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ACMG STATEMENT

Points to consider for the next-generation-sequencing-based detection of copy-number abnormalities (CNAs) and balanced chromosomal rearrangements in neoplastic disorders: A statement of the American College of Medical Genetics and Genomics (ACMG)

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Introduction

Neoplastic disorders are biologically driven by large-scale chromosomal abnormalities and/or small sequence variants, including single-nucleotide variants (SNVs) and small insertion-deletions (indels). Historically, clinical genomics laboratories have utilized multiple individual assays, including G-banded analysis, fluorescence in situ hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA), and chromosomal microarray analysis (CMA) for the detection of copy-number abnormalities

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(CNAs) and balanced rearrangements. Additionally, Sanger sequencing was historically used to detect SNVs/Indels in neoplastic disorders. Although identification of CNAs has historically relied upon cytogenetic or targeted approaches, clinical practice is evolving to increasingly use next-generation sequencing (NGS) technologies, which allow for more comprehensive and genome-wide analysis to inform clinical decision making.

NGS has become a routine diagnostic tool in clinical laboratories for the identification of SNVs/Indels, evolving from evaluation of single genes to gene panels followed by exome sequencing (ES) and genome sequencing (GS). DNA sequencing approaches may complement G-banded analysis and microarray analyses for the detection of CNAs and balanced structural variants (bSVs). There has been a proliferation of technologies, target regions, and bioinformatic approaches to infer and quantify CNAs, bSVs, and SNVs/Indels from genomic sequencing data. Common approaches include shallow GS, inference from targeted gene panels, and targeted capture of rearrangement hotspots and immune cell receptor loci. This has led to substantial challenges in comparing results across different sequencing tests, particularly when attempting to quantify CNAs or to resolve bSVs to precise genomic breakpoints. The latter issue is further heightened by the emergence of RNA sequencing as a highly effective strategy to detect fusion genes but not retaining DNA breakpoint information. The detection of CNAs and bSVs from NGS has been recently incorporated into the validation of gene panels, ES, and GS, but these variant types are more challenging to identify than SNVs/Indels. Because the goal of any clinical test is to provide accurate and high-quality information, it is imperative to understand what assay was performed, what type of data can be detected, and what are the limitations of the assay.

Detection of constitutional CNAs by NGS is increasingly used in clinical diagnostic testing accounting for 3% to 5% of positive diagnoses.[1] However, detection of larger rearrangements such as CNAs and bSVs has been challenging because of limitations including short-read lengths (150-300 bp) and guanine-cytosine (GC) bias. Some of these limitations can be overcome by software methods[2] or specialized library preparation methods to favor longer-insert reads or long-read sequencing (LRS) (>10 kb), such as Pacific Biosciences (PacBio) and Oxford Nanopore Technologies, although these assays are not used routinely in clinical laboratories. Given the time-sensitive nature for reporting results from such tests, the need to use a single methodology to detect all clinically significant genomic variants has become of paramount importance. With the increasing use of NGS and advanced bioinformatics tools, it is now possible to detect SNVs/Indels, CNAs, and bSVs from a single assay. However, the detection of CNAs and bSVs and their accurate interpretation in the context of neoplastic diseases require several considerations that will be discussed.

Here, we will discuss the bioinformatics tools needed to call CNAs and bSVs and review the differences in the algorithms used to call these aberrations. We will also note the nuances in the definitions of gains vs amplifications detected in NGS data and briefly touch upon other emerging technologies to detect these genomic abnormalities (eg, optical genome mapping [OGM] and LRS).

Types of variants reported by NGS assays

CNAs

NGS-based sequencing assays can detect many types of CNAs, including gains, amplifications, and losses (Table 1). Additionally, copy-neutral loss of heterozygosity (CN-LOH) and complex CNAs, such as chromothripsis, can also be detected. The sensitivity of the detection is highly dependent on the depth and breadth of coverage. The size, location, and types of CNAs have been previously described in a joint consensus recommendation from the American College of Medical Genetics and Genomics (ACMG) and the Cancer Genomics Consortium (CGC).[3] For neoplastic tissues, one must consider potential subclonal aberrations, tumor content within the received specimen, and altered ploidy states when determining the appropriate terminology for reporting (Table 1). Additionally, although single-cell sequencing is available in the research setting,[4] this methodology remains limited in the clinical setting. Therefore, it is important to remember that NGS-based assays are performed using bulk DNA extraction and may represent an aggregate of different copy states observed in multiple subclones present in varying quantities. Therefore, previously established normal cutoffs using a different assay, such as FISH, which can assess aberrations at the single-cell level, may need to be reevaluated for NGS-based assays. During the clinical validation, each laboratory must establish its own criteria for calling various copy-number states, particularly thresholds for amplification.

Copy-neutral loss of heterozygosity

CN-LOH is defined as an allelic imbalance without an associated copy-number change resulting from duplication of an allele with concurrent deletion of the other allele.[5] In neoplastic tissues, CN-LOH is critical because it may serve as a second hit to a driver alteration observed in either the germline or somatic tissues.[6-9] In many cases, the presence of CN-LOH may result in biallelic inactivation of a tumor suppressor gene which drives tumorigenesis. Validation of an NGS-based assay for neoplastic tissues should reliably identify CN-LOH. In addition to providing important clinical information, these data may also aid in interpretation by resolving the presence of altered ploidy states or providing evidence that subclonal events may be present. Similar to that with CNAs, the degree of LOH may be affected by other factors, such as diminished tumor content. Depth of

Table 1 Recommendations for CNA definitions

Type of CNA	Definition	Recommendation
Gain	Gain of genomic material relative to the baseline ploidy state	Use "gain" when describing an increase in genomic material less than the cutoff for amplification. Use "relative gain" when describing an increase in genomic material relative to an altered baseline ploidy.
Amplification	High copy gain of genomic material above the baseline ploidy	Use "amplification" to describe high-level gains. Each laboratory should define cutoffs for reporting amplification which may be tumor type dependent. Recommend values ~5-fold increase over the baseline ploidy.
Loss	Loss of genomic material relative to the baseline ploidy state	Use "loss" when describing a single copy loss of genomic material. Use "biallelic loss" when describing a 2-copy loss of genomic material relative to a diploid profile. Use "relative loss" when describing a loss in genomic material relative to an altered baseline ploidy.
Copy Neutral Loss of Heterozygosity	Allelic imbalance without an associated copy-number change	
Chromothripsis	Alternating copy states across a region or chromosome	Use "chromothripsis" when there are at least 10 alternating copy states across a region.

coverage and extent of regions covered is especially important in detection of CN-LOH. For germline studies, optimal results for GS were observed at 30x depth or higher.[1] Additional studies are needed to determine the recommended minimum depth of coverage for GS of tumor samples.[10-15] Lower depth and breadth of genome-wide coverage results in reduced sensitivity, making it more challenging to detect subchromosomal events.

Homologous recombination deficiency (HRD) markers

Large genomic events are key biomarkers used to assess HRD in cancer, which can indicate the level of genomic instability within a tumor and potentially predict sensitivity to PARP inhibitors. Currently, 2 FDA-approved HRD assays—MyChoice CDx and FoundationOne CDx—serve as benchmarks for validating other laboratory developed HRD tests.[16,17] FoundationOne CDx detects HRD from NGS data by evaluating the level of LOH without considering other genomic instability markers.[18,19] MyChoice CDx calculates HRD using a combination of LOH, telomeric allelic imbalance, and large-scale state transitions.[20] In addition, different measurements of LOH have been used in different HRD assays. MyChoice CDx uses a single-nucleotide polymorphism array to define and count the number of LOH regions of >15 Mb but < whole chromosome.[21] FoundationOne CDx measures the percentage of focal LOH in the tumor genome.[16,19] Currently, there is no consensus on how to detect and quantify HRD and no standardized method for measuring LOH levels.[22] Different assay platforms and measurements of HRD may lead to discordant results. Therefore, when validating an in-house HRD test, laboratories should select a ground truth data set that most closely resembles the characteristics of the in-house test and establish laboratory-specific thresholds for interpretation of positive and negative results.

Balanced structural variation

G-banded analysis remains the gold standard for detection of bSVs. Given the low resolution (10 Mb), particularly in the setting of oncology chromosome preparations, karyotyping does not enable identification of the disrupted genes. Utilization of FISH allows for a targeted analysis of gene rearrangements; however, this testing depends on the probe design and may not provide information on the gene fusion partner, nor does it provide resolution to determine which protein domains are lost or retained. Gene panels and ES are only capable of detecting unbalanced events in which gain or loss of genomic material is present but have limited value if the breakpoints are in intronic sequences due to limited coverage. However, balanced and unbalanced SVs may be detected using appropriate bioinformatic tools for GS. In some cases, an apparently balanced rearrangement may not be fully balanced, and small regions of focal gain or loss may be detectable within a critical gene by NGS data.

Patterns of CNAs

In the setting of neoplastic tissues, reporting patterns of CNAs is critical in clinical interpretation. For example, codeletion of 1p and 19q is pathognomonic for oligodendroglioma and therefore, considered an essential diagnostic criterion for this entity within the World Health Organization (WHO) Classification of Tumors of the Central Nervous System.[23] Literature has also demonstrated that a

whole chromosome gain of 7 and a whole chromosome loss of 8 and 11 are associated with a favorable outcome in standard-risk group 4 medulloblastoma,[24,25] whereas in neuroblastoma, the presence of whole chromosome gains confers favorable outcomes when compared with the presence of segmental aberrations, which are associated with a poorer prognosis.[26,27] For multiple myeloma, the International Myeloma Working Group acknowledges the presence of hyperdiploidy with trisomies of odd numbered chromosomes being associated with a more favorable prognosis.[28] Comprehensive, genome-wide evaluation of CNAs and LOH is recommended to allow for the interrogation of diagnostic and prognostic implications in the tumor specimen under study.

Bioinformatic algorithms for detecting CNA from NGS assays

Methods for the detection of CNAs and bSVs from NGS data are described in detail in other research and review articles[29-34] and herein are only briefly summarized. After the initial step of "reading" the raw sequence data, the secondary analysis begins with alignment of these data in the FASTQ format to a reference genome using BWA-MEM.[35] The aligned files are stored as a binary alignment map or a compressed version (CRAM) file. Redundant reads or PCR duplicates can be identified and removed using Picard[36] and Sambamba[37] so that they can be eliminated from downstream analysis. Using the Genome Analysis Toolkit (GATK) Best Practices workflow (https://gatk.broadinstitute.org/hc/en-us/sections/360007226651-Best-Practices-Workflows) allows additional steps to improve data quality before variant calling. Numerous bioinformatics algorithms have been developed to identify CNAs. These can be divided into 2 broad categories. First are "read-depth" methods in which the deviation from the expected number of reads across a region can indicate changes in copy number (gain and losses). These methods do not look at the actual sequence but just the number of reads. The second class of methods analyzes the sequence and can be further divided into 2 groups: pair-end mapping and split-read analysis. These methods have the advantage of theoretically being able to also detect bSVs in addition to copy-number variations (CNVs). However, their effectiveness for application in neoplastic samples is limited due to heterogeneity in the sampled cells. Examples of some common bioinformatic pipelines for calling CNAs from NGS data include the following: CNVKit,[38] ExomeCNV,[39] ExomeDepth,[40] GATK-CNV (https://gatk.broadinstitute.org/), VarScan2,[41] and XHMM.[42] Algorithms for calling bSVs rely on pair-end and/or split-read analysis and include DELLY,[43] Lumpy,[44] Manta,[45] and Pindel.[46] Both CNA and bSV detection can be challenging, which may be due, in part, to the location of the CNAs or bSVs because they preferentially occur in regions with repetitive sequences, which may be difficult to sequence. Furthermore, short-read fragments may not be sufficient to resolve complex variants. The use of long-read sequencing may overcome some of these limitations, including reduction of alignment ambiguity and reduced GC bias.

Interpretation challenges

In addition to variability in primary data generation and secondary CNA and bSV calling, tertiary interpretation of these variants is not as mature as for subexonic SNVs/Indels. Although somatic cancer-associated SNVs/Indels are currently well supported by clinical-grade databases and knowledgebases, such as ClinVar,[47,48] OncoKB,[49,50] and CIViC,[51] that incorporate thousands of disease-specific biological and clinical interpretations, CNAs and bSVs lag both in the number and depth of annotated entities. Furthermore, cancer subtypes are often defined in clinical and laboratory guidelines by patterns of CNAs and bSVs but are not readily searchable through computational variant annotation systems. Although disease and domain experts who manually review CNA and bSV profiles are familiar with these co-occurring structural variants, there is a lack of representation standards and annotations for coupled CNAs and bSVs that could enhance the clinical interpretation and reporting of these patterns across genetic laboratories. This is particularly true as comprehensive assays have further enabled pan-cancer genome testing. Recent machine learning approaches have demonstrated the power of integrated clinical and genomic annotation,[52] further highlighting the need for highly structured CNA and bSV databases, ideally backed by a continually growing resource of clinical genome profiles and interpretations such as those amassed by AACR GENIE.[53] Given the high level of genomic and technical expertise of existing teams building and maintaining SNV interpretation databases, we recommend incorporating somatic CNA and bSV interpretation and CNA pattern definitions into these systems rather than deploying new stand-alone databases.

Relevant testing methodologies

Gene panels

Gene panels were developed as a cost-effective assay to interrogate specific genes of interest to the tumor type or cancer predisposition. Targeted capture and enrichment of genes of interest are sequenced with a very high depth of coverage (~500-1000x). Typically, these panels are designed to target the coding region of genes of interest, from a few to hundreds of genes. One challenge for calling CNAs from gene panels is the identification of the most appropriate bioinformatic algorithm. Although numerous CNA detection tools are available for NGS data, many of these are designed for the more comprehensive data generated by ES and GS.[33,54] Detection of CNAs from gene panels typically utilizes algorithms that rely on changes in read depth, which are proportional to the number of copies

of that specific chromosome segment.[31] The high depth of coverage for gene panels may allow for higher resolution and increased sensitivity for identifying smaller, exon-level deletions and duplications in genes of interest; however, it has reduced sensitivity for calling larger CNAs, particularly compared with ES or GS.[54-56] Importantly, gene panels using a read-depth-based algorithm for CNA calling are limited in their ability to derive LOH information.

ES

Similar to gene panels, ES utilizes a targeted capture approach but sequences the coding region of the genome (~2% of the total genomic sequence) at a relatively high depth of coverage (typically >100x). Although ES has increased coverage across the genome relative to gene panels, which improves the calling of large CNAs, it still has similar challenges as targeted gene panels given the lack of coverage across noncoding regions. The nonuniform distribution of probe coverage may affect CNA detection depending on the bioinformatic algorithm. For example, read-depth-based CNA callers may be influenced by biases introduced by repetitive sequence, GC-rich content, exome capture, and PCR amplification.[57] To improve CNA calling in ES, a copy-number variant backbone spike-in may be used for which oligonucleotide probes are tiled across the human reference genome. Depending on the patient population the laboratory serves, custom copy-number variant backbones could provide additional probe coverage across genes of interest. With either gene panel or ES, capture probes targeting regions with known single-nucleotide polymorphisms having high variant allele frequency in the population can be favored to build the genome-wide backbone. This will improve the ability to detect CNAs, CN-LOH, and LOH events. ES has a high sensitivity in reporting larger CNAs (full gene to whole chromosome events), whereas the ability to detect smaller intergenic and intragenic events may rely on the bioinformatic pipeline used for analysis.[29,58] That said, given the reduced probe coverage across noncoding regions, breakpoints must be estimated in these regions.

GS

GS is currently the most comprehensive NGS test which can detect CNAs, bSVs, and SNVs/Indels in the clinical laboratory. Although the depth of coverage is much lower than targeted gene panels and ES, the relatively uniform distribution of probes across both coding and noncoding regions of the genome improves sensitivity, accuracy, and resolution of CNA detection, with a sensitivity demonstrated to be similar to CMA in the constitutional setting.[59] Nonetheless, the lower depth of coverage poses limitations in identifying subclonal variation and resolving copy-number states when the sample is estimated to contain diminished tumor content.[60] Similar to ES, GS has limited resolution to accurately identify CNA breakpoints in regions of repetitive genomic sequence and GC-rich content. Identification of the most appropriate bioinformatic tool is critical as algorithms may function differently between GS, ES, and gene panels.[33]

Emerging methodologies

LRS

LRS technologies, such as PacBio single-molecule real-time sequencing and Oxford Nanopore Technologies nanopore sequencing, generate long insert sizes of 10-200 kb, depending on the chemistry.[61] As previously discussed, short-read sequencing technologies are subject to biases, which may influence the calling of CNAs and other structural variation. In contrast, LRS generates reads that span problematic regions of genomic sequence that challenge short-read sequencing technologies, such as repetitive and GC-rich sequences.[61-63] Bioinformatic approaches to CNA detection tend to be developed specifically for the underlying technology, and interpretation may be challenging. Despite promising data suggesting potential utility of LRS in the oncology setting,[64] the high error rate (8%-20%), reduced performance with degraded formalin-fixed paraffin-embedded (FFPE) samples, and cost limit the use of this technology in the clinical setting.[61,62]

OGM

Although OGM is not a sequencing assay, it is slowly finding a home in the clinical laboratory setting for detection of genome-wide numerical and structural rearrangements. OGM requires ultrahigh molecular weight DNA, which, through an enzymatic reaction, fluorescently labels specific sequence motifs in the genome. These labeled long DNA molecules are linearized in nanochannels and imaged. The color patterns are compared with a reference contig to identify the presence of different types of SVs, including balanced and unbalanced CNAs, gene fusions, and other complex rearrangements. Previous studies have shown high sensitivity, specificity, and resolution in both the germline and somatic settings.[65-69] Currently, OGM has demonstrated utility in the setting of hematologic malignancy, whereas adoption to solid tumors has been slow because of the need to isolate ultrahigh molecular weight DNA and the inability to utilize FFPE tissues.[70-73] Clinical implementation of OGM is expanding the toolbox for identification of CNAs and SVs in hematologic malignancies.[74]

Points to consider for laboratories—Validation

- CNAs and bSVs may be called from various NGS-based methodologies, including gene panels, ES, and GS (Table 2). These assays may involve sequencing of only a tumor specimen or use a paired sequencing approach of both the tumor specimen and representative germline sample. Although a paired analysis will allow the laboratory to determine the etiology of called variants as either germline or somatic in nature, a tumor-only analysis will only infer etiology and

requires additional orthogonal testing to confirm if a variant is observed in the germline. Points to consider and recommendations for reporting germline variation in the setting of a tumor-only analysis have been previously published.[75-78]

- The advantages and limitations of each assay and bioinformatic pipeline utilized for CNA and bSV calling should be well understood before validation to ensure that the assay is optimal for the patient population (ie, pediatric/adolescent young adults and/or adult) served by the laboratory. Clinical validation needs to consider both the wet lab and dry lab (ie, bioinformatics) processes.
- Clinical validation of LDTs or modified FDA-cleared/approved tests is typically performed using guidelines set forth by the College of American Pathologists (CAP) Molecular Pathology checklist (https://www.cap.org/laboratory-improvement/accreditation/accreditation-checklists). Laboratories are therefore required to determine analytical performance of the assay, including accuracy, precision/reproducibility, reportable range, reference intervals, and analytical sensitivity (lower limit of detection) (https://www.wadsworth.org/regulatory/clep/clinical-labs/obtain-permit/test-approval). Additionally, clinical validity of the assay should be evaluated as part of the validation processes, including clinical sensitivity, clinical specificity, positive and negative predictive values, and clinical utility.
- LDT approval by the New York State Department of Health Clinical Laboratory Evaluation Program may also be necessary (https://www.wadsworth.org/regulatory/clep/clinical-labs/obtain-permit/test-approval). These guidelines expand on the CAP requirements and necessitate validation of additional parameters for full approval.
- Validation of CNAs should include all expected variant types (eg, gains of various copy states, including amplifications, heterozygous and homozygous loss, and CN-LOH) and sizes (eg, whole chromosome/arm, segmental, and focal), which will be reported by the assay. Any methodology to detect CNA/bSV that is already validated in the laboratory or interlaboratory comparisons can be used. The orthogonal testing does not have to provide a whole-genome review. Using samples with multiple previously detected CNAs identified by clinically validated MLPA or chromosomal microarray of various types and sizes may be a cost-effective way to fulfill the number of required samples for validation.
- Validation of CNAs should be performed using the minimum input DNA for the NGS assay.
- Given the expected differences in performance metrics, all tissue preservation methods/matrices to be tested (ie, snap frozen, FFPE, fine needle aspirates, fresh blood or bone marrow, decalcified bone, etc) will require validation.
- If validating a paired tumor and normal assay, consideration for use of the germline comparator and/or a panel of normals as part of CNA calling should be considered. This may be dictated by the bioinformatic pipeline used for CNA calling.
- If using a panel of normals for data analysis, ~50 normal, sex-matched samples representing different specimen types are recommended (with 10 as the minimum). The panel of normals should represent the sample types undergoing testing. Patient ancestry should also be considered.[79,80] Importantly, if the test includes the sex chromosomes, male samples are necessary (to get values for Y). The normal samples should be as similar as possible to the cases (ie, similar specimen processing).
- An important consideration for CNA reporting from neoplastic tissue is the cutoff of tumor content estimation (often by pathology review of an H&E slide). Because NGS assays represent bulk sequencing, the copy-number profile will reflect CNAs called in both the neoplastic tissue and intermixed nonneoplastic tissue. A sample with an estimated low tumor content will have aberrantly diminished copy-number calls diluted by the presence of a predominately diploid copy state of the nonneoplastic tissue. During clinical validation, it is important to perform dilution studies to determine the performance of the CNA caller at diminished tumor content. It is critical that different types of CNAs (focal to whole chromosome events) are evaluated as each type of CNA may perform differently as the tumor content diminishes, typically the smaller the event, the more challenging it is to detect it at a low percentage without introducing a high degree of false-positive calls. The cutoff for CNA calling by NGS should be determined based on the accuracy of these dilution studies for all copy-number events. Of note, an estimated tumor content is also established for SNV calling and the tumor content cutoffs may differ between the 2 variant types.
- For targeted gene panel testing, the laboratory should validate the performance to detect abnormalities (eg, CNAs, bSVs, and SNVs/Indels) that are being reported. Recommend following guidelines for validation of targeted NGS testing, which have been described in a joint consensus of AMP/CAP.[81]
- During validation, the laboratory should establish the resolution or the smallest size of reportable CNAs (lower limit of detection) specific for the assay. This metric may vary between assays and labs. One could consider a size threshold, number of bins, number of probes, or different measurable metrics derived from the assay. Resolution of the assay should be provided within the report.
- Determining the optimal orthogonal testing to determine concordance remains a challenge when validating NGS assays as there is not a single comparable gold standard assay to use for concordance. The

assay(s) used for orthogonal confirmation should also be clinically validated by the individual laboratory. Microarray-based assays allow for genome-wide copy-number calling but differences in probe coverage or density may result in discrepancies. MLPA, FISH, or qPCR techniques for CNAs may also be considered. Orthogonal confirmation of CNA events should be determined by the individual laboratory, including the appropriate methodologies for each type and size of CNA identified.

Points to consider for laboratories—Preanalytical/quality control

- Many factors can influence the data quality generated by NGS assays, including DNA quality and average depth and breadth of coverage. Fragmented or degraded DNA may result in smaller than expected insert sizes and fewer aligned and on-target reads. CNA callers look for changes in the depth of coverage across a region to accurately call regions of copy-number change. Reduced depth and/or breadth across regions of interest may impact the accuracy in CNA calling.
- Each laboratory needs to establish both run-level and sample-level quality control (QC) metrics for CNA calling from NGS for either panel, ES, or GS. It is recommended that both positive and negative (also known as a no template control [NTC]) controls are included for each library preparation that undergoes sequencing. It is recommended by some regulatory bodies to sequence both positive and negative controls. The negative control or NTC confirms the absence of reportable events and allows for monitoring if contamination has occurred during library preparation or sequence. A positive control should include multiple types and sizes of CNAs for which the assay is validated to report. Given the complexity of NGS testing, rotating various positive controls that evaluate different types of CNAs may be warranted. The CAP NGS Work Group has established recommendations for acceptance and rejection criteria for NGS assays.[82]
- Commercial QC material for somatic CNAs that includes a breadth of CNA types and sizes would be optimal as a positive control. However, there is sparse availability of reference materials with somatic CNAs that cover a wide breadth of variant types. Rotation of multiple controls may overcome this challenge. For example, SeraCare offers the Seraseq Solid Tumor CNV Mix with the option to include 3, 6, or 12 copies of 12 genes. The Horizon Structural Multiplex Reference Standard also includes amplification of *MET* and *MYCN*. However, neither of these reference materials includes regions of loss, thus limiting their utility as the sole positive control. The Coriell Institute for Medical Research also has reference materials from many different types of cell lines from patients which could be queried for potential use as QC. The laboratory should determine, during assay validation, the thresholds for passing or failing a control sample. One could consider using a standard deviation cutoff or presence or absence of an event at the expected copy-number state.
- There are currently no standards to determine if a CNA analysis passes or fails at the sample level. For CMAs, many methods have been used depending on the software and platform used. These methods measure the amount of variance of the log ratio between neighboring probes. For example, Agilent software uses the derivative log-ratio spread (DLRS) score.[83] The DLRS value describes the variance of the absolute log2 ratio from probe to probe, averaged across the entire genome. The ThermoFisher software, ChAS, uses Median of the Absolute values of all Pairwise Differences (MAPD) score and Bionano VIA software uses Robust Variance QC measure. In all these cases, a higher score indicates a sample with increased probe-to-probe noise, which may result in challenges distinguishing real CNAs from noise. Any of these measures can be implemented for NGS in which a probe is replaced by a read-depth bin. A lab should establish its own acceptable cutoff realizing that this is not a perfect QC measure because a low score indicating small variance can be caused by other quality issues, such as poor capture.
- Depending on the assay, bioinformatic pipeline, and validated sample types (ie, snap frozen, FFPE, etc), the laboratory may consider utilizing a positive control consisting of a high-quality genomic DNA sample and an FFPE sample.
- If the laboratory is performing a paired tumor/normal assay, sample provenance and/or clinical history should be reviewed before data analysis, including the possible history of allogeneic bone marrow transplantation. The presence of admixture or contamination in a germline/tumor sample may present with genome-wide LOH. Review of the LOH B-allele frequency (BAF) plot may show 3 tracks, which should prompt further investigation by the laboratory.
- If needed, orthogonal confirmation of bSVs by reverse transcription-polymerase chain reaction may be performed if the split-reads representing the event are identified.

Points to consider for laboratories—Interpretation and reporting

- For clinically significant CNAs, the report should include at a minimum the following: genomic coordinates and genome build, cytoband, size of the event, type of CNA called (eg, gain, amplification, loss, biallelic loss, CN-LOH), classification, disease-associated gene content, interpretation of the

findings as a whole and/or important patterns observed, defined reporting criteria, and limitations of the assay.
- Genes critical to clinical interpretation should be highlighted in the report to allow for greater visibility and clarity.
- When interpreting copy-number calls, estimated tumor content must be considered because diminished tumor content may affect the amplitude of the called copy-number event and LOH patterns. For example, a biallelic loss may appear as a single copy loss in the setting of low tumor content.
- Currently, ISCN does not provide guidance for nomenclature to report complex profiles in neoplastic tissues.
- Classification of CNAs from neoplastic tissues are described by joint consensus recommendations from the ACMG and the CGC.[3] This is a 4-tiered system to categorize single copy-number events and patterns of CNAs characteristic for a tumor type. These standards also address reporting a germline CNA that predisposes to cancer.
- CNAs should be evaluated for clinical significance based on the tumor type under study and the mechanism of pathogenicity for the genes involved. The laboratory may consider the histopathological diagnosis and other comprehensive cytogenetic or molecular testing to determine the most appropriate tumor type for classification.
- In oncology, single CNA events and pattern of CNAs often provide clinically significant information to the clinician. Coverage across potential regions of interest is important to highlight during interpretation because targeted assays, for example, may not provide coverage to identify all regions of interest. If a region is not covered by an assay, this should be disclaimed in the report because additional testing may be warranted.
- For some tumor types, historical knowledge is based on cytogenetics, which may be challenging for those trained predominately in molecular techniques. Clinical-grade databases and knowledgebases, such as ClinVar (https://www.ncbi.nlm.nih.gov/clinvar),[47,48] cBioPortal (https://www.cbioportal.org/),[84,85] and COSMIC (https://cancer.sanger.ac.uk/cosmic)[86] for SNVs and small indels are widely available. However, there are no computable databases for cancer-specific genomic patterns.
- Interpretation should utilize resources that have been stringently curated, such as the WHO Blue Books (https://tumourclassification.iarc.who.int), professional guidelines, such as the National Comprehensive Cancer Network (https://www.nccn.org/), clinical trial reports, and literature.

Points to consider for laboratories—Disclaimers

- In addition to describing the methodology of the assay and meaningful observed genomic variation, the clinical report should also describe potential pre-analytical, analytical, or post analytical factors that may influence the interpretation of the results. A report should consider listing gene(s) with low depth of coverage or probe coverage for which a negative result would not be conclusive, and orthogonal testing may be recommended if this region is critical for that tumor type.
- A limitation of CNA calling from NGS-based assays is the challenge in appropriately identifying altered ploidy in the setting of a balanced copy state (ie, tetraploidy with chromosomes demonstrating a balanced 4-copy state). Although some analysis tools may display both a BAF and variant allele frequency plots which aid in determining copy state, NGS-based CNA callers only present LOH calls using the BAF. In the setting of a balanced tetraploid tumor, the copy-number data may appear to be centered correctly at both the diploid and tetraploid baselines; thus, the laboratory is unable to accurately determine the appropriate ploidy without the use of additional orthogonal testing, such as FISH. It is recommended to report these tumors with a disclaimer to this effect and mention that the copy-number state is inferred based on the global genomic profile. Follow-up orthogonal testing may be recommended if accurate copy-number determination is necessary for clinical management.
- Tumor samples harboring multiple clones or subclones can also pose a challenge with accurate calling of CNA. Because NGS does not evaluate samples at a single-cell level, all clones are taken into consideration when determining the presence of a CNA. This may result in a copy-number profile with CNAs that does not match an expected pattern of copy-number amplitude and LOH, thus rendering accurate copy-number determination challenging.

Points to consider for laboratories—Proficiency testing

- According to CAP, laboratories should enroll and participate in biannual proficiency testing for each assay offered on the test menu. If a commercial proficiency test is not available for the assay, the laboratory may enroll in an alternative assessment, such as joint proficiency testing with a peer institution.
- The CNVST challenge provided by CAP is designed for NGS assays reporting CNAs in solid tumors. A comparable challenge for hematologic malignancies is not yet available.

Table 2 Advantages and limitations of various cytogenetic methodologies in somatic testing

	G-banded Analysis	FISH	CMA	OGM	GS
Analyte	Chromosome in dividing cells	DNA (in interphase nuclei and metaphase)	DNA	DNA	DNA
Genome coverage	Whole	Targeted	Whole	Whole	Whole
Distinction of individual cell clones	Yes	Yes	No	No	No
Analysis bias	Yes	Yes (when cultured)	No	No	No
Turnaround time	3 to 7 days	4 hours to 2 days	3 to 7 days	7 to 10 days	7 to 14 days
Unmapped region detection	Yes	Yes	No	Some Alu and LINE elements	No
Multiplexing capability	Low	Low	High	Low to Medium	High
Analytical sensitivity	1%[a]-3% (out of 20 metaphases)	1%-10%	10%-20%	5%-20%	20%-30%
bSVs	Yes	Yes	No	Yes	Yes (long- or short-read deep sequencing)
CNAs	Yes	Yes	Yes	Yes	Yes
SNVs	No	No	No	No	Yes
Disease status	Diagnosis Relapse Disease monitoring	Diagnosis Relapse Disease monitoring	Diagnosis Relapse	Diagnosis Relapse	Diagnosis Relapse
Well established	Very	Very	Very	Slightly	Slightly
Cost	$$	$$	$$$	$$$	$$$$$

[a]Depending on the clinical context, the presence of a recurring abnormality in a single metaphase may be considered evidence of an abnormal clone.

- If an assay includes reporting of both SNVs/Indels and CNAs, an alternative assessment that evaluates the wet lab and bioinformatic processes for all variant types may be considered.

Points to consider for clinicians

- The specific tumor type under study is required for interpretation of the clinical significance of specific CNAs; thus, close collaboration between the laboratory performing the testing and the clinician/pathologist responsible for reporting the integrated diagnosis is critical.
- Clinicians/pathologists should be aware of the coverage of the assay used at their site, both in general and in each specific instance because targeted assays, for example, may not provide coverage to characterize all regions of interest. Before acting on negative results, reports should be reviewed for disclaimers regarding potential regions of interest not covered by an assay because of technical and/or assay design reasons.
- Clinicians/pathologists should be aware of the challenges in appropriately identifying altered ploidy in the setting of a balanced copy state (see above) when using NGS-based assays. In the setting of a balanced tetraploid tumor, the laboratory is unable to accurately determine the appropriate ploidy without the use of additional orthogonal testing, such as FISH. Note should be made of these potential disclaimers. Follow-up orthogonal testing may be recommended if accurate copy-number determination is necessary for clinical management.
- Tumor samples harboring multiple clones or subclones pose a specific challenge with accurate calling of CNAs because clonal alterations are averaged in bulk NGS-based assays. Because many prognostic CNA alterations (eg, *MYC* amplification) were originally determined based on FISH, if subclonal, they may be missed or less accurately assessed with NGS-based assays.

Summary

In neoplastic tissues, the accurate detection of CNAs, bSVs, and SNVs/Indels are critical for clinical interpretation and patient management. NGS technologies are becoming more routinely used as a diagnostic tool for molecular characterization of neoplastic tissues. Thus, it is important to understand the specifics of the assay regarding bioinformatic processing, reportable variant types, and interpretation, as well as variables that may affect these data, including the type of methodology used, bioinformatic caller, tumor heterogeneity, clonal aberrations, tumor content within the tested specimen, and others.

Conflict of Interest

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