



SPECIAL ARTICLE

Recommendations for Next-Generation Sequencing Germline Variant Confirmation

A Joint Report of the Association for Molecular Pathology and National Society of Genetic Counselors

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Clinical laboratory implementation of next-generation sequencing (NGS)—based constitutional genetic testing has been rapid and widespread. In the absence of widely adopted comprehensive guidance, there remains substantial variability among laboratories in the practice of NGS. One issue of sustained discussion in the field is whether and to what extent orthogonal confirmation of genetic variants identified by NGS is necessary or helpful. The Association for Molecular Pathology Clinical Practice Committee convened the NGS Germline Variant Confirmation Working Group to assess current evidence regarding orthogonal confirmation and to establish recommendations for standardizing orthogonal confirmation practices to support quality patient care. On the basis of the results of a survey of the literature, a survey of laboratory practices, and subject expert matter consensus, eight recommendations are presented, providing a common framework for clinical laboratory professionals to develop or refine individualized laboratory policies and procedures regarding orthogonal confirmation of germline variants detected by NGS. (*J Mol Diagn* 2023, 25: 411–427; <https://doi.org/10.1016/j.jmoldx.2023.03.012>)

The introduction of next-generation sequencing (NGS) is one of the most important technological advances in the history of clinical laboratory genetics. Since its inception in the early

2000s, NGS has moved from an innovative research tool to a common and effective method for clinical molecular diagnosis.^{1,2} Compared with traditional DNA genotyping and

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The Next Generation Sequencing Germline Variant Confirmation Working Group of the Clinical Practice Committee, Association for Molecular Pathology (AMP), with organizational representation from the National Society of Genetic Counselors (K.D.F.H.). The AMP 2021 Clinical Practice Committee consisted of Jane Gibson (Chair), Fatimah Nahhas, Steven Sperber, Rashmi Goswami, Michael Kluk, Susan Hsiao, David Eberhard, Joseph Yao, Blake Buchan, Joshua Coleman, Elaine Gee, Andres Madrigal, and Jack Tung.

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sequencing methods, NGS has been demonstrated to be more flexible and scalable, and both time and cost-effective. However, NGS has limitations. Among other challenges, the accuracy of NGS variant calls can vary considerably by method, variant type and size, genomic region, and specimen type and quality.³ Appropriate concerns about delivering false-positive (FP) NGS results led to an early reliance on confirmatory assays to reduce this possibility, and confirmatory testing remains recommended by many current NGS clinical practice guidelines for germline (constitutional) testing,^{4–6} albeit with limited detail provided by those recommendations.

However, confirmatory testing adds time, complexity, and cost to clinical laboratory workflows. As it became recognized that the relative likelihood of an NGS FP result was at least somewhat systematic and predictable,^{3,7} laboratories became interested in decreasing the burden of orthogonal confirmation by focusing on those NGS-based variant calls that they believe are at risk of being FPs, while not necessarily confirming other variant calls that confidently appear to be true positives (TPs). Although the adoption of NGS technologies for clinical practice has been rapid, the development of specific guidelines related to such confirmatory testing has lagged, contributing to inconsistent policies between laboratories and raising potential patient safety concerns. In this guideline, we provide recommendations for orthogonal confirmation practices that, in concert with existing guidelines, provide additional specifics and thus may help promote standardization, transparency, and quality improvement among laboratories.

The Need for Guidance

To date, several professional organizations have addressed orthogonal confirmation in germline testing with position papers or recommended best practices. In one early effort, the US CDC convened the Nex-StoCT workgroup, which in 2012 published a set of principles and guidelines for quality assurance in NGS-based clinical testing. They recommended “confirmatory testing of all clinically actionable variants detected by NGS.”⁸ A similar recommendation that “all disease-focused and/or diagnostic testing include confirmation of the final result using a companion technology” was included in the 2013 NGS standards published by the American College of Medical Genetics (ACMG).⁹ This guideline further noted that laboratories “must have developed extensive experience with NGS technology … before deciding that result confirmation with orthogonal technology can be eliminated.” The guideline provided no further guidance on what would constitute extensive experience or what data would be required to validate a laboratory workflow that did not include confirmation of all reported variants.

In 2015, the College of American Pathologists published NGS standards stating that each laboratory performing NGS “must have a policy in place that clearly documents

indications for confirmatory testing and/or documents how their assay validation determined that such testing was not required.”⁴ In its 2022 version of the Molecular Pathology checklist, the College of American Pathologists states, “The laboratory performs confirmatory testing of NGS results as indicated in its policy and records correlation of the NGS confirmatory results” and notes that laboratories must determine during validation whether and when confirmatory testing is indicated. EuroGentest and the European Society of Human Genetics presented NGS guidelines in 2016, including in the Supplementary Material, “At the time being, it is advisable to confirm all reported variants to make sure that no samples swap occurred and/or to validate the informatics pipeline.”⁵ Each of these publications acknowledged that their positions regarding orthogonal confirmation of NGS variants were influenced by the relatively recent adoption of NGS technology and laboratories’ lack of robust experience with its performance. These publications anticipated that the necessity of confirmation would decrease as NGS technology and bioinformatics evolved. The ACMG updated its NGS standards in 2021, suggesting that “laboratories should establish and make available a confirmatory testing policy for each variant class, based on a workflow that identifies variants based on laboratory-specific quality metrics drawn from a large and diverse data set as well as visual inspection of read alignments. In the absence of a validated approach, laboratories should continue orthogonal confirmation.”⁶ Similarly, the 2020 ACMG technical standards for *CFTR* testing state, “Laboratories should determine … whether orthogonal confirmation is required for the reporting of certain variants and what criteria will be used to make that determination. It is recommended that *CFTR* reports based on NGS methods clearly state whether or not a reported variant was confirmed using an alternate methodology; if orthogonal confirmation is not employed, a brief description of the criteria used to make that determination in the methods section of the report is recommended.”¹⁰

Consistent with the College of American Pathologists and ACMG recommendations, a variety of individual clinical laboratories embarked on studies to determine which variant calls require orthogonal confirmation and which may not.^{11–17} These studies were encouraging, finding that NGS variant calls that meet strict quality standards are typically TPs. However, these studies were remarkably different in design, size, statistical analyses (if any), variant types considered, quality metrics used, and general approach.¹⁵ Inconsistency in laboratory practices related to orthogonal confirmation was also observed in a survey conducted by this Working Group and a subsequent, detailed literature review (see *Results*). Collectively, these findings indicate that, in the absence of widely adopted guidelines, laboratory professionals may remain unaware of which metrics can (or cannot) be used as reliable indicators of quality to responsibly reduce confirmation. There is no consensus on how to best establish and validate specific thresholds for these

metrics. Equally important, there is limited consensus on the validation requirements for confirmatory assays or the appropriate documentation to provide users of clinical genetic tests regarding confirmation. As NGS chemistry and bioinformatics pipelines continue to evolve, a long-term view of how such decisions are made and re-evaluated over time is required.

At present, many clinical laboratories have years of experience with thousands to millions of tests performed, and the literature regarding clinical NGS testing has matured. Taking into consideration these advancements, the Association for Molecular Pathology (AMP) convened a Working Group charged with generating a set of recommendations by which laboratories might efficiently develop and implement rigorous orthogonal confirmation policies, with the primary goals of helping the community align its practices, promoting transparency, and improving quality of testing.

Technical Background

Next-Generation Sequencing

In comparison with Sanger sequencing, NGS produces relatively short reads, and each of these reads can have a relatively high raw data error rate. NGS compensates for these challenges by producing numerous reads covering each targeted position. Because of the large volume of data produced and the complexities of those data, NGS relies on elaborate bioinformatics software, databases, and algorithms to identify genetic variants in each DNA specimen. As described in other AMP/College of American Pathologists guidelines,¹⁸ these bioinformatics systems also compute a variety of quality metrics that can help indicate the technical reliability of each variant call. Using these metrics, calls that are highly likely to be FPs are often filtered out. However, the remaining calls, after filtering, can still include a mixture of TP and FP results.¹⁵

Clinical Considerations of NGS Error Rates

FP errors can have substantial impact on medical care and patient outcomes.³ In some clinical circumstances, positive (whether TP or FP) genetic test reports directly influence decisions regarding prophylactic surgeries, pregnancy termination, medical interventions, or choice of therapy. FPs may also lead to misdiagnosis and premature closure of a search for the true cause of a patient's disease.

With the rapid evolution of NGS, error rates have improved considerably. At the same time, NGS has enabled increasingly broad use of genetic tests, and these new tests often analyze large sets of genes in each patient. This utilization trend presents many challenges, one of which is that the probability of a clinically significant FP can increase, even as the NGS methods improve technically.

Consider, for example, an NGS test that produces only one FP every 1,000,000 base pairs (1 Mb) tested. This low error rate is consistent with some, but not all, recent NGS performance studies.¹⁹ In protein coding regions, humans have one germline DNA variant approximately every 2 kb (this rate is higher in noncoding regions). Thus, a test producing 1 FP per Mb would have an analytic (or technical) positive predictive value (PPV) of 99.8% and an analytic FP rate of 0.0001% on a per-bp basis. This performance level may seem high but may not be adequate. For example, *BRCA1* and *BRCA2* total roughly 10 kb (10,000 bp) of protein coding sequence, and thus one analytic FP would be expected for every 100 patients tested for these two genes at this FP rate. If the test is expanded to include a gene panel spanning 200 kb, then one FP would be expected for every five patients tested. In an exome sequence spanning 30 Mb, 30 FPs would be expected in each patient's results.

The apparent clinical significance of an FP variant call is a key factor in considering the degree of concern that FPs present. Many NGS FP errors are single-nucleotide variants (SNVs),¹⁹ which, following annotation and interpretation, may or may not appear to be clinically significant. It is important to recognize that FP SNVs will usually not be filtered out by processes that use population databases to remove common polymorphisms. Insertions and deletions (indels) are less common in human DNA than SNVs, although indel errors represent a disproportionately large fraction of all NGS errors. FP indels in protein coding regions are far more likely to appear to be loss-of-function changes (eg, frameshifts) compared with FP SNVs. For these reasons, the chance of an FP variant call appearing clinically significant may be high, depending on the mechanism of disease for the condition and gene(s) in question.

To understand the impact of FPs, one should consider clinical PPV,²⁰ not just analytic PPV. Clinical PPV takes into account both variant interpretation and the patient population undergoing testing. To continue with the *BRCA1/2* example used above, assume that one analytic FP error occurs every 100 patients, and that half of these FPs may appear to be pathogenic, resulting in one potentially significant FP every 200 patients tested. In general, when *BRCA1/2* testing is applied to patients with a personal or family history strongly suggestive of hereditary breast or ovarian cancer, approximately 10% of patients in most (not all) ethnic groups are found to be truly positive.^{21,22} Thus, the clinical PPV of this example test would be roughly 95% in these patients—1 of 21 positive results would be false. In a screening context, however, where if 0.5% of patients are truly positive, the clinical PPV would be only 50%. In other words, a positive result from this example screening test is as likely to be false as it is to be true. A gene panel used in a screening context would have an even lower clinical PPV.

In summary, even if the rate of analytical FPs is low, the increasingly large numbers of genes and patients tested by NGS could lead to a substantial number of FP clinical reports.

Technical Considerations of NGS Errors

All laboratory methods are subject to error. Established techniques, such as Sanger sequencing, are often referred to as gold standards, which is appropriate only insofar as the limitations of these methods are recognized. As NGS is newer and rapidly evolving, its limitations may be less well understood, although this too is changing. Certain NGS methods may now be equally if not more accurate than Sanger sequencing for certain classes of variants,^{23–25} which prompts a valid question: Could confirmation of NGS results using Sanger sequencing reduce overall accuracy? Properly utilized, as described herein, the answer is no, because Sanger and NGS are orthogonal techniques, meaning that the errors they make are in most cases uncorrelated (see *Recommendation 4*). However, because neither method is perfect, blindly assuming one method, in this case Sanger, to be correct when the two methods disagree could increase error rates, as has been demonstrated.²⁶ Instead, if discrepancies are properly investigated and resolved, then the specificity of the combined process (NGS + Sanger confirmation) will be better than that of either method alone.

All laboratory technologies have sensitivity/specifity trade-offs. As sensitivity increases, specificity decreases (ie, FP rate increases). In clinical NGS, it can be desirable to optimize sensitivity, for example, to help detect large and complex types of indels, to detect variants in homologous or repetitive regions, or to identify variants represented by few NGS reads (eg, mosaic mutations or variants in regions that are difficult to cover for biochemical reasons), all of which can be prevalent causes of disease.^{27–29} Adopting methods and adjusting parameters to sensitively detect these variant types are also likely to increase FP rates. Having a confirmatory testing regimen in place to detect and remove any such FPs can thus help optimize not just specificity but also (indirectly) sensitivity.^{13,15,16}

Certain types of NGS variant calls are far more likely to be FPs compared with others.^{13,15,16} Laboratories may be able to establish strict criteria that separate high-confidence TP variant calls from those that are less confident and that may or may not be TPs. It is important to recognize that these strict criteria are separate from quality filters, which are used to remove variant calls that are highly likely to be FPs. The role of these distinct types of criteria is illustrated in *Figure 1*. Various studies have demonstrated that simple technical criteria, such as NGS read depth, do not alone adequately separate the high-confidence TP and candidate TP categories. Ideally, a single robust variant call quality score would do so, although the scores produced by the most commonly used variant callers (as of the time of this publication) have similarly been shown to be inadequate alone. Rather, combinations of criteria appear far more effective.^{15–17}

With any laboratory technology, including NGS, FP errors can be of two types: systematic or random. Random

errors can occur any time or any place, whereas systematic errors preferentially occur under particular circumstances (eg, within repetitive sequences). In modern NGS, systematic errors can represent a large fraction of all NGS FPs,³ which presents both an opportunity and a complication. The opportunity: As the factors that underlie systematic errors can be quantified by appropriate studies, it becomes possible to identify variant calls that are of greatest risk of being systematic FPs and to ensure that these variants are subjected to confirmation. The complication is that systematic errors, by definition, recur, meaning that observing the same or similar variants in multiple specimens using the same technique provides little confidence that the variant call is in fact real.¹⁵ To know if such variant calls are TPs, an orthogonal method is required.

Limitations of This Publication

Our recommendations are intended to apply to clinical tests that detect germline (constitutional) DNA variants using NGS. We briefly describe special considerations that may apply to certain other variant types detected by germline testing (eg, germline mosaic and mitochondrial variants, variants that require haplotype analysis to be correctly interpreted). Other clinical DNA tests are not in scope, including tumor sequencing and liquid biopsy tests, both intended to detect somatic mutations, as well as noninvasive prenatal screening, intended to detect fetal variants in maternal blood.

Our recommendations are intended to apply to short-read NGS platforms and chemistries that are commonly used in clinical laboratories as of this writing [eg, Illumina (San Diego, CA) and Ion Torrent (Thermo Fisher Scientific, Waltham, MA)]. We have not considered in detail NGS platforms that may be less common in clinical laboratories, platforms that are no longer marketed, or any of the newer single-molecule long-read platforms. Our recommendations are also not intended to apply directly to non-NGS platforms (eg, copy number or single-nucleotide polymorphism genotyping arrays) when used for initial variant detection (eg, in pharmacogenomic variant panels). Regardless, many of the concepts and approaches we describe are expected to apply to these other technologies. We note that non-NGS platforms and long-read platforms may be appropriately used as orthogonal technologies to confirm NGS-detected variants, as we describe below.

Materials and Methods

Working Group Composition, Charge, and Scope

The NGS Orthogonal Confirmation Working Group was established by the AMP Clinical Practice Committee in collaboration with the Genetics Subdivision Leadership. Members included laboratory geneticists, molecular pathologists, bioinformaticians, and genetic counselors from the United States with expertise in clinical sequencing for the

The chart illustrates the technical criteria for variant confirmation. It features three horizontal rows representing different categories of variant calls, ordered from highest quality at the top to lowest at the bottom. To the left of the rows, a vertical arrow points upwards, labeled 'Highest' at the top and 'Lowest' at the bottom, indicating increasing quality metrics. To the right of the chart, two arrows point towards the rows: a black arrow pointing to the bottom row is labeled 'Filters, lower thresholds'; a black arrow pointing to the top row is labeled 'Validated strict criteria'.

Variant call category	Recommendation
High-confidence true positives	May not require confirmation
Candidate true positives	Require confirmation
High-liability false positives	Filtered out from clinical reporting

Figure 1 Technical criteria. Traditionally, a single set of criteria (or filters) has been used to separate high-quality next-generation sequencing variant calls from those that may be analytic false positives. In the recommended framework, laboratories should use two levels of criteria: filters (**bottom arrow**) intended to remove variant calls that are likely false positives and that should not be reported; and much stricter criteria, intended to separate the highest confidence calls, likely true positives, from those of lower quality that may or may not be true. Using two (or more) levels of criteria with orthogonal confirmation can improve both specificity and, indirectly, sensitivity (see *Technical Background*). Criteria in both cases should be validated and may include quality metrics, genomic context, and other factors (see *Recommendation 5*).

molecular diagnosis of constitutional disorders. This expert Working Group was charged with devising recommendations for orthogonal confirmation of sequence variants and copy number variants (CNVs) identified through the use of next-generation sequencing technologies specific to constitutional (germline) variants. All appointed subject-matter expert Working Group members complied with the AMP conflicts of interest policy, which required disclosure of financial or other interests that may have an actual, potential, or apparent conflict throughout the course of the project.

Survey and Stakeholder Input

The Working Group developed a survey to assess current practices for variant confirmation by clinical laboratories performing germline testing. This survey was posted to the AMP's membership mailing list (CHAMP), social media, and other communication channels. The survey was not limited to AMP members and was open from March 23 to April 27, 2018. In addition, draft recommendations were presented at the 2018 AMP annual meeting and discussed by Working Group members and conference attendees in an open forum.

Literature Review

A scoping review was performed to identify published literature describing the confirmation of variants identified by NGS. In addition, literature was evaluated that established the performance characteristics of NGS for the identification of germline variants relative to a reference method. The search strategy, which used a collection of keywords synonymous with the concepts of NGS, Sanger sequencing, and confirmation, was applied to multiple bibliographic databases and Google Scholar entries with publication dates of 2011 to October 24, 2018

(Supplemental Table S1). Articles with title and/or abstract containing these keywords were loaded into the web-based Covidence system (Melbourne, VIC, Australia), which was used to manage the review process.

In the first review phase, Working Group members reviewed abstracts in a double-blinded manner, with a third member serving as a tiebreaker when needed. Included articles were required to either directly evaluate the performance characteristics of NGS relative to an orthogonal method for identifying germline sequence variants; or present a standard, guideline, or suggested practice regarding orthogonal confirmation of germline sequence variants identified by NGS. Articles were excluded if they addressed only somatic (tumor) sequencing or RNA sequencing, were not performed on human samples, were not peer reviewed, were not in English, had <50 data points, utilized obsolete platforms, or were a commentary, conference abstract, or secondary source, such as a review article.

Abstracts identified as potentially relevant in phase 1 were re-evaluated by a similar double-blinded process in phase 2 using the full article text. Publications meeting criteria in phase 2 were then passed onto phase 3, where predefined data elements were recorded for each article using Survey Monkey (San Mateo, CA), again by two workgroup members. Discrepancies in the data extracted were resolved through discussion and consensus between the individuals performing data extraction.

Development of Recommendations

The Working Group met on a bimonthly basis by conference call to review published evidence and develop the recommendations and article. Face-to-face Working Group meetings were held during the AMP 2018 and 2019 Annual Meetings. On the basis of the results of the systematic evidence review, the survey, stakeholder input, and the

cumulative practice experience of the members of the Working Group, the recommendation statements were developed by expert opinion consensus of the Working Group.

A public open comment period on the eight draft recommendation statements was held from December 10, 2021, through December 31, 2021. The public comment was administered online via Survey Monkey (Momentive Inc., San Mateo, CA). The open comment period was publicized via AMP society communications announcements via multiple outlets (eg, email, member listserv announcements, social media) and in cooperation with the National Society of Genetic Counselors.

The website received 1451 comments in total (agree, agree with comment, disagree with comment, and neutral/not applicable responses were captured). All draft recommendation statements achieved between 75.1% and 94.7% agreement (agree + agree with comment) ([Supplemental Tables S2–S5](#)). The Working Group reviewed all comments received. Following panel discussion, the Working Group members determined whether to maintain the original draft recommendation as is, revise it with minor language change, or consider a major recommendation change. Resolution of all changes was obtained by consensus of the Working Group using nominal group technique (rounds of email, virtual meeting discussions, and multiple edited recommendations) among the group members. The final recommendation statements were approved unanimously by the group with a formal vote. The Working Group considered the risks and benefits throughout their considered judgment process. Formal cost analysis or cost-effectiveness was not performed.

Results

Survey Results

Forty-six responses to our survey were received from members of 40 unique laboratories, with 76% of respondents identifying as laboratory directors. The volume of germline tests performed by these laboratories varied widely from none to several hundred thousand tests per year. For pathogenic or likely pathogenic variants, approximately 50% of respondents report always confirming variants (50% for SNVs, and 54% for indels), whereas an additional 40% reported sometimes confirming these variants (37% for SNVs, and 41% for indels). The remaining approximately 10% of respondents indicated that their respective laboratories never confirmed variants (11% for SNVs, and 4% for indels). For those laboratories that do not universally confirm variants, quality score, depth of coverage, variant allele fraction, genomic context, and variant size were the most common characteristics used to determine criteria for confirmation. The percentage of laboratories that confirm CNVs was somewhat higher than it was for SNVs or indels, with approximately 70% of respondents indicating that they

always confirm pathogenic CNVs using an orthogonal method.

A few survey results were of particular interest. For example, a few respondents reported confirming variants with orthogonal methods that are not validated. Also, there was a wide range of responses to the question regarding approaches toward sequence variants for which the NGS result cannot be confirmed because of repeated failure of the confirmation assay. In this open response question, laboratory responses varied from reporting such results as equivocal, uninterpretable, or inconclusive to reporting the NGS result without confirmation.

Overall, respondents in our survey indicated that NGS and orthogonal confirmatory methods were highly concordant, with 72% responding that <1% of variants demonstrated discordance. In discordant cases, further investigation is usually undertaken, with most laboratories responding that they are almost always able to resolve the cause of the discrepancy. The most common causes of NGS FPs were reported to be pseudogene interference and/or difficult genomic contexts, whereas allele dropout for Sanger sequencing was the most common cause of confirmatory assay false-negative calls. A substantial increase in turnaround time was noted for cases requiring confirmation, with 46% of survey takers responding that confirmation adds an extra 7 to 14 days to turnaround time. Confirmation also increased cost for most of the respondents (91%). Given these experiences, most respondents (67%) said that their laboratories have changed their variant confirmation policies over time or are planning to do so. On the basis of our survey data, variant confirmation procedures vary widely in clinical practice. Although this input from the clinical laboratory genetics community was solicited in 2018, there have not been published observations of a shift to alignment to common practices, and anecdotal evidence suggests that large differences between laboratories' procedures and policies, such as those identified in our survey, remain. Therefore, this Working Group sought to develop recommendations to help standardize variant confirmation practices for clinical genetic testing.

Literature Review Results

A total of 2738 publications were returned from the literature search following the deduplication of references. Following dual review for title/abstract screening and for full-text review, 84 met all inclusion criteria for data extraction ([Figure 2](#)).^{30,31}

As previously noted, publications from four professional organizations that have previously weighed in on germline NGS confirmation were reviewed. These publications agree that confirmation should be performed for reported germline NGS variants unless validation studies support the elimination of the requirement to confirm in certain circumstances. However, these publications define neither the level of performance required to forego confirmation nor the

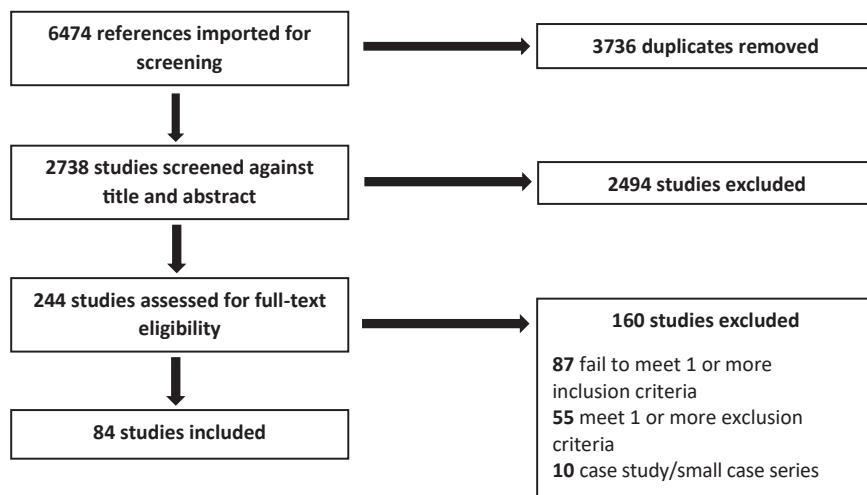


Figure 2 PRISMA diagram for scoping literature review. A PubMed search strategy using a combination of Medical Subject Headings and keywords for the concepts of next-generation sequencing, Sanger sequencing, and confirmation was generated, and searches were performed in multiple bibliographic databases and Google Scholar. A publication limit of 2011 to the present was applied to reflect the exclusion criteria. With approval of the team, the librarian customized the search using controlled vocabulary (when available) and keywords in all the previously listed databases. A publication type limit to exclude conference papers was applied when available. See [Supplemental Table S1](#) for full search details. Given Google Scholar's demonstrated value in retrieving relevant studies³⁰ but its limited transparency and exportation options, only the first 200 citations were included. We ran the searches on October 24, 2018, and exported all the resulting citations into an EndNote X8 (Clarivate Analytics, Philadelphia, PA) library, and duplicates were removed.³¹ The resulting collection of citations was then imported into Covidence (Melbourne, VIC, Australia) for screening. Given the exclusion criteria of the review, no additional efforts were conducted to include gray literature. On January 12, 2022, we reran the search in all bibliographic databases and imported the unique citations into Covidence for screening to identify any additional relevant literature published outside our scoping review but before publication. Included articles were required to either directly evaluate the performance characteristics of next-generation sequencing (NGS) relative to an orthogonal method for identifying germline sequence variants; or present a standard, guideline, or suggested practice regarding orthogonal confirmation of germline sequence variants identified by NGS. Articles were excluded if they addressed only somatic (tumor) sequencing or RNA sequencing, were not performed on human samples, were not peer reviewed, were not published in the English language, had <50 data points, utilized obsolete platforms, or were a commentary, conference abstract, or secondary source, such as a review article. Of the 160 full-text studies excluded, 87 failed to meet one or more inclusion criteria, 55 met one or more exclusion criteria, and 18 were case studies or small case series.

design or size of such studies. These groups recognize the trend for NGS accuracy to increase over time and thus that the need for confirmation might change.

Several clinical laboratories published internal validation studies examining the utility of orthogonal confirmation, although these publications represented a small fraction of the articles we reviewed. Most commonly, these articles concluded that orthogonal confirmation of NGS results is sometimes necessary but not always required. However, there was significant variability in how these studies came to this conclusion. The sizes of the data sets examined varied greatly (from <100 variants to >100,000), and some studies appeared statistically underpowered to support their conclusions. Only a few studies included any statistical analyses, and some studies appeared to exhibit overfitting (eg, from setting thresholds, and then evaluating the performance of those thresholds using the same data set). Although multiple publications described robust data sets for SNVs, indels were less well represented, and there was little data regarding CNVs. All of these studies examined the TP rate of variant calls meeting strict quality thresholds, but only some studies examined sub-threshold calls to determine how many of these other calls were also TPs. There was significant variability in the quality metrics used:

some studies used a single metric, others used large sets of metrics, whereas others generated synthetic scores that combined metrics. The overall rate of NGS FPs varied considerably, likely reflecting variant calling and filtering differences that could impact sensitivity. The rate at which FP variant calls may have appeared in NGS-based clinical diagnostic reports was generally not determined, nor did most articles attempt to define an acceptable FP rate.

A few publications addressed whether confirmation of NGS results by Sanger sequencing was always helpful. These studies showed that discrepant results between the two techniques could be either Sanger or NGS errors, with allele dropout usually causing the Sanger errors. In some studies, all discrepancies were Sanger errors—this appeared to be the case when NGS quality thresholds were strict.

Most of the publications we reviewed addressed the subject of NGS accuracy, but not necessarily the specific question of orthogonal confirmation. For example, a common study design was to perform NGS on samples previously tested by Sanger sequencing and to use concordance to calculate the sensitivity (or, equivalently, the false-negative rate) of NGS. Studies using this design could not always measure NGS specificity (or FP rate). An alternative design was to perform NGS and to then orthogonally

Table 1 Summary of Recommendations for Clinical Laboratories Offering Germline Testing Using NGS

Recommendation 1	Clinical laboratories offering germline testing using NGS should establish a written policy regarding orthogonal confirmation of NGS results.
Recommendation 2	Laboratories' orthogonal confirmation policy should be overseen and approved by a qualified and appropriately certified medical professional with training and experience in NGS.
Recommendation 3	Laboratories' confirmatory methods, platforms, and associated bioinformatics should be validated and maintained under appropriate regulatory oversight, as for other aspects of the test.
Recommendation 4	Laboratories' confirmatory methods should be orthogonal. Discrepant results between NGS and a confirmatory assay should be investigated and resolved, rather than accepting any one method to be always correct.
Recommendation 5	Laboratories should perform confirmatory testing for reported germline variants with significant clinical implications, except for variant calls meeting technical criteria rigorously demonstrated to ensure high positive predictive value from NGS alone.
Recommendation 6	Laboratories should clearly articulate their specific policies, criteria, and methods regarding orthogonal confirmation in written materials readily available on request.
Recommendation 7	Laboratories' clinical test reports should summarize orthogonal confirmation policy in every report, and when exceptions to the policy are made, these should be clearly indicated.
Recommendation 8	Special considerations apply to certain NGS-based test types and findings.

See text for important details regarding each of these recommendations. Laboratories should perform confirmatory testing for reported variants with significant clinical implications.

NGS, next-generation sequencing.

confirm the detected variants to calculate specificity. Other designs included testing of reference materials, such as those characterized by the Genome in a Bottle Consortium (see *Recommendation 5*) and comparison of different NGS platforms.

Recommendations

Following analysis of the survey data, literature review findings, considering public feedback provided, and robust subject matter expert discussions, the Working Group developed a consensus set of best practice recommendations (summarized in **Table 1** and discussed in additional detail below).

Recommendation 1: Clinical Laboratories Offering Germline Testing Using NGS Should Establish a Written Policy Regarding Orthogonal Confirmation of NGS Results

Each clinical laboratory should determine whether and under what circumstances orthogonal confirmation of NGS results is required. We recommend that the NGS orthogonal confirmation policy be formalized, signed by the laboratory director(s), and maintained as part of the official laboratory documentation.

Variables to consider when establishing a policy include test content (eg, single gene versus exome sequencing), NGS platform, variant type, variant classification, quality metrics, clinical impact, and other factors. Because of the many differences between laboratory procedures and tests, it is not possible for this Working Group to craft general recommended policy templates for orthogonal confirmation. Rather, we recommend each laboratory develop its own

policy following the recommendations and considerations described herein.

Laboratories should take reasonable steps to address as many as possible of those variables that are likely to impact the predictive value of the test(s) offered and should use these data to write and approve a policy that applies uniformly. In some laboratories, whether orthogonal confirmation is performed in a particular circumstance is left to the discretion of the molecular professional interpreting the results, with some being more conservative than others. It is better practice to ensure that patient samples are treated consistently.

Recommendation 2: Laboratories' Orthogonal Confirmation Policy Should be Overseen and Approved by a Qualified and Appropriately Certified Medical Professional with Training and Experience in Next-Generation Sequencing

Developing an appropriate policy for orthogonal confirmation requires the careful consideration of highly technical data in the context of clinical molecular genetics testing. It is our recommendation that an appropriately board-certified and experienced clinical molecular professional direct the development, finalization, and implementation of the policy.

The individual(s) responsible should have extensive experience in NGS and applicable clinical laboratory regulations and should oversee the development of the policy in its entirety, from determining which data and variables to consider through final approval of the document. It is appropriate, at the clinical molecular professional's discretion, to include other stakeholders and laboratory members (eg, molecular pathologists, laboratory geneticists, laboratory technologists and supervisors, research and development staff, and bioinformatics staff) in the policy's

development, and much of the technical work may best be delegated to those with day-to-day bench and informatics expertise. However, the final responsibility for implementing a scientifically and clinically sound policy is that of the clinical molecular professional.

Distributed laboratory testing models, where separate parties may be involved in analytical testing and result interpretation, may pose logistical challenges for confirmation testing. Confirmation testing should be considered when entering a distributed laboratory relationship, and the responsibility for the validation, performance, and interpretation of confirmation testing should be clearly defined.

Recommendation 3: Laboratories' Confirmatory Methods, Platforms, and Associated Bioinformatics Should be Validated and Maintained Under Appropriate Regulatory Oversight as for Other Aspects of the Test

Technologies such as Sanger sequencing or digital PCR are often used as orthogonal methods for confirmation of variants identified by NGS. Because the procedure of variant confirmation is part of the clinical test, analytical validation for each confirmatory method should be performed either independently or as part of the overall validation of the NGS-based test. Validation should be performed under the appropriate regulatory and accreditation guidelines and may include assay design, wet laboratory methods, and associated bioinformatics and software programs.

Because of practical limitations, particularly the need to confirm novel variants, it is acceptable for analytical validation to be platform or method specific. For example, a methods-based validation of Sanger-based amplicon sequencing would be acceptable instead of validation of individual primer pairs—an impractical and onerous task. Such a methods-based approach should include an end-to-end validation of the entire confirmation workflow, including PCR primer design, wet laboratory PCR amplification assays, and manual or software programs for reading Sanger sequencing data. If multiple PCR amplification protocols are used (eg, GC-rich PCR and long-range PCR), then each PCR method must be validated separately. Because individual primer pairs are not validated in this approach, it is vital that additional investigation into any discrepancies is performed where the NGS result differs from the confirmation result. Such investigations may include a review of the primer design (eg, identifying regions of homology and potentially interfering variants) and review of amplification and sequence data. For variants that are recurrently in need of confirmation using an alternative method if they are challenging to resolve by NGS alone (examples include but are not limited to identification of certain repeat expansions and recurrent variants within a known region of homology), a variant-specific validation using the preferred alternative technology is recommended.

Some laboratories may choose to send out variant confirmation to other laboratories. Send-out of the complete or partial analytical testing process (eg, only the wet bench or analysis portions) must be sent to a laboratory meeting equivalent requirements as determined by the Centers for Medicare & Medicaid Services, accredited by a recognized organization, or certified by an appropriate government agency. The laboratory director of the primary laboratory is responsible for the selection and evaluation of the referral laboratory and must retain records demonstrating that the referral laboratories meet the requirements of the relevant governing bodies for the performance of clinical genetic testing. The primary laboratory should record detailed protocols and worksheets for specimen handling, specimen transfer, and maintenance of chain of custody between the primary and referral laboratory.

The laboratory director or designee should review and approve all validations relevant to the methods conducted within the laboratory. The director/designee should review all validations relevant to methods performed in referral laboratories, if applicable or as required by the Centers for Medicare & Medicaid Services or its accreditation programs.

Recommendation 4: Laboratories' Confirmatory Methods Should be Orthogonal: Discrepant Results between NGS and a Confirmatory Assay Should be Investigated and Resolved, Rather Than Accepting any One Method to be Always Correct

The aim of confirmatory testing is to uncover FPs and prevent them from being reported. It is important to remember that many NGS FP errors are systematic^{15,32} (ie, likely to be repeated) and that no assay is 100% accurate (see *Technical Background*). Thus, confirmatory assays should be chosen such that the error profile of the confirmatory assay correlates as little as possible with the error profile of the primary NGS assay. The concept of two assays that operate by distinct means and have minimally overlapping error profiles is commonly known as orthogonality.

Choosing confirmatory assays requires careful judgement by the laboratory director. Although achieving complete orthogonality may not be possible, obvious problems can be avoided. For example, simply repeating the primary NGS assay does not provide any degree of orthogonality and would likely result in systematic FPs being reported. We recommend that confirmation assays use neither the same core sequencing technology nor the same library preparation method. Reanalyzing the same raw data with different bioinformatics pipelines is also strongly discouraged as a confirmatory method.

Sanger sequencing of amplicons is frequently used in current practice for orthogonal confirmation of sequence variants identified by NGS, which often uses hybridization-based targeting. Similarly, microarrays, quantitative PCR, or

multiplex ligation-dependent probe amplification is often used to confirm CNVs detected by NGS. Given the methodological differences, one expects these strategies to have a significant degree of orthogonality.

Because no assay is 100% accurate, when results between primary and confirmatory assays conflict, neither assay should be assumed to be correct. Instead, the cause of the discrepancy should be investigated and resolved to the greatest extent reasonably possible before including or excluding a variant from a report (Figure 3). The investigation should involve examining the underlying data for the primary and confirmatory assays and may also include repeated testing or a third (validated) testing method. Laboratories should consider sensitivity limitations of the confirmatory assay as a potential cause of discrepancy and employ another method for specific patients or variants as needed. For example, a copy number microarray lacking sufficient probe coverage in the region of interest may produce a false-negative confirmation result of a TP CNV detected by NGS. Similarly, Sanger sequencing may provide false-negative confirmation results because of allele dropout.²⁶ Testing of an incorrect sample in either the initial NGS testing or the confirmation process (ie, a sample swap) is another potential cause of discrepancy that should be ruled out during the investigation. When a definitive resolution between the primary and confirmatory assays cannot be achieved, the decision to report or omit the variant in question should be made by an experienced clinical molecular professional. If a variant with discrepant cross-method results is reported, the results of the confirmatory assay(s) should be described in the report along with an interpretation of the likelihood that the variant is a TP.

Recommendation 5: Laboratories Should Perform Confirmatory Testing for Reported Germline Variants with Significant Clinical Implications, Except for Variant Calls Meeting Technical Criteria Rigorously Demonstrated to Ensure High Positive Predictive Value from NGS Alone

The ultimate decision by the laboratory regarding which variants require orthogonal confirmation will depend on two types of criteria, which both should be clearly described in the laboratory's confirmation policy and applied individually to each variant call that may be included in a test report.

1. Technical criteria: How likely is this variant call to be an analytic TP versus FP based on the available data?
2. Medical criteria: How likely is this variant to have a significant clinical impact on the patient's care?

The impact of these two types of criteria is shown in Figure 4.

Each laboratory should develop its own technical and medical criteria, guided by the considerations described in *Technical Background*. Of particular importance is the fact

that NGS-based tests may have a significant chance of reporting FP results even when low analytical FP rates are achieved—this fact distinguishes NGS from more focused types of genetic tests. There is currently no generalized standard for what constitutes an acceptable FP rate. For each test offered, this should be guided by the clinical PPV in the population indicated for testing and the medical impact(s) of the test's results.

We describe recommendations for these two criteria types separately.

Technical Criteria

Strict criteria may be developed to identify variant calls that are highly unlikely to be FPs (Figure 1). Although read depth, variant type, variant allele fraction, genomic context, and many current quality score calculations have been shown to be inadequate alone for this purpose, combinations of such criteria have been shown to be effective (see *Technical Background*). These combinations may include the following elements.

- Quality metrics: Variant calling tools often produce many quality metrics for each variant call. The commonly used GATK variant caller, for example, produces multiple scores that guidelines recommend¹⁸ and that are each informative in identifying the highest confidence variant calls. Aggregate scores may not work as well as considering multiple underlying quality metrics separately.
- Variant type: SNVs are far more prevalent than indels in human DNA. Indel variant calls tend, however, to have much higher error rates than SNV calls and are more likely to be interpreted as pathogenic in the many genes for which loss of function is a disease-causing mechanism. It is typically easier to accumulate large data sets with orthogonal data for certain variant types (eg, SNVs) to determine which calls may or may not require confirmation. For other variant types (indels and CNVs), a laboratory may not have an adequately large data set to determine criteria in a statistically sound manner. For these reasons, variant type can be one important criterion in determining which variants require confirmation. Optimal quality thresholds for SNVs and indels are likely different.
- Genomic context can be an important criterion for the identification of regions that are prone to FPs and that may not be adequately flagged by common quality metrics. These regions often include low-complexity repeats, such as homopolymers and short tandem repeats, mobile elements, and genes with highly similar paralogs or pseudogenes. During validation, laboratories may identify regions of the genome in which variants identified by NGS are unreliable and should always be subject to orthogonal confirmation.
- Variant allele fraction is an important quality metric with complex implications. Variant calls at skewed allele fractions (heterozygotes far from 50:50 variant allele

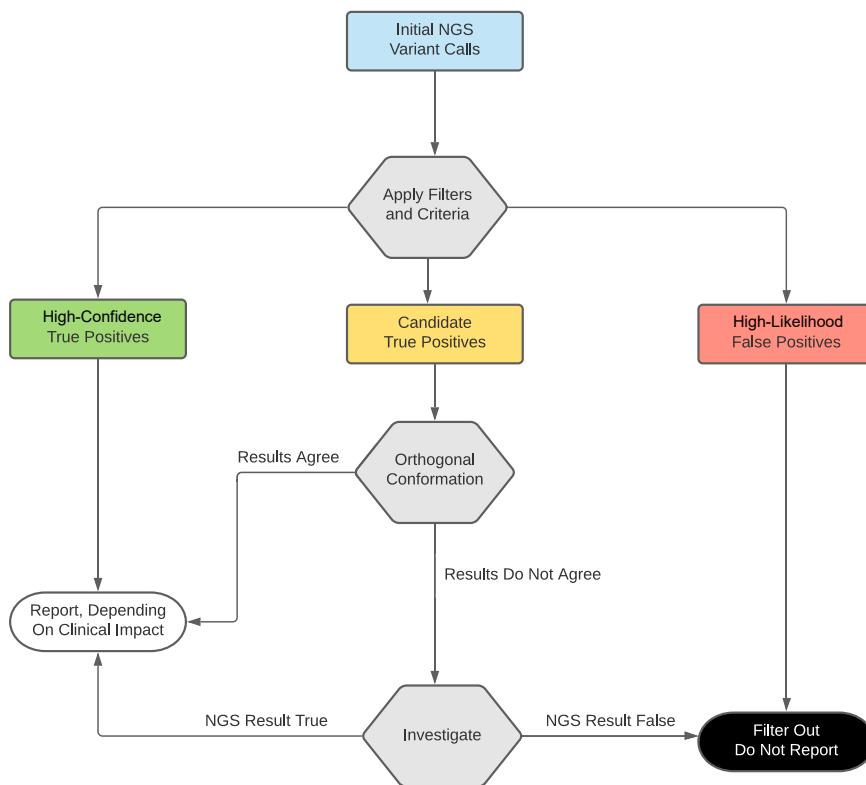


Figure 3 The role of orthogonal confirmation in next-generation sequencing (NGS) processes. Orthogonal confirmation can be used to determine which of the candidate true-positive variant calls from an NGS run (Figure 1) are confidently true positives as opposed to false positives. Because no technique is perfect however, discrepancies between NGS and the confirmatory test should be investigated to determine which of the two methods is more likely correct (see *Recommendation 4*).

fraction in germline DNA) are often FPs, although they may be TPs subject to a technical artifact (eg, reference bias or mismapping). Alternatively, they may be mosaic TPs (see *Recommendation 8*).

There are criteria we do not recommend without extensive additional validation.

- Repeated confirmations of the same variant in multiple samples. Some guidelines have suggested that laboratories may stop confirming a particular variant after it has been confirmed as a TP a certain number of times, in either a validation study or routine practice (New York State Department of Health, https://www.wadsworth.org/sites/default/files/WebDoc/Germline_NextGen_Validation_

Technical Impact		
Clinical Impact	Unknown, moderate, or high likelihood that the variant is an analytic false positive	Validated high likelihood that the variant is an analytic true positive
High chance of significant clinical impact, assuming the variant is a true positive	Orthogonal confirmation recommended	Confirmation may not be needed
Low chance of significant clinical impact, assuming the variant is a true positive	Confirmation may not be needed	Confirmation may not be needed

Figure 4 Role of medical and technical criteria. Both clinical impact and technical criteria should be considered in each laboratory's policies for determining whether any particular variant call should be orthogonally confirmed.

Guidelines_FINAL_2020_rev_09092021.pdf, last accessed August 31, 2022). Unfortunately, systematic errors are prevalent in NGS, and calls of the same variant in different specimens can have remarkably different levels of quality. Studies have shown that repeated confirmations can have limited predictive power regarding the accuracy of subsequent observations of the same variant.¹⁵

- Manual review of NGS data as an alternative to confirmation. Manual review of NGS data can identify certain cases where the variant calling software has made an FP error. Laboratory policy may allow such variants to be removed without using a secondary assay. However, the converse may not be true: Failure to see a problem in manual review does not necessarily mean that the variant call is confident. Biochemical and mapping issues that can cause FPs are not always readily apparent in manual review.

Analysis of existing data sets containing NGS variant calls, their quality metrics, and corresponding orthogonal data is necessary to establish which metrics are informative and which thresholds are appropriate. There can be many substantial and subtle differences in the NGS processes used by different laboratories, and there often are important differences among the targets examined by different test(s). Each laboratory should gather such a data set using its own procedures and use those data to develop its own confirmation criteria, rather than attempting to use either data or criteria from another laboratory.

Optimization algorithms (which may be heuristic, statistical, or machine-learning based) have been shown to be useful for determining which combinations of quality metrics and thresholds are most informative.^{14–17} These methods can require large input data sets. Well-characterized samples, such as the seven currently released by the Genome in a Bottle consortium (<https://www.nist.gov/programs-projects/genome-bottle>, last accessed August 31, 2022),^{33–35} are widely available and can be valuable in such studies. An important caveat to these specimens, however, is that many clinically important genes are not yet fully characterized by the consortium.^{3,15,28} Unfortunately, these uncharacterized regions have the highest NGS error rates. Another caveat is that most variants available in the Genome in a Bottle data sets, particularly in protein-coding regions, are SNVs. We strongly recommend against applying criteria determined by optimization methods to variants with types, locations, or characteristics that are not well represented in the input data sets. Using a mixture of both well-characterized reference and clinical specimens in the input data may help alleviate such issues.

Once technical criteria are established, they should be validated, as is required for other aspects of the test. It is important that these validation studies are performed rigorously. Among other considerations, a separate data set must

be used in validation from that used to establish confirmation criteria, to avoid overfitting (alternatively, methods such as randomized training/test splits may be employed). Additional detailed recommendations for studies used to determine and validate technical criteria are provided online (AMP Validation Resources, <https://www.amp.org/resources/validation-resources>, last accessed August 31, 2022).

After establishing analytical criteria and performing sufficient validation, laboratories may use these criteria to exclude certain variant calls from requiring confirmation following the process shown in Figure 3. These criteria should be included in the policy described in Recommendation 1.

Medical Criteria

When technical evaluation suggests that there is a risk of a reportable variant being an analytic FP, orthogonal confirmation is recommended when such an FP could have a detrimental impact on the quality of diagnosis, treatment, or management of a current or future medical condition in the patient. This will often be the case for pathogenic and likely pathogenic findings in high or moderate penetrance genes related to the patient's indication for testing. By contrast, benign variants generally have no clinical impact and are often not reported. Confirmation of benign variants is thus usually not warranted.

Other types of findings may have a significant clinical impact, and confirmation of these variant calls may be important. Medical criteria to consider when setting laboratory policy for which calls do, or do not, require confirmation include but are not limited to the following.

- Uncertain variant interpretations: Although variants of uncertain significance (VUSs) should not usually influence clinical decisions, some VUSs may be later reclassified as pathogenic or likely pathogenic. Analytic FPs could thus be problematic if the variant was not confirmed because of its initial VUS classification. If laboratory policy is that some or all VUSs are not confirmed because of their classifications, this policy and the implications of it should be made clear to ordering clinicians. If a VUS is later reclassified as pathogenic or likely pathogenic, the laboratory should apply its current standard policy to the variant to determine whether confirmatory testing is required before issuing an amended report.
- Primary versus secondary findings: Although primary findings (ie, those directly related to the patient's indication for testing) typically have the greatest clinical impact, secondary (sometimes called incidental) findings can also have a significant impact. Consider, for example, a pathogenic *BRCA1* secondary finding in an exome sequence of a patient undergoing diagnosis for a cardiovascular condition. Variants in genes recommended by the ACMG^{36,37} or in genes of pharmacogenetic

relevance³⁸ are common types of secondary findings that may be reported in clinical germline testing and that can merit orthogonal confirmation.

- Carrier findings: Monoallelic pathogenic variants uncovered in recessive genes can have significant clinical impact in reproductive medicine. Also, in some patients with such a carrier finding, a second pathogenic variant in the same gene may later be uncovered using a different test method, making the initial finding directly relevant to their health care.
- Penetrance: Both high- and low-penetrance pathogenic variants can suggest significant changes in clinical care under current medical practice guidelines, despite the relatively low disease risk that low-penetrance variants may confer.

Laboratories should weigh carefully the risks when deciding which variants require confirmation on medical grounds. Laboratory policies should be clear to ordering clinicians so that they can confidently know which variants are, and are not, subject to confirmation in any clinical report (see *Recommendation 6*).

Recommendation 6: Laboratories Should Clearly Articulate Their Specific Policies, Criteria, and Methods Regarding Orthogonal Confirmation in Written Materials Readily Available on Request

Laboratories should make available both written materials regarding orthogonal confirmation policies and written documentation summarizing the confirmation policy validation results, including whether the laboratory has excluded certain variants from confirmation on technical grounds, per *Recommendation 5*. Providing this information is consistent with federal standards for laboratories in the United States (Federal Register, Clinical Laboratory Improvement Amendments, <https://www.govinfo.gov/content/pkg/USCODE-2011-title42/pdf/USCODE-2011-title42-chap6A-subchapII-partF-subpart2-sec263a.pdf>, last accessed August 31, 2022):

The laboratory must, on request, make available to clients a list of test methods employed by the laboratory and, as applicable, the performance specifications established or verified as specified in §493.1253. In addition, information that may affect the interpretation of test results (eg, test interferences) must be provided on request. Pertinent updates on testing information must be provided to clients whenever changes occur that affect the test results or interpretation of test results.

Transparency instills confidence in the laboratory's practices. The written documentation should be current, including updates and amendments, and include the laboratory's standard criteria for which reported variants are or are not subjected to confirmatory testing. Note that general statements (eg, variants that meet high quality standards) do not alone satisfy this recommendation. The written documentation should also include actions taken for variant calls

for which the initial NGS data and the confirmation test data disagree. Furthermore, the documentation should include actions taken for exceptions to the standard policy, including scenarios in which confirmation cannot be performed for a technical reason, such as inadequate specimen availability or because a confirmatory assay has failed to produce reliable data. Please see *Recommendation 7* for memorialization of exceptions in clinical test reports.

Recommendation 7: Laboratories' Clinical Test Reports Should Summarize Orthogonal Confirmation Policy in Every Report, and when Exceptions to the Policy Are Made, These Should be Clearly Indicated

Laboratories should include a summary of their standard orthogonal confirmation policy within the report. This could be a concise summary or a reference to the laboratory's standard criteria for determining which reported variants are subject to confirmatory testing. If the laboratory does not have a policy for confirmation or does not routinely perform orthogonal confirmation, this should be stated.

As described in *Recommendation 5*, there may be scenarios in which confirmation cannot be performed for technical reasons, such as inadequate specimen availability or because a confirmatory assay has failed to produce reliable data. All exceptions to a laboratory's standard criteria for variant confirmation should be clearly documented in the clinical report.

Example reporting language:

This variant did not pass the established NGS confidence thresholds and is therefore subject to confirmation by an orthogonal method as per the laboratory's standard confirmation policies. Repeated Sanger sequencing confirmation attempts failed to produce interpretable data. We recommend that this variant be confirmed in a clinical laboratory that has a validated gene-specific method.

Recommendation 8: Special Considerations Apply to Certain NGS-Based Test Types and Findings

We note a few special circumstances that laboratories should carefully consider when determining their confirmation policies.

Sample Identity Confirmation

Orthogonal assays can serve dual purposes of confirming that a variant is an analytic TP and ensuring that it is present in the correct patient's DNA sample. Confirmation assays can thus detect sample or data mix-ups during NGS, particularly when the confirmation assay is performed on a separate aliquot of the patient's initial specimen. If laboratories do not perform confirmation on either some or all clinically significant variants, then alternative positive sample-tracking methods are highly recommended.

Examples include single-nucleotide polymorphism assays, which can be compared with NGS results to confirm sample identity, or the use of spike-in oligonucleotides.³⁹ These mechanisms have the additional advantage of tracking samples with both negative or positive findings.

Indel Resolution

For certain variants detected by NGS, it can be clear from the NGS data that a variant is present, although the exact diploid sequence of that variant may be unclear. Confirmation assays can serve the important role of providing additional data to determine the exact nature of the indel, which can be important for variant interpretation. This is useful for complex indels and het-alt sites (heterozygous sites where neither allele is the reference allele).

Location Resolution

Short-read NGS platforms cannot always accurately determine the specific location of variants that are observed in genes that have homologous pseudogenes or gene family members. For example, variants mapping to the *PMS2* gene may in fact be arising from the *PMS2CL* pseudogene, which is 99% sequence identical to *PMS2* in particular regions of the gene. Confirmation assays using, for example, long-range PCR or long-read sequencing can both determine which locus a potentially pathogenic variant is in and determine whether it is in fact an analytic TP.

Mosaic Variants

Variants observed at a low variant allele fraction in the NGS results present specific considerations. Variants may appear at low allele fractions for many reasons, either biological or technical, including the following.

- True mosaicism.
- Clonal hematopoiesis of indeterminate potential, which can be observed in lymphocyte-derived DNA from blood, buccal, and saliva specimens.
- Mismapping of reads from homologous regions (eg, pseudogenes).
- Reference bias: The fact that NGS reads containing a different allele than that which is present in the reference genome may not readily map to the correct genomic location.
- Systematic errors.

If laboratories clinically report variants at low allele fractions, then appropriate procedures to attempt to resolve the cause of the allelic imbalance are important. Reporting language is also critical, particularly when there is ambiguity as to the root cause of the low allele fraction. Confirmation technologies, particularly Sanger sequencing, may have reduced sensitivity for actual mosaic variants, and thus one could see relatively common cases where a TP observed by NGS is a false negative in the confirmatory assay.

Mitochondrial Variants

Because of heteroplasmy, variants in the mitochondrial genome may be present at low allele fractions. In addition to the challenges associated with mosaic variants mentioned above, the mitochondrial variants can be multi-allelic, having over two alleles present in a DNA mixture, a situation similar to that encountered in tumor sequencing because of heterogeneity. Laboratory confirmation policies should detail how such situations are handled and reported.

Haplotyping/Phasing

Clinical interpretation of certain variants can require haplotyping (ie, determining the *cis* or *trans* phase relationship of multiple variants in the same gene or region). This step can be important in recessive genes (to determine when biallelic, compound heterozygous variants have been observed) and in genes in which multiple *cis* variants are required to identify specific alleles (eg, star alleles in the *HLA* or cytochrome genes). Short-read NGS platforms typically cannot determine phase over genomic regions larger than a few hundred base pairs. Haplotypes and phase over larger distances may be determined through the following.

- Mendelian analysis (when family members are available for testing).
- Imputation or other statistical inference methods.
- Certain confirmatory assays, such as long-range PCR or long-read sequencing.

Laboratories should be clear when phasing is known through confirmatory testing, when it is inferred, or when it is unknown. If the phase relationship of variants is unknown and haplotyping is important, the test report should clearly mention that the provider should consider family member testing or additional assays to adequately interpret the variants.

Familial Sample Testing

Often in genetic testing, familial samples are tested along with the proband's sample to aid variant classification (ie, determining cosegregation and/or phase). If family members are tested using NGS and receive personalized reports, then orthogonal confirmation should be performed on variants reported to these individuals following the same criteria as are used for the proband. Because many NGS FPs are systematic, not random, simply observing the same variant in multiple individuals, whether related, is strongly discouraged as a confirmation technique.

Tumor Testing

Tumor-only sequencing may detect germline variants,⁴⁰ although current tumor tests vary whether and how such variants are reported. Guidelines on this topic continue to evolve.^{41,42} In some cases, the patient is referred to a different laboratory for germline follow-up testing by NGS, and in this circumstance the germline laboratory may

consider the prior tumor test as confirmation for any germline variants that are uncovered. Whether this is appropriate depends on the germline laboratory director's judgement as to whether the two tests are orthogonal (see *Recommendation 4*). For example, if the two tests use the same sequencing platform and capture method, systematic errors may be repeated. The fact that the tumor test and the germline test use different specimens does not alone make the tests orthogonal.

Discussion

NGS has transformed the field of clinical molecular genetic testing. Its scalability and broad utility across a range of applications and testing indications has brought about its rapid transition from an innovative technology used only in a handful of well-resourced laboratories to the core method of much of current clinical molecular diagnostics. Traditionally, reporting of sequence variants by clinical molecular diagnostic laboratories has been limited to variants identified with high confidence such that the reporting of an FP was thought to be a rare event. However, the frequency of reported FP variants has not been quantified, and no exact threshold for an acceptably low FP rate has been established in the field. Advances in high-throughput sequencing technology have enabled the identification of additional genetic variant types with lower specificity than that of simple substitutions and small indel calls. Examining larger portions of the genome in larger numbers of patients increases the risk of FPs. Thus, we expect that confirmation testing will continue to serve as an important tool to limit the reporting of FPs to maintain high confidence in the results of NGS-based constitutional genetic testing.

The evidence-based consensus recommendations provided within this article provide a framework for clinical laboratory professionals to develop or refine individualized laboratory policies and procedures regarding orthogonal confirmation of germline variants detected by NGS. With the utilization of NGS across a wide range of platforms, bioinformatics pipelines, and indications for testing, it is not possible to establish universally applicable thresholds for variant and test performance metrics, but these recommendations support the application of fundamental principles of clinical testing to orthogonal confirmation: That policies be established on the basis of rigorous scientific study by trained and credentialed personnel; that they be applied uniformly to the tests of a given assay within the laboratory; that all test methods, both primary and confirmatory, be appropriately validated; and that policies and methods (and any exceptions to them) relevant to the ordering provider are communicated clearly in the report or in accessory materials made available on request.

This initial version of AMP recommendations is based on a review of the supporting literature, survey of practices among clinical molecular genetics laboratories, and expert

consensus opinion. This document summarizes the current state of knowledge, and its recommendations are based on the technology available as of the time of this publication. We acknowledge that as the method and bioinformatics underlying NGS-based variant detection develop, revisions to recommendations such as these will be needed to guide quality patient care.

Disclaimers

The Association for Molecular Pathology (AMP) Clinical Practice Guidelines and Reports are developed to be of assistance to laboratory and other health care professionals by providing guidance and recommendations for particular areas of practice. The Guidelines or Reports should not be considered inclusive of all proper approaches or methods, or exclusive of others. The Guidelines or Reports cannot guarantee any specific outcome, nor do they establish a standard of care. The Guidelines or Reports are not intended to dictate the treatment of a particular patient. Treatment decisions must be made on the basis of the independent judgment of health care providers and each patient's individual circumstances. The AMP makes no warranty, express or implied, regarding the Guidelines or Reports and specifically excludes any warranties of merchantability and fitness for a particular use or purpose. The AMP shall not be liable for direct, indirect, special, incidental, or consequential damages related to the use of the information contained herein.

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Supplemental Data

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