Next-generation sequencing (NGS) of clinical oncology specimens has become more widely available over the past several years as growing scientific literature points to the importance of identifying targetable genetic changes and predictive/prognostic molecular markers. Multi-gene panel analyses and whole exome sequencing studies in both epithelial[1,2] and hematologic[3] malignancies have solidified the concept of tumor heterogeneity and clonal evolution as disease progresses and relapses. Additionally, unlike competing technologies such as polymerase chain reaction (PCR) or Sanger sequencing, NGS can detect most genomic alterations in all clinically relevant cancer genes in a single assay[4].

The Center for Personalized Diagnostics initially launched with two clinical NGS panels, a custom Hematological Panel (Heme-NGS Panel) and an off-the-shelf Cancer Panel for FFPE specimens (Solid-NGS Panel) (see **Figure 1**). These panels cover clinically relevant or high interest regions of the selected genes; not all genes or exons are fully covered. Amplicons are generated by probe hybridization followed by extension and ligation. The targeted regions are further amplified using PCR with universal dual-indexed primers, allowing up to 96 samples to be pooled for sequencing.
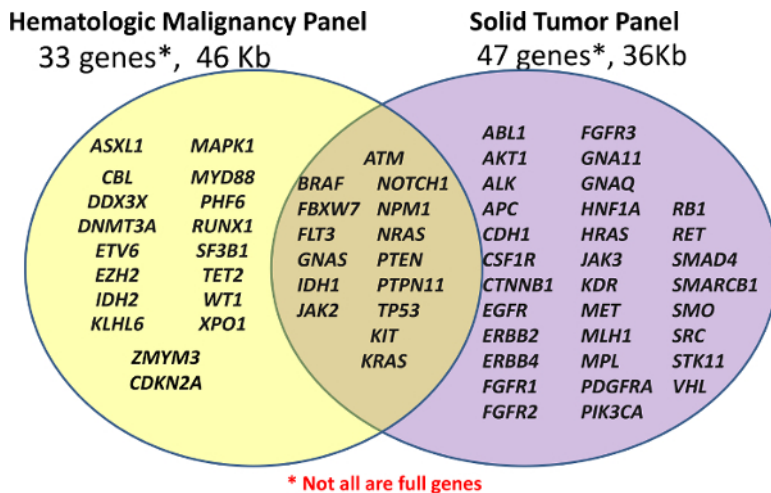
Figure 1: List of Genes Covered in the Panels. Library preparation is performed using either the custom Hematological Panel (Heme-NGS Panel) of 33 genes or the off-the-shelf Amplicon Cancer Panel (Solid-NGS) of 47 genes. Not all of the genes or exons are covered in full, as some amplicons may only cover certain hotspots. Please click here to view a larger version of this figure.

The content for the Heme-NGS Panel was derived from multiple sources, but centers around 16 genes mutated in acute myeloid leukemia (AML) previously described as demonstrating a high level of clinical utility[5]. The Solid-NGS Panel is commercially produced with the targeted regions based upon commonly mutated genes in cancer as reported in the Catalogue of Somatic Mutations in Cancer (COSMIC) database[6].

Several key steps characterize the overall workflow for clinical NGS. After the clinician orders the test, a pathologist determines adequacy of the specimen following analysis for tumor percentage and sample volume. In our institution, we require at least 10% tumor due to the background sequencing error rate ("noise") of the technology and the efficiency of the targeted approach. If the tissue is adequate for testing, genomic DNA is extracted. This DNA is then subjected to multiple quality control (QC) steps. If the DNA passes QC, an amplicon library is generated and sequenced. The resulting data is analyzed through an in-house bioinformatics pipeline. Following bioinformatics analysis, variants are manually reviewed and interpreted for pathogenicity before incorporation into a clinical report. Below we describe two cases that went through this rigorous workflow and ultimately led to changes in clinical management.

Case 1 - Acute Myeloid Leukemia

A bone marrow biopsy from patient A was diagnostic for AML, without maturation. Cytogenetic studies were sent on the bone marrow specimen and demonstrated a normal female karyotype. There were 95% circulating blasts present, so a peripheral blood specimen was sent for personalized diagnostic testing on the Heme-NGS Panel.

Acute myeloid leukemia (AML) is a hematological malignancy of the myeloid lineage of white blood cells. The detection of gene mutations in AML has become increasingly important for prognosis and treatment, with recurrent gene mutations recognized as important in pathogenesis and prognosis[7]. Mutations in *NPM1* and *CEBPA* have been associated with a favorable prognostic risk, while internal tandem duplications (ITDs) in *FLT3* have been associated with a less favorable outcome[8]. A growing body of evidence supports a pathogenic role for these and other mutations in AML[9].

Case 2 - Lung Adenocarcinoma

A biopsy of a left supraclavicular mass from patient B demonstrated pulmonary adenocarcinoma. Biopsy material from the formalin-fixed paraffin-embedded (FFPE) lymph node mass was sent for genomic testing (Solid-NGS Panel) as rolls/curls with greater than 50% tumor, to identify whether a mutation was present for targeted therapeutic intervention.

Lung cancer is the leading cause of cancer related mortality in the United States and is divided into two main types, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC can be further defined as either adenocarcinoma or squamous cell carcinoma, based on the histology of the lesion. Lung adenocarcinoma is the most common subtype of lung cancer, seen in both smokers and non-smokers, and is the most common form of lung cancer for non-smokers[10]. Molecular studies of lung adenocarcinomas have identified mutation in multiple oncogenes[11]. The most common driver mutations identified in smokers are mutations in *KRAS* and *BRAF*. The most common mutations in non-smokers are mutations in *EGFR*, and rearrangements involving the genes *ALK*, *RET* and *ROS1*. Lung tumors have been described with an in-frame exon 20 insertion in the gene *ERBB2* (*HER2/neu*). The most common abnormality in *HER2/neu* is an amplification of this locus in breast cancer for which a targeted therapy is available (trastuzumab: a humanized monoclonal antibody against *HER2/neu*). The *HER2/neu* exon 20 insertion that is observed in 2 - 4% of lung adenocarcimomas[12] has shown partial response to combination therapy with HER2/neu and mTOR inhibitors (neratinib and temsirolimus, respectively)[13].

Protocol

This protocol comprises the salient steps of two validated laboratory developed tests for the genomic profiling of solid and liquid tumors, respectively. The testing performed in the laboratory is done in accordance with the requirements of the Clinical Laboratory Improvement Amendments (CLIA) of 1988.

1. DNA Extraction from Peripheral Blood or Bone Marrow

1. Determine how much blood or bone marrow to take based on **Table 1**.

Sample/WBC	Amount to be treated as 1 ml of blood
Bone Marrow	250 µl
Blood WBC 12,000 – 50,000	1 ml
Blood WBC 50,000 – 100,000	500 µl
Blood WBC 100,000 – 200,000	200 µl
Blood WBC > 200,000	100 µl
* For Blood WBC < 12,000, take 2 ml of blood	

Table 1: Blood/Bone Marrow Volume to Use Chart. Since the white blood cell count will vary from sample to sample, it is difficult to specify a specific volume of blood to use. Therefore, the amount of blood to use for the assay must be determined by looking at the white blood cell count (WBC) prior to starting the assay. Although less blood is utilized, it should still be treated as if its 1 ml since the volume of blood used is reduced because the number of cells present is greater than normal.

2. Follow the commercially available kit's protocol to isolate the genomic DNA.

2. DNA Extraction from Formalin-fixed Paraffin-embedded (FFPE) Tissue

1. Based on the tumor region the pathologist circled on the H&E slide, line up the unstained slides with the guide H&E slide and outline a similar area for extraction. For macro-dissection, process only one specimen/patient's set of slides at a time.
2. Heat the slides on a 45 °C heat block to slightly melt the paraffin. Carefully scrape the tissue within the lines that are marked on the slide, using a new scalpel for each specimen to be extracted. Place the wax scrapings into the appropriately labeled 1.5 ml tube. Be careful because the scraped wax is very electrostatic and may jump out of the tube.
3. Add 320 µl of Deparaffinization Solution for every five to six 5 µm sections (25 - 30 µm total). For example, if a tube containing 3 sections of a 10 µm roll/curl is going to be processed, then use 320 µl, but if 5 sections at the same thickness were obtained then use 640 µl.
4. Vortex vigorously for at least 10 sec and perform a quick spin in a microcentrifuge to remove the tissue/wax from the sides and cap and into the solution. Incubate at 56 °C for 3 min., and then incubate at RT for 5 - 10 min.
5. Following the RT incubation, add 180 µl of ATL buffer for every 320 µl of Deparaffinization Solution added. Mince the tissue ten times using a sterile mini-pestle using a new pestle for each specimen. Ensure there is no tissue stuck to the pestle, as it can be very sticky. Vortex the suspension vigorously for 3 sec, then centrifuge at maximum speed for 1 min.
6. Add 10 µl of Proteinase K to the lower clear phase. Mix gently by pipetting up and down to ensure the tissue is resuspended. Do NOT vortex. Incubate at 56 °C overnight with shaking at 400 - 500 rpm.
7. The next morning, check if the tissue is fully dissolved (this has occurred if the lower solution is clear). If the lower solution is not clear, then vortex vigorously for 3 sec and centrifuge at maximum speed for 1 min. Add an extra 5 - 10 µl of Proteinase K and incubate at 56 °C for an additional 30 - 60 min.
8. Incubate at 90 °C for 1 hr to help reverse the formaldehyde cross-linking. Allow samples to cool to room temperature for 5 - 10 min and then briefly centrifuge each tube to consolidate the liquid.
9. Transfer the lower clear phase into a labeled 1.5 ml tube. If there are multiple tubes from the same patient specimen (such as the case if multiple rolls are being used), recombine the lower phases into one tube at this point. Note: Transferring small amounts of the Deparaffinization Solution should not interfere with the purification procedure, but there is a risk if a large amount is transferred.
10. Add 2 µl of RNase A solution. Vortex gently or invert 25 times and quick spin in a microcentrifuge. Incubate at RT for 5 min.
11. Add 200 µl of Protein Precipitation Solution. If doing one or two rolls, use 200 µl. If doing three rolls at once, then use 400 µl. Vortex vigorously at high speed for 30 sec to uniformly mix the lysis buffers. Incubate on ice for 5 min or the samples can stay on ice for up to an hr.
12. Centrifuge at 5,000 x g for 5 min. The precipitated protein should form a tight, white pellet. Pour the supernatant into a labeled 1.5 ml tube, and then incubate the samples on ice for at least 3 min. Centrifuge at 5,000 x g for 3 min.
13. Add 200 µl of 2-propanol (Isopropanol) for every 180 µl of Buffer ATL added earlier to a labeled 1.5 ml tube (it might be necessary to use a 2 ml tube). For example, if doing three rolls add 600 µl of isopropanol. Add 1 µl of glycogen for every 180 µl of Buffer ATL added earlier to the isopropanol and invert the tube several times to mix.
14. Carefully add the supernatant from the Protein Precipitation step into the Isopropanol mixture. Mix the tube by inverting gently at least 50 times.
15. Centrifuge at maximum speed for 3 min. The DNA will be visible as small white pellet at the bottom of the tube.
16. Pour or aspirate off the supernatant into the appropriate waste tube. Keep a separate 1.5 ml waste tube for each specimen in case the pellet comes dislodged so it will not be lost or mixed with the waste of other specimens. Drain the tube on a paper towel and ensure the majority of the Isopropanol is removed.
17. Add 300 µl of freshly made 70% ethanol. Invert the tube gently several times to wash the pellet. Try to ensure the pellet comes dislodged to ensure a more thorough cleaning.
18. Centrifuge at maximum speed for 5 min. and then carefully remove the ethanol. The pellet may be loose, so pour or aspirate slowly and watch the pellet. Remove the excess ethanol from the inside of the tube, without touching the pellet. Allow the samples to air dry for 5 - 15 min, being careful to not over dry the sample.
19. Add between 25 - 100 µl of DNA Hydration Solution to each sample based on the size of the DNA pellet and the starting amount of tissue. Vortex the tubes vigorously and briefly spin in a microcentrifuge. Incubate for 1 hr at 65 °C to fully rehydrate the DNA.

3. Genomic DNA Quality Control

Note: There are three independent steps for DNA quality control (QC). See **Table 2** for further explanation of why each QC step is performed.

Instrument	Result	Indication	Ideal Range
DropSense96	A260/A230	Identification of chemical contaminants (*e.g.,* ethanol)	1.50 – 2.2
DropSense96	A260/A280	Identification of protein contaminants	1.60 – 2.2
DropSense96	Concentration	DNA quantification	> 1 ng/µl
TapeStation	DNA Smear	Determination of DNA integrity (*e.g.*, degradation/fragmentation of extracted DNA)	50% > 1,000 bp
Qubit 2.0	Concentration	More accurate DNA quantification	> 1 ng/µl

Table 2: DNA QC Expected Results. All of these values are taken into account before running permitting a sample to proceed to the library preparation stage.

1. Following the manufacturer's protocol, run 2 µl of the extracted DNA on a fluorometer to obtain the working concentration (ng/µl) of the sample.
2. Run 1 - 2 µl of each sample on a UV/VIS spectrophotometer to check the quality of the sample (A260/A230 and A260/A280 ratios) according to the manufacturer's instructions.
3. For FFPE samples: Following the manufacturer's instructions, run 1 µl of each sample on a microfluidic gel-electrophoresis system to assess degradation/fragmentation of the DNA. See **Figure 2** for examples.
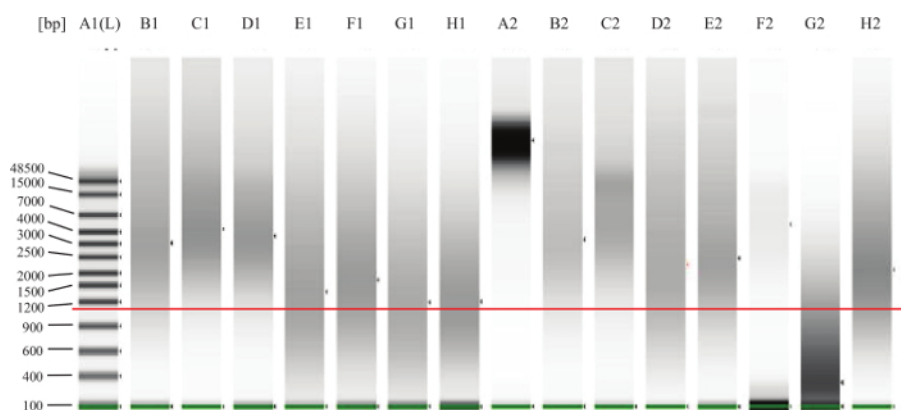
Figure 2: Genomic DNA QC Gel Example. The green line over the lower bands is to indicate the lower marker. The added-in red line is to indicate approximately 1,000 bp. Lane A2 represents fully intact DNA, as one would expect from fresh tis*sue* (*e.g.*, peripheral blood or bone marrow). Lanes B1, C1, D1, B2, C2, E2 are examples of good, intact FFPE DNA. The DNA in lane F2 appears to be intact, but at too low a concentration. The DNA in lane G2 is degraded or fragmented and will not work in the assay. Lanes E1, F1, G1, H1, D2, H2 represent DNA which falls in the 'gray zone' meaning that the assay might work well, but some of the DNA could be too damaged or cross-linked and thus it will not perform well in the assay. [Please click here to view a larger version of this figure.](#)

4. Amplicon Library Preparation

1. Hybridization of Oligo Pool and Extension-Ligation of Bound Oligos
   1. In the Pre-PCR room, add the necessary volume of Low EDTA TE, 5 µl of the Panel's Oligo Tube (*e.g.*, Solid-NGS Panel or Heme-NGS Panel), and 100 - 250 ng of genomic DNA to each corresponding well in a semi-skirted 96 well-plate labeled as the Hybridization (HYB) plate.
   2. Add 40 µl of Oligo Hybridization for Sequencing Reagent 1 (OHS1) to each sample in the HYB plate. Gently pipette up and down at least 5 - 6 times to mix. Change tips after each column to avoid cross-contamination.
   3. Seal the HYB plate with adhesive aluminum foil and centrifuge at 1,000 x g for 30 sec.
   4. Incubate the HYB plate in the pre-heated hybridization incubator at 95 °C for 1 min. Set the temperature of the hybridization incubator to 40 °C. Continue incubating as long as it takes the incubator to decrease from 95 °C to 40 °C (~ 90 min). Note: This gradual cooling is critical for proper hybridization.
   5. During the last 15 - 20 min of the hybridization incubation, pre-wash the Filter Plate Unit (FPU). Prepare only the wells to be used in the current assay, *i.e.*, only use fresh/unused wells of a previously opened filter plate, but never re-use wells that have been used. Note: This should be clear based upon the Kit Number on the filter plate, the markings on the filter plate, and the sealant used across the plate.
      1. Using a multi-channel pipette, add 45 µl of Stringent Wash 1 (SW1) to each well.

CAUTION: Contains formamide. Cover and centrifuge the FPU at 2,250 x g for 3 min at 20 °C. Check every single well for residual liquid (> 15 µl/well).

2. If residual liquid exists, rotate the FPU 180° and repeat the centrifuge step again for an additional 3 min. If the residual liquid spins through the second time, then proceed to the next step, if not then there may be a defect in the filter plate and the current plate will need to be replaced.

6. Once the hybridization incubator has cooled to 40 °C, centrifuge the plate at 1,000 x g for at least 30 sec at 20 °C to collect condensation. Transfer the entire volume of each sample from the HYB plate onto the center of the corresponding pre-washed wells of the FPU. Change tips after each column to avoid cross-contamination. Cover the FPU and centrifuge at 2,250 x g for 3 min at 20 °C.
7. Add 45 µl of SW1 and centrifuge at 2,250 x g for 3 min at 20 °C. Repeat for a total of two washes. Rotate the FPU 180° and centrifuge again at 2,250 x g for 3 min to completely remove all the SW1.
8. Dissemble the FPU. Discard all the flow-through (containing formamide) into the proper waste container. Reassemble the FPU using a different MIDI Waste Collection Plate. Add 45 µl of Universal Buffer 1 (UB1) to each sample well and centrifuge at 2,250 x g for 3 min at 20 °C. CAUTION: Contains formamide.
9. Add 45 µl of Extension Ligation Mix 3 (ELM3) to each sample well on the FPU plate and pipette up and down 3 times to mix. Note: The Extension-Ligation reaction takes place on the filter plate membrane.
10. Seal the FPU plate with adhesive aluminum foil and incubate the entire FPU assembly in a pre-heated 37 °C incubator for 45 min.

2. **Indexing and PCR Amplification**
   1. Aliquot the indexes being used to the corresponding wells in the Indexed Amplification Plate (IAP) by arranging the primers in the Index Plate Fixture, arranged in the following manner: i5 primer tubes (white caps, clear solution) vertically, aligned with rows A through H, i7 primer tubes (orange caps, yellow solution) horizontally, aligned with columns 1 through 12. Using a p10 multi-channel pipette, add 4 µl of i7 primers (yellow solution) to each row of the IAP and add 4 µl of i5 primers (clear solution) to each column of the IAP.
   2. On ice or a cooling block, prepare the PCR Master Mix by adding 0.5 µl of DNA Polymerase 1 (TDP1) to 25 µl of PCR Master Mix 2 (PMM2) per sample. Invert, quickly vortex, and briefly centrifuge the PCR Master Mix to mix.
   3. Add 22 µl of the PCR Master Mix to each well of the IAP and pipette up and down 3 times to mix. Change tips between wells. Keep the IAP at 4 °C.
   4. After the 45 min Extension-Ligation reaction (step 4.1.10), remove the FPU from the incubator and carefully remove the aluminum foil seal. Cover with the lid and centrifuge at 2,250 x g for 3 min at 20 °C.
   5. Add 25 µl of 50 mM NaOH to each sample well on the FPU. Pipette up and down at least 6 times; ensure the pipette tips come in contact with the membrane. Change tips after each column. Incubate at room temperature for 5 min.
   6. Transfer samples eluted from the FPU to the IAP as follows:
      1. Using a p100 multi-channel pipette set to 20 µl, pipette the NaOH on the FPU plate up and down at least 6 times. Slightly tilt the FPU plate to ensure complete aspiration off the plate.
      2. Transfer 20 µl from the FPU to the corresponding column of the IAP. Gently pipette up and down at least 6 times to thoroughly combine the DNA with the PCR Master Mix. Seal the IAP with adhesive film and centrifuge at 1,000 x g for 1 min at 20 °C.

   7. Bring the PCR Plate into the Post-PCR Room and load the plate onto a thermal cycler. Run the PCR program consisting of a heat denaturing step at 95 °C for 3 min; followed by 25 cycles of 95 °C for 30 sec, 62 °C for 30 sec, 72 °C for 60 sec; followed by a final extension of 72 °C for 5 min; finishing with a hold at 10 °C. If not proceeding to the next stage following the completion of PCR, the plate can remain on the thermal cycler overnight, or it can be stored at 2 to 8 °C up to two days.

3. **PCR Purification and Bead-Based Normalization**
   1. Remove the magnetic purification beads, Elution Buffer (EBT), and gel-electrophoresis reagents from the 4 °C fridge and place at RT at least 20 min before the next stage.
   2. Once the PCR is complete, centrifuge at 1,000 x g for 1 min at 20 °C to collect condensation. Transfer 1 µl of each PCR reaction to strip tubes/plate wells containing 4 µl of water to dilute the samples 1/5. Pipette up and down to mix.
   3. Add 2 µl of the diluted PCRed samples to 2 µl of microfluidic-gel buffer. Seal the strips/plate. Shake at 1,800 rpm for at least 30 sec and centrifuge at 1,000 x g for 30 sec. Following manufacturer's protocol, run the mixture on a microfluidic gel to assess if the library preparation yielded an acceptable library (see **Figure 3**).
   4. Vortex the magnetic purification beads until they are well-suspended and the color appears homogeneous. Add 45 µl of the beads to each sample.
   5. Seal the plate with clear adhesive film and shake the plate at 1,800 rpm for 2 min. Incubate at room temperature without shaking for 10 min.
   6. Place the plate onto a magnetic stand. Once the supernatant has cleared, carefully remove and discard the supernatant. If any beads are inadvertently aspirated into the tips, dispense the beads back to the plate and let the plate rest on the magnet for 2 min and confirm that the supernatant has cleared.
   7. With the plate on the magnetic stand, add 200 µl of freshly prepared 80% ethanol to each sample well. Move the plate back and forth a few times. Incubate the plate on the magnetic stand for 30 sec. Carefully remove and discard the supernatant. Repeat for a total of two washes.
   8. Remove the excess ethanol by briefly centrifuging the plate at 1,000 x g to drive down any ethanol on the tube sides, placing the plate back onto the magnetic stand, and using a p10 multi-channel pipette set to 10 µl to remove the ethanol. Allow the beads to air-dry for 5 - 8 min.
   9. Using a p100 multi-channel pipette, add 30 µl of EBT to each well. Pipette up and down a few times to ensure the beads come off the side of the tube. Seal the plate with clear adhesive film and shake the plate at 1,800 rpm for 2 min.
   10. Incubate at room temperature without shaking for 3 min. If there are samples in which the beads are not completely resuspended, gently pipette up and down to resuspend the beads.
   11. Place the plate on a magnetic stand. Using a p100 multi-channel pipette, transfer 20 µl of the supernatant to a whole new plate called the Library Normalization Plate (LNP). Transfer the remaining ~ 10 µl of the individual sequencing libraries to a separate plate called

the Remaining Cleaned-Up Library Plate (RCLP). Store this plate along with the final library prep, as it can be used as a reserve for a second sequencing run, if necessary. If not proceeding to the next stage, the LNP and RCLP can be stored at -15 to -25 °C.

12. Vigorously vortex and resuspend the Library Normalization Beads 1 (LNB1). It is critical to completely resuspend the LNB1 bead pellet at the bottom of the tube. Prepare the Normalization Mix, by mixing 8 µl of LNB1 with 44 µl of Library Normalization Additives 1 (LNA1) per sample. Vigorously vortex the Normalization Mix for 10 - 20 sec.
    CAUTION: LNA1 contains formamide.

13. With intermittent inversion and vortexing of the Normalization Mix, add 45 µl to each sample of the LNP. Seal the plate with clear adhesive film and shake the plate at 1,800 rpm for 30 min. This 30 min incubation is critical for proper library normalization as incubations of greater or less than 30 min may affect library representation and cluster density.

14. During the 30 min incubation, prepare the reagents for sequencing by thawing the reagent cartridge and Hyb Buffer (HT1) [CAUTION: Both contain formamide]. In addition, get ice for a later step and ensure a heat block suitable for 1.5 ml centrifuge tubes is set to 96 °C.

15. When the 30 min mixing step is complete, place the LNP on a magnetic stand. Once the supernatant has cleared, use a multi-channel pipette to carefully remove and discard the supernatant into the appropriate waste container.

16. Remove the LNP from the magnetic stand and wash the beads with Library Normalization Wash 1 (LNW1) as follows:
    1. Add 45 µl of LNW1 to each sample well.
       CAUTION: Contains formamide. Seal the plate with clear adhesive film and shake the plate at 1,800 rpm for 5 min. Repeat for a total of two washes. Ensure to remove all the LNW1 after the second wash.

17. Remove the LNP from the magnetic stand and, using a multi-channel pipette, add 30 µl of 0.1 N NaOH (less than a week old) to each well to elute the sample. Seal the plate with clear adhesive film and shake the plate at 1,800 rpm for 5 min.

18. During the 5 min elution, add 30 µl of Library Normalization Storage Buffer 1 (LNS1) to each well to be used in a new plate named the StoraGe Plate (SGP).

19. Place the LNP on the magnetic stand. Once the supernatant has cleared, transfer the 30 µl elution to the LNS1 in the SGP. Change tips between samples to avoid cross contamination.

20. Seal the plate with adhesive film and centrifuge at 1,000 x g for at least 30 sec.

21. Add 5 µl of each sample to be sequenced to a labeled Pooled Amplicon Library (PAL) 1.5 ml tube. Vortex the PAL to mix and briefly spin down in a microcentrifuge.

22. Depending on what sequencing chemistry being used (V2 or V3) add 4 - 10 µl of the PAL to 590 - 596 µl of HT1. Generally, add 5.8 µl of PAL to 595 µl of HT1 for V2 chemistry and 8.5 µl of PAL to 592 µl of HT1 for V3 chemistry. Label this tube as the Diluted Amplicon Library (DAL) tube.

23. Vortex the DAL and briefly spin down in a microcentrifuge. Incubate the DAL at 96 °C for 2 min. Invert the DAL tube 3 times and put the DAL on ice for at least 5 min, while preparing the sequencer for sequencing. After the 5 min, the DAL is ready to be loaded.

24. If finished with the SGP, seal the plate with adhesive aluminum foil film and label it with the date and plate ID. Store the sealed SGP and PAL at -15 to -25 °C.
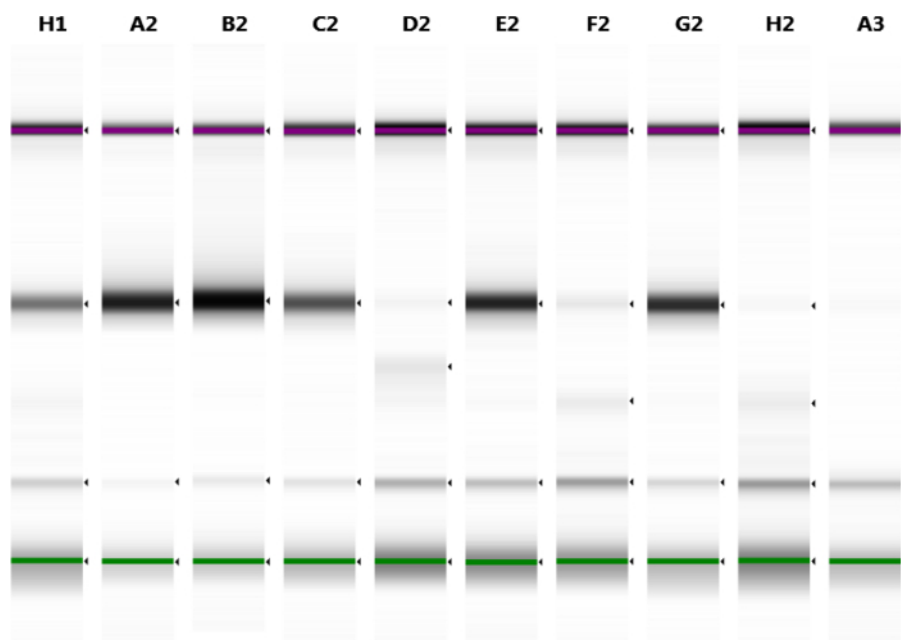
Figure 3: Library Prep QC Gel Example. The green line over the lower bands is to indicate the lower marker and the purple line over the upper bands is to indicate the higher marker. Everything worked well for lanes H1, A2, B2, C2, E2, and G2. The library prep did not work optimally, for lanes D2, F2, and H2, but results will still be obtained they just might not have adequate coverage. For A3, the library prep barely worked and most likely this DNA sample was not adequate for the assay. The lower bands above the lower marker are the unused primers, because the aliquot is taken directly from the PCRed well. The NTC sample should only have the unused primer band, and nothing else. Please click here to view a larger version of this figure.
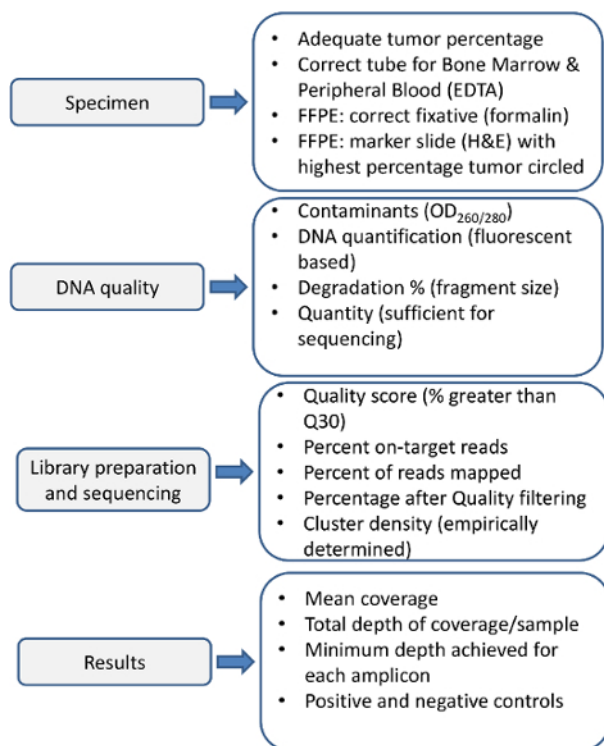
5. Sequencing

1. Ensure a correct SampleSheet.csv has been made for the run. See Supplementary **Figure 1** for an example.
2. Rinse and dry the flow cell and add 600 µl of the DAL to the thawed reagent cartridge.
3. Prepare the sequencer for sequencing by following the on-screen prompts.

6. Data Analysis

1. Execute the bioinformatics pipeline. Utilize the in-house, custom designed bioinformatic pipeline to identify mutations, insertions, deletions and amplifications[18].
2. After the pipeline has completed, check the log files for errors/warning, as any significant errors/warnings aid in the QC of the pipeline processing.
3. Analyze the run statistics (**Table 3**) to ensure the sequenced library has passed the lab determined QC metrics (**Figure 4**). Manually review each variant by viewing the .bam files in a genomic data viewer (*e.g.*, the Integrative Genomics Viewer[16] (IGV)).
   NOTE: Only the variants within the validated allele-frequency range and above the minimum depth of coverage after quality filtering are reported (using Human Genome Variation Society's (HGVS) nomenclature). For final reporting, each exonic variant was categorized into: pathogenic, probably disease associated, variant of unknown significance (VUS), likely benign, and benign. All variants above the reporting criteria of 5% allele frequency, except those deemed benign variants and synonymous changes, were reported.

Figure 4. Overview of Quality Control Steps for NGS. Quality control of each step in the process is necessary to ensure the sequencing will yield results such that pre and post sequencing metrics are considered. Appropriate specimen treatment is essential for high quality DNA. Blood and bone marrow in inappropriate fixatives can yield low quality DNA. Inappropriate fixation of solid tumor specimens can degrade DNA (*e.g.*, fixation in B5). DNA quality should be assessed for protein and RNA contamination by spectrophotometric means, and accurately assessed for the amount and integrity of DNA. The sequencing metrics need to be empirically determined in the sequencing laboratory and followed for each sequencing reaction and every specimen. Prior to reporting the sequencing results for each specimen should be assessed for coverage, depth, and adequate performance of positive and negative controls. Please click here to view a larger version of this figure.

Representative Results

Case 1 - Heme-NGS Panel

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The DNA extracted from leukemic peripheral blood was of sufficient quality and quantity (176 ng/µl) for the Heme-NGS Panel. The overall mean depth of coverage was 4,933x (above the minimum mean depth of coverage of 1,000x). Additional run statistics are in **Table 3**. Of the 8 regions below 250x coverage, 3 were due to improper trimming of the primers (*i.e.*, the primer sequence was not properly removed due to sequencing errors), 1 was a known artifact of the assay, and the other 4 were partial regions of exons of different genes with no reportable variants. Although our clinical protocol only includes reporting variants covered by at least 250 reads, all data with at least 100 reads are imported into the database for review.

Data processing from the bioinformatics pipeline detected three reportable, disease-associated mutations; a missense mutation in *FLT3*, a missense mutation in *IDH2*, and a frameshift mutation in *NPM1*. The exonic variants with their allele frequencies are described in **Table 4**. A common mutation in *FLT3* is the *FLT3*-internal tandem duplication (ITD) which is not automatically called by our pipeline and requires a visual inspection of exon 14. Visual inspection of exon 14 of *FLT3* showed no insertion or duplication in the submitted specimen. The *FLT3* I836del was at 1% allele frequency and was not included in the final report because it fell below the validated minimum allele frequency of 5%. This mutation is not on the same DNA molecule as the *FLT3* D835Y change (*i.e.*, observed in the same amplicon region, but not in "cis" in any of sequencing reads) and it was only observed by manual review of the .bam files when verifying the p.D835Y change. The lower allele frequencies of the two *FLT3* mutations suggests these mutations may represent heterogeneity and/or clonal evolution; however, the difference could be due to assay performance for that amplicon or a single-nucleotide polymorphism (SNP) near or overlapping the primer sequence that affected the amplification of this allele.

The Heme-NGS Panel results for Case 1 with AML identified mutations in *FLT3*, *IDH2*, and *NPM1*, three commonly mutated genes in AML. Mutations in *FLT3* are observed in ~ 25% of adult patients with AML (COSMIC database[17]) and are either internal tandem duplications (ITDs) or missense mutations in the tyrosine kinase domain. The *FLT3-ITDs* are the more common mutation and are associated with poor response to standard chemotherapy, while the prognostic significance of *FLT3* kinase domain point mutations, as seen in this AML patient, has an unclear impact on prognosis[14]. Isocitrate dehydrogenase 2 (NADP+), mitochondrial (*IDH2*) is a gene that encodes an epigenetic modifier that is commonly mutated in AML. Mutations in epigenetic modifiers are relatively common in AML, with mutations in *IDH1* and *DNMT3A* representing other mutations in this class of genes that lead to gene dysregulation. Mutations in the gene for nucleophosmin (*NPM1*) are one of the most common acquired mutations in AML and are generally considered to be a good prognostic factor (in the absence of a *FLT3-ITD*).

Co-mutations of *NPM1* and the *IDH2* have been described in the literature as a favorable prognostic indicator[5], with a 3-year overall survival of 89%. This represents a significant survival benefit when compared to the overall 3-year survival of wild type *NPM1* and *IDH2* of 31%. For example, standard-of care practice includes mutation analysis for *NPM1* and *FLT3-ITD* mutations. In this scenario, the detection of only an *NPM1* mutation would fail to appropriately stratify patient for risk, as the secondary mutations can be favorable (*e.g., IDH2*) or unfavorable (*e.g., TET2*), reducing confidence for alleviating a bone marrow transplant.

Case 2 - Solid-NGS Panel

DNA extracted from the FFPE tissue was of sufficient quality and quantity for the Solid-NGS Panel, with a concentration of 252 ng/µl and only 4% of the DNA below 1,000 base pairs (bp). After data analysis the mean depth of coverage was 9,167 reads (well above our minimum depth cutoff of 1,000 reads) with no regions below 250 read depth. Additional QC metrics are shown in **Table 3**.

Data processed through the bioinformatics pipeline detected two disease-associated mutations: an in-frame insertion in exon 20 of *ERBB2* (*Her2/neu*) and a missense mutation in *TP53*. All the exonic variants with their allele frequencies are represented in **Table 5**. The insertion in *ERBB2* actually represents a tandem duplicated (*Her2/neu*) sequence, as reflected in the nomenclature. Identification and confirmation of the in-frame insertion required manual review of the sequencing reads through IGV. The detection of mutation frequencies of greater than 50%, as seen for both the *TP53* and the *Her2/neu* mutations is suggestive of a loss of heterozygosity (LOH) event (see discussion).

The Solid-NGS Panel results for Case 2 with lung adenocarcinoma detected mutations in *ERBB2* (*HER2/neu*) and *TP53*, two genes not commonly tested for as part of the current standard-of-care for lung cancer patients. *HER2/neu* encodes a tyrosine kinase receptor similar to another commonly mutated gene in lung cancer, *EGFR*. Activating *HER2/neu* exon 20 insertions are observed in 2 - 4% of lung adenocarcinomas, account for the majority of *HER2/neu* mutations observed in lung cancer, and are typically seen in tumors without mutations in other driver genes such as *EGFR* and *ALK*[12]. There are several lines of evidence showing potential for various treatment options for patients with activating *HER2/neu* insertions, including partial response to combination therapy with *HER2/neu* and mTOR inhibitors[13] and significant disease control with the monoclonal antibody Trastuzumab in conjunction with chemotherapy[15]. Discovering a *TP53* change is not uncommon in cancer, but at this time there are no actionable therapies.

	Case 1	Case 2
Total Starting Reads	2,215,926	2,129,110
Percentage of Reads Mapping	98.42%	98.29%
Percentage of Reads on Target	99.01%	97.29%
Percentage of Reads on Target After Filter	97.60%	95.45%
Percentage of Usable Reads	94.87%	91.79%
Percentage of Bases above 250x Coverage	98.40%	100%
Percentage of Bases above 1000x Coverage	95.90%	99.70%
Coverage Below 250x - Amplicon Number	8	0

Table 3: Sequencing Run QC Metrics. This is a summary of the most important run statistics, not including the mean coverage, that are used for data review to determine if a library prep sample has passed QC. The whole process is successful if all the percentages are above 90%, but it is possible, with carryover of SW1 or UB1 in the FPU washing step or primer cross-talk, to have lower 'Percent on Target' in the range of 80 - 90%. If the 'Percent Mapped' is too low, that would indicate a contamination of bacteria or other DNA, as all samples should align to human. When any of these specifications are below 80%, the sample is flagged for further review to help determine how to proceed and improve the process.

Gene	Position	Ref	Alt	Effect	cDNA Change	Protein Change	Ref Total # of Reads	Alt Total # of Reads	Allele Frequency	Categorization	Reported
NPM1	chr5.170837548	*	+CAGA	FRAME_SHIFT_INSERTION	c.863_864insCAGA	p.W288fs	1892	859	45.4	Pathogenic	Y
FLT3	chr13.28592637	*	-ATG	CODON_DELETION	c.2508_2510delCAT	p.I836del	8600	86	1.00	Pathogenic	N
ATM	chr11.108183167	A	G	NON_SYNONYMOUS_CODING	c.A5948G	p.N1983S	7314	7294	99.73	Benign	N
FLT3	chr13.28592642	C	A	NON_SYNONYMOUS_CODING	c.G2503T	p.D835Y	8415	2715	32.26	Pathogenic	Y
IDH2	chr15.90631934	C	T	NON_SYNONYMOUS_CODING	c.G419A	p.R140Q	5303	2622	49.44	Pathogenic	Y
ASXL1	chr20.31022959	T	C	NON_SYNONYMOUS_CODING	c.T2444C	p.L815P	3625	3611	99.61	Benign	N
TET2	chr4.106196951	A	G	NON_SYNONYMOUS_CODING	c.A5284G	p.I1762V	7008	3586	51.17	Benign	N
SF3B1	chr2.198265526	A	G	SYNONYMOUS_CODING	c.T2631C	p.G877G	7516	7510	99.92		N
NOTCH1	chr9.139397677	C	A	SYNONYMOUS_CODING	c.G5124T	p.S1708S	1067	506	47.42		N
NOTCH1	chr9.139397707	G	A	SYNONYMOUS_CODING	c.C5094T	p.D1698D	1108	563	50.81		N

Table 4: Case 1 Results. Detected pathogenic, disease associated, variant of unknown significance (VUS), and likely benign exonic variants above the reporting criteria of 5% allele frequency are listed. Please click here to download this table.

Gene	Position	Ref	Alt	Effect	cDNA Change	Protein Change	Ref Total # of Reads	Alt Total # of Reads	Allele Frequency	Categorization	Reported
ERBB2	chr17.37880996	*	+ATACGTGATGGC	CODON_INSERTION	c.2313_2324dupATACGTGATGGC	p.Y772_A775dup	4107	2712	66.03	Pathogenic	Y
ATM	chr11.108138003	T	C	NON_SYNONYMOUS_CODING	c.T2572C	p.F858L	13035	4594	35.24	Benign	N
TP53	chr17.7577114	C	T	NON_SYNONYMOUS_CODING	c.G824A	p.C275Y	8619	6246	72.47	Pathogenic	Y
TP53	chr17.7579472	G	C	NON_SYNONYMOUS_CODING	c.C215G	p.P72R	2987	443	14.83	Benign	N
KIT	chr4.55593464	A	C	NON_SYNONYMOUS_CODING	c.A1621C	p.M541L	28442	3742	13.16	Benign	N
RET	chr10.43613843	G	T	SYNONYMOUS_CODING	c.G2307T	p.L769L	2631	2624	99.73	Benign	N
GNA11	chr19.3119239	C	T	SYNONYMOUS_CODING	c.C771T	p.T257T	6054	1182	19.52		N
IDH1	chr2.209113192	G	A	SYNONYMOUS_CODING	c.C315T	p.G105G	12289	2353	19.15		N
FGFR3	chr4.1807894	G	A	SYNONYMOUS_CODING	c.G1959A	p.T653T	3295	3270	99.24		N
PDGFRA	chr4.55141055	A	G	SYNONYMOUS_CODING	c.A1701G	p.P567P	14597	14556	99.72		N
KIT	chr4.55602765	G	C	SYNONYMOUS_CODING	c.G2586C	p.L862L	13784	1782	12.93		N
APC	chr5.112175770	G	A	SYNONYMOUS_CODING	c.G4479A	p.T1493T	29601	15132	51.12		N
EGFR	chr7.55249063	G	A	SYNONYMOUS_CODING	c.G2361A	p.Q787Q	7379	6387	86.56		N

Table 5: Case 2 Results. Detected pathogenic, disease associated, variant of unknown significance (VUS), and likely benign exonic variants above the reporting criteria of 5% allele frequency are listed. Please click here to download this table.

Supplemental Figure 1: An example of an amplicon-based SampleSheet.csv. This sheet conveys to the sequencer what chemistry to run (in this case Amplicon), what workflow (*e.g.,* GenerateFastq), what Application and Assay (*e.g.,* FastqOnly), how many bases (or reads) to sequence (in this case 186 bp x 186 bp), and lastly what samples are associated with certain indexes (in this case dual indexing). The parts that are highlighted in yellow can be changed to whatever the experimenter wants, but in this case the lab uses these parameters. Please click here to download this figure.

Discussion

As the two NGS tests described in this manuscript are offered clinically, the most important practical consideration is quality control. Specifically, close consideration must be paid to the quality and quantity of extracted DNA. This is especially important for FFPE samples which are often highly degraded with variable DNA yield. An isopropanol precipitation method was developed in order to maximize DNA yield from FFPE samples as column-based methods were found to sometimes lead to DNA shearing with limited elution volumes. Therefore, most of the time when a specimen yields too low concentration or is too degraded for the assay, it is most likely due to the tissue size, type, or fixation and not the extraction process. For blood/bone marrow specimens, if there is an extraction failure, it is usually due to a sample being hemodilute (*i.e.*, not having sufficient number of white blood or tumor cells in that draw) or chemo ablated.

During validation, cutoffs for acceptability of DNA quality and quantity should be established. The recommended input of 100 - 250 ng is often used in the assay; however, if the DNA quality is good, then lower input amounts can be successful. In addition, if the DNA quality is poor (*i.e.*, the amount of amplifiable DNA is less than 100 - 250 ng) then higher input amounts can improve the quality of the sequencing results (since the amount of amplifiable DNA will reach the recommended input). Metrics for DNA quality and quantity should be applied to each sample before advancing the DNA into library preparation. Those samples in a "gray zone" (see **Figure 2**) should be run at the discretion of the laboratory director or designee. Currently the best way to predict if the DNA will not perform well during sequencing is to perform a qPCR-based assay that allows for the quantification and quality assessment of input DNA. This approach addresses the bioavailability of different sized fragments in the specimen, through the amplification of different sized fragments (*e.g.,* 100 bp, 150 bp, 200 bp and 300 bp) and comparison yield.

Currently, library preparation involves a large number of manual steps where a misstep at one of several junctures can cause the library to either fail or to be of poor quality. The microfluidic gel analysis is the only QC step to check a library prep issue before sequencing. Accordingly, there are several critical steps where extra mindfulness can increase the probability of a successful reaction. It is imperative to guarantee the correct sample and oligonucleotide pool are used for each specimen. Ensuring and properly recording that each sample contains one of 96 unique combinations of dual-indexed PCR primer pairs reduces chance for a sample mix up. Also, it is important to ensure the filter plate (FPU) drains correctly; if it does not drain appropriately this can cause the extension-ligation step of the library preparation to perform suboptimally and lead to poor quality sequencing data. After library QC, it is critical to ensure that the LNB1 beads are fully resuspended and that the LNB1/LNA1 solution is well mixed before adding it to the samples as the concentration of this mixture is used to determine the molarity of the library. Finally, if the bead elution step leads to a suboptimal amount of library eluting off the beads it will decrease clustering density and possibly cause the library to not obtain adequate mean coverage. Conversely, an excess of library will lead to poorer quality reads. Therefore, it is important to be consistent at the bead-based normalization step to ensure optimal pooling and clustering of the libraries on the sequencer.

In addition to library preparation, it is critical to validate a bioinformatics pipeline that will produce accurate mutation calls from the raw, de-multiplexed fastq files. Choosing a custom solution may be time-consuming as there are many open source and commercially-available aligners, variant callers, and NGS software packages that one would have to sift through. Custom algorithms will need to be designed to extract essential performance statistics, identify unique recurring mutations that eluded most open source tools, and determine copy number status over each of the loci. During the validation process of a bioinformatics pipeline, it is important to determine the reportable cutoffs for variants that meet or exceed both a minimum depth of coverage after quality filtering (*e.g.*, a minimum of 250 reads) and a minimum allele frequency (*e.g.*, 4%). Since this a multiplexed amplicon-based assay, it is important to determine the minimum mean depth of coverage (*e.g.*, 1,000x) that the library needs to achieve to be able to get the lowest performing amplicon to the minimum depth of reads. In addition, the multiplexed nature of the assay does cause off target effects and these 'artifacts' will need to be discovered and fully vetted before launch. Another important limitation to the described assay is a need for samples to contain greater than 10% tumor in order to achieve the validated minimum allele frequency.

The detection of low frequency, 1%, *FLT3* insertions is evidence that manual review is still desirable in this process. Even with an allele frequency cut-off of 5%, some important mutations maybe missed and thus manual review will be essential to ascertain these variants. For *FLT3-ITDs*, visual inspection of exon 14 is performed for all AML patients to ensure a low level or large insertion/duplication does not go unnoticed. In addition, *HER2* exon 20 insertions that are commonly next to the primer sequence, need manual intervention. Despite having a robust bioinformatics pipeline, some variants could go overlooked which is just the nature of having a hard cutoff for most the stats mentioned above. Better bioinformatics would be needed to help alleviate this issue, as will better library preparation and/or sequencing methodologies, because it is more advantageous to have good quality data at even lower cutoffs that contain fewer artifacts and false positives.

The detection and interpretation of allele frequencies can be difficult due to the difficulty in tumor percentage determination and amplification bias of some regions of the genome. In addition, allele frequencies over 50% can be detected, as observed in case 2. This is interpreted as a loss of heterozygosity (LOH) event, either due to loss of the normal allele, leading to the apparent increase of mutant reads, a gain of the mutant allele (*e.g.*, 2 mutant and one normal copy) or other mechanisms. These mechanisms can be elucidated by utilizing array comparative genomic hybridization (aCGH[19]) and/or a SNP genotyping array.[20]

The current target enrichment methodologies rely on full-day procedures of either inefficient hybrid capture or multiplexed PCR techniques that result in the need for more sequencing coverage of a single sample and more off target sequencing reads. Additional applications for NGS molecular oncology expected in the near future will include easier library preparation methods that can be fully automatable and be able to process samples with very low amounts of input DNA (*i.e.*, less than 1 ng) as well as samples with highly degraded DNA. To address these challenges, most methods will presumably be PCR based, either being a multistep PCR approach or a massively-parallel singleplex PCR approach. In addition, molecular barcoding of individual amplicons has been shown to dramatically reduce background sequencing noise and will enable testing of samples with lower proportions of tumor cells to achieve lower allele frequencies and move towards capturing circulating tumor cells.

Detection of disease-associated mutations in cancer specimens has been standard of care for decades. Historically, genes were often tested sequentially, one gene/exon at a time, with the identification of a mutation leading to the end of the testing sequence. The advent of NGS has allowed for a less biased approach to sequencing multiple genes associated with many cancers in parallel leading to the identification of multiple mutations that are associated with neoplasia. The clinical utility of NGS for the detection of somatic mutations in cancer is increasingly evident. Indeed, NGS-based analysis of tumor samples represents a new paradigm that challenges traditional, single gene testing, but the clinical utility is very clear. Clinical laboratories today have the exciting opportunity to wed careful method validation and test interpretation with the application of this powerful technology.

Disclosures

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