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SPECIAL ARTICLE

Designing and Implementing NGS Tests for Inherited Disorders

A Practical Framework with Step-by-Step Guidance for Clinical Laboratories

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Comprehensive next-generation sequencing (NGS) tests are increasingly used as first-line tests in the evaluation of patients with suspected heritable disease. Despite major technical simplifications, these assays still pose significant challenges for molecular testing laboratories. Existing professional guidelines and recommendations provide a framework for laboratories implementing such tests, but in-depth, concrete guidance is generally not provided. Consequently, there is variability in how laboratories interpret and subsequently implement these regulatory frameworks. To address the need for more detailed guidance, the College of American Pathologists with representation from the Association for Molecular Pathologists assembled a working group to create a practical resource for clinical laboratories. This initial work is focused on variant detection in the setting of inherited disease and provides structured worksheets that guide the user through the entire life cycle of an NGS test, including design, optimization, validation, and quality management with additional guidance for clinical bioinformatics. This resource is designed to be a living document that is publicly available and will be updated with user and expert feedback as the wet bench and bioinformatic landscapes continue to evolve. It is intended to facilitate the standardization of NGS testing across laboratories and therefore to improve patient care. *(J Mol Diagn 2019, 21: 369—374; https://doi.org/10.1016/j.jmoldx.2018.11.004)*

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Over the past decade, molecular diagnostic testing has experienced a dramatic expansion in complexity and scope. Comprehensive next-generation sequencing (NGS) tests, ranging from large, disease-focused gene panels to whole exome or genome assays, are now firmly established in clinical practice and are increasingly used as first-line tests in the evaluation of patients with suspected inherited disease.

Despite major technical simplifications, these tests still pose significant challenges for molecular testing laboratories and require a high degree of specialized expertise. Guidelines and recommendations have been released by professional organizations and provide an excellent foundational framework for laboratories that seek guidance on implementing such tests (Table 1).[1–12]

The College of American Pathologists (CAP) has generated accreditation checklist requirements specific to clinical laboratories that implement NGS-based testing, addressing preanalytical, analytical, and postanalytical phases of laboratory testing.[13] These checklist requirements are designed to ensure analytical and clinical validity, development of a quality management program, and compliance with the Clinical Laboratory Improvement Amendments regulations.

Although these resources have filled major knowledge gaps, they do not provide concrete, step-by-step guidance, and there is variability in how laboratories implement regulatory frameworks. An example is the CAP checklist item MOL.36015, which states that "Analytical validations must consist of a baseline methods-based validation that establishes the test's general performance for the detection of the sequence variant type(s) that the test is designed to identify. For tests that are offered for specific clinical indications (eg, diagnostic gene panels) the laboratory should investigate whether any analyte, disease, or gene-specific needs exist that necessitate inclusion of additional specimens or reference samples in the validation. For example, this may include samples containing prevalent pathogenic variants, especially if they are technically difficult to accurately detect (eg, large indels)."[13] This requirement does refer to a second checklist (MOL.36115), which provides further detail. However, the checklist is formulated to establish requirements for a wide variety of NGS tests and indications; therefore, neither one of these requirements provides comprehensive guidance. In this example, the laboratory director must determine the number and types of specimens to include in the laboratory's NGS test validation. Although this

Table 1 Existing Guidelines for NGS Testing (Inherited Disorders)

Year	Organization/entity	PMID	Reference	Title
2010	EuroGentest	20664632	1	A standardized framework for the validation and verification of clinical molecular genetic tests
2012	CDC	23138292	2	Assuring the quality of next-generation sequencing in clinical laboratory practice
2012	AMP	22918138	3	Opportunities and challenges associated with clinical diagnostic genome sequencing: a report of the Association for Molecular Pathology
2013	ACMG	23887774	4	ACMG clinical laboratory standards for next-generation sequencing
2014	CLSI	NA	5	MM09: nucleic acid sequencing methods in diagnostic laboratory medicine, 2nd edition
2014	CAP	24650895	6	Methods-based proficiency testing in molecular genetic pathology
2015	CDC	26154004	7	Good laboratory practice for clinical next-generation sequencing informatics pipelines
2015	CAP	25152313	8	College of American Pathologists' laboratory standards for next-generation sequencing clinical tests
2015	NYS	NA	NA	Guidelines for validation submissions of next generation sequencing (NGS) assays under the NYS testing category of genetic testing— molecular*
2016	ESHG EuroGentest	26508566	9	Guidelines for diagnostic next-generation sequencing
2017	CAP	28322587	10	Development and validation of targeted next-generation sequencing panels for detection of germline variants in inherited diseases
2017	CAP	28362156	11	Development and validation of clinical whole-exome and whole-genome sequencing for detection of germline variants in inherited disease
2018	FDA	NA	NA	Considerations for design, development, and analytical validation of next-generation sequencing (NGS) — based *in vitro* diagnostics (IVDs) intended to aid in the diagnosis of suspected germline diseases†
2018	AMP/CAP	29154853	12	Standards and guidelines for validating next-generation sequencing bioinformatics pipelines: a joint recommendation of the Association for Molecular Pathology and the College of American Pathologists

*Available at *https://www.wadsworth.org/sites/default/files/WebDoc/20809000015/Germline_NextGen_Validation_Guidelines.pdf*.

†Available at *https://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/UCM509838.pdf*.

ACMG, American College of Medical Genetics and Genomics; AMP, Association for Molecular Pathology; CAP, College of American Pathologists; CDC, Centers for Disease Control and Prevention; CLSI, Clinical Laboratory Standards Institute; ESHG, European Society of Human Genetics; FDA, US Food and Drug Administration; NA, not applicable; NYS, New York State.

approach can provide flexibility for a laboratory director, the lack of detailed guidance can make this process difficult to navigate for laboratories that are new to NGS and leads to a high degree of variability among NGS laboratories.

The US Food and Drug administration has identified this variability as a major area of improvement and has recently issued final guidance for NGS-based *in vitro* diagnostics that is intended to assist test developers directly and to inform the development of consensus standards by community experts (Table 1). Approaches to standardize NGS testing are actively discussed in the laboratory community. Proposals include use of performance standards (eg, fixed thresholds for NGS metrics such as a required analytical sensitivity for detecting germline variants) and design concept standards that define approaches and principles to provide guidance about how specific testing scenarios influence performance requirements, rather than prescribe fixed standards. Although consensus is still being developed, it appears likely that both approaches will be needed.

To provide additional specificity to existing guidelines and recommendations, the CAP, in collaboration with the Association for Molecular Pathologists, assembled a working group to create a practical resource for laboratories performing NGS-based testing with an initial focus on design, development, optimization, validation, and quality management of a test for variant detection in the setting of inherited disease. NGS tests allow inclusion of unprecedented numbers of genes. With the expanded scope comes an increased need to integrate clinical requirements with the technical capabilities of the platform to ensure that the test is appropriately designed for the intended use. However, the enormous diversity of clinical testing scenarios and technology platforms makes all-encompassing guidance challenging. The working group therefore focused on major concepts and the most frequently used NGS selection methods (hybridization capture and amplicon sequencing). This resource consists of a set of practical, ready-to-use worksheets that were structured to cover each step in the lifecycle of an NGS test and to expand the scope significantly compared with existing guidance documents, which are traditionally less focused on test design considerations. The worksheets presented here cover test design, optimization, validation, and quality management. Bioinformatics aspects, which are integral components of every step in the lifecycle of an NGS-based laboratory developed test, are addressed within each document as well as a separate bioinformatics guidance worksheet. To limit complexity, this initial set of worksheets was designed to address NGS testing for germline disorders because practices and requirements for somatic testing have significant differences.

These worksheets are intended to complement existing professional recommendations, as well as the CAP accreditation checklists, with the goal of fostering standardization of NGS test design and development approaches across laboratories and thereby improving patient care. They are not intended to be prescriptive, but rather they focus on key concepts and emphasize how various factors influence performance requirements. Examples of such design concept standards or dynamic standards are the definition of an acceptable false-positive rate (which depends on whether a laboratory orthogonally confirms variant calls), the need to fill in missing data when NGS fails (which is influenced by gene-specific characteristics such as the existence of mutation hotspots), or the need to supplement NGS with additional, non-NGS assays when complex variants in a particular gene are a frequent cause of disease. The working group did not define concrete performance standards (such as coverage threshold or thresholds for commonly used quality metrics) and did not include guidance on variant interpretations and reporting.

The worksheets are available for download (College of American Pathologists, *www.cap.org/member-resources/precision-medicine/next-generation-sequencing-ngs-worksheets*) and will be regularly updated by the CAP, using solicited user feedback to expand scenarios covered and to accommodate the rapidly changing technical landscape.

Methods

The working group consisted of experts in NGS, bioinformatics, quality management, and accreditation requirements from academic and commercial entities. Members included clinical laboratory directors, bioinformaticians, clinicians, and individuals engaged in developing CAP's accreditation guidelines. Regular meetings were held by phone to review and iteratively improve the worksheets.

Results

The working group created five Excel worksheets (Microsoft, Redman, WA), each containing background, instructions, and step-by-step guidance with examples for the different phases of NGS test development and implementation. These worksheets cover i) test design, ii) optimization, iii) validation, iv) quality management, and v) bioinformatics. The working group recognized that bioinformatics was an integral part of all phases, but a separate worksheet was created because the community had recognized bioinformatics processes as a complex entity that required separate attention, as evidenced by the publication of published focused recommendations[12] and the existence of a separate section in the CAP checklist.[13] The workgroup did not address variant prioritization, interpretation, and reporting.

Test Design

This worksheet focuses on the strategic considerations that a clinical laboratory director may consider before test development, including selection of genes and diseases as well as technical criteria to closely examine the genes and associated genomic regions in a test. This information will help the laboratory understand the variant spectrum for a particular

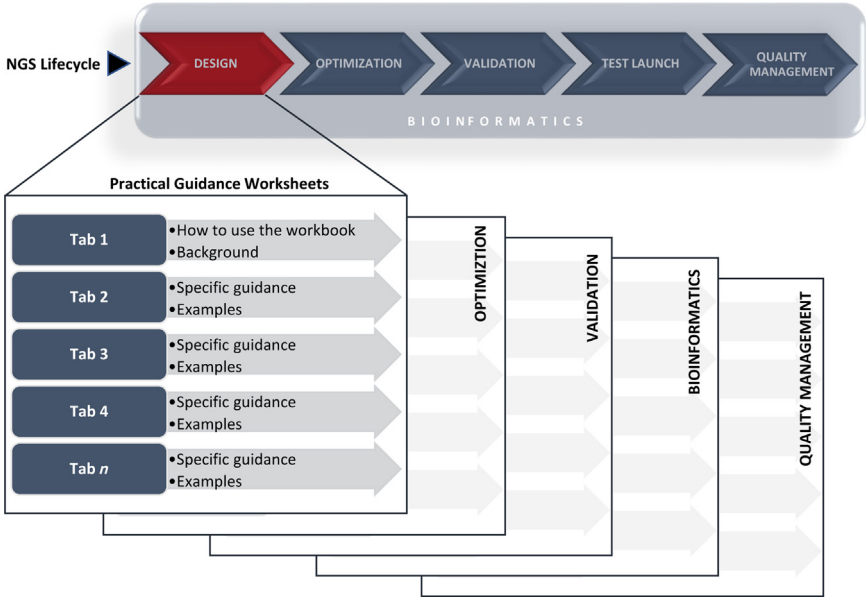

Figure 1 Next-generation sequencing (NGS) lifecycle and worksheet architecture. Standard phases of NGS test development and operations are shown on top. Below, the general architecture of workbooks that were designed to cover each phase is outlined. Each workbook included several tabs with practical, step-by-step guidance as well as background, instructions on how to use, and examples.

disease or gene panel and will influence the selection of technology that will facilitate optimal variant detection. For example, being aware of regions of high homology or GC content will allow laboratories to use focused enrichment strategies to ensure adequate coverage of the tested genes or alternative bioinformatics pipelines to capture challenging variation.[10]

Test Optimization

This worksheet translates the test design requirements to an initial assay design, including defining the coverage over the target regions, capture, and sequencing methodologies and any supplementary assays as needed. A target calculator estimates the size of the genomic region to be covered and, given a particular sequencing instrument/kit combination, how many samples can be sequenced per run and how many samples can be comfortably processed per week. The worksheet will also trigger considerations surrounding contamination and specimen identity and determination of procedures regarding confirmatory testing of variants called by NGS. After the test is optimized, acceptance and rejection criteria for each part of the process may be determined from iterative runs.

Test Validation

Typically, a new test validation is performed to cover all steps included in the test. Traditionally, this includes all steps from DNA extraction to the final result (clinical report), although clinical NGS tests are increasingly modularized such that parts of the process (eg, sequencing, bioinformatics, or clinical interpretation) are outsourced to another clinical entity. Regardless of whether a laboratory performs all or part of the testing process, the laboratory offering the test is responsible for documenting that the entire process was properly validated. Typical analytical performance metrics, including analytical sensitivity, specificity, reproducibility, and repeatability, are described along with associated formulas, suggested reference materials, and calculators. In addition, example worksheets on validation study design and subsequent data analysis are included. From the validation data, a laboratory may derive inferences on the limitations of the test in the detection of certain variant types. Such limitations along with any

Table 2 Architecture and Content of the NGS Guidance Worksheets

Worksheet	Tab name
Test content design considerations	Gene disease variant info
	Gene technical info
	Region list
Assay design and test optimization	Assay requirements
	Initial assay design
	Assay details
Test validation	Performance metrics
	General resources
	Example sample list
	Example validation grid
	Example performance metrics
	Assay limitations
Quality management	Test QC checklist
	Test QC monitoring
	Documentation list
Bioinformatics and IT considerations	Determining compute infrastructure
	Infrastructure considerations
	Tertiary analysis
	Validation log

IT, information technology; QC, quality control.

recommendations for additional testing are typically provided in the test report to ensure that clinicians are informed.

Quality Management

This worksheet provides an overview of the procedure monitors for the preanalytical, analytical, and postanalytical phases of NGS-based testing. These monitors are used at the time of patient testing and typically include checkpoints to test whether the reagents, equipment, and informatics pipelines are delivering accurate results. Many of these metrics in each phase of testing (preanalytical, analytical, and postanalytical) are monitored on a periodic basis such as quarterly or annually to track for trends and corrective actions. Although examples are provided in the worksheet, laboratories may choose monitors that most closely reflect individual procedures and protocols. Proficiency testing and laboratory development quality assessment programs are used to assess overall laboratory performance and are necessary to identify errors during the analysis and interpretation of an assay. Formal proficiency testing programs for NGS-based sequencing and exome/genome interpretation are now available through CAP.

Bioinformatics and Information Technology

This worksheet provides an overview on critical considerations for the compute infrastructure as well as selection and validation of informatics approaches for tertiary processing of samples. Criteria for determining the appropriate computing infrastructure are described. Requirements for cost, privacy, and vendor selection may also be influenced by the laboratory setting (private versus hospital based). Data retention, storage, and maintaining appropriate security measures are increasingly critical for the laboratory budget and for ensuring patient privacy. Finally, selection of appropriate tools for annotation, filtration, prioritization, and analysis influence the interpretation for patient data and therefore are critical to validate. Different approaches toward validation of these pipelines are described.

Figure 1 outlines the general worksheet architecture. To the extent possible, all worksheets follow the same concept by breaking down the relevant content into discrete subtopics, each represented by a separate tab. The next section describes the worksheet architecture in more detail, using one topic (test validation) as a representative example.

Worksheet Content

TAB-1: Before You Begin

This tab is part of every worksheet and contains a general introduction of what is covered on subsequent tabs. For test validation, this includes background on the validation approach recommended by the CAP (checklist requirement MOL.30785), overview and definitions of standard Clinical Laboratory Improvement Amendments performance metrics (such as analytical sensitivity, specificity, inter- and intra-run variability), approaches to derive these performance metrics from validation data, and commonly used sources for reference materials.

TAB-2 to TAB-*n*

These tabs cater to the specific needs of each topic covered by the different worksheets and provide step-by-step guidance with examples. For test validation, these include i) a sample list of assay specifications that summarize all settings and variables of the test, ii) a general resource tab, iii) a sample grid that provides a representative validation schema, iv) an example of a how to construct an informative list of validation samples accompanied by critical annotations, v) a detailed performance summary sheet, and vi) a set of questions designed to catalog assay limitations and prompt defining follow-up action if needed. Table 2 summarizes the content of all worksheets.

Discussion

The worksheets presented here provide detailed guidance on how to design, validate, and implement NGS tests for germline variant detection in inherited disorders. In addition to the discussion of test optimization and validation, the working group thought it was important to provide dedicated resources for test design, quality management, and bioinformatics and information technology considerations.

The intent of the worksheets is to address the gap between existing published guidelines and recommendations and the detailed implementation guidance needed in the clinical laboratory. This type of guidance is especially needed in clinical laboratories when first establishing NGS-based testing.

The worksheets are also designed to complement the CAP checklist, which is structured to provide general requirements to laboratories and to facilitate laboratory inspections. The working group recognizes that these documents do not address all NGS technologies or all aspects of the representative technologies selected. Although some consolidation has already occurred, the current NGS landscape is still highly complex and fast evolving. As such, the working group intended this work to provide baseline guidance for commonly used platforms and processes. We envision regular updates and expansions as this field continues to evolve. An important future goal is to migrate from Excel document–based guidance to a more dynamic and interactive web-based framework.

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References

1. Mattocks C, Morris M, Matthijs G, Swinnen E, Corveleyn A, Dequeker E, Müller CR, Pratt V, Wallace A; EuroGentest Validation

Group: A standardized framework for the validation and verification of clinical molecular genetic tests. Eur J Hum Genet 2010, 18:1276–1288
2. Gargis AS, Kalman L, Berry MW, Bick DP, Dimmock DP, Hambuch T, et al: Assuring the quality of next-generation sequencing in clinical laboratory practice. Nat Biotechnol 2012, 30:1033–1036
3. Schrijver I, Aziz N, Farkas D, Furtado M, Gonzalez AF, Greiner TC, Grody WW, Hambuch T, Kalman L, Kant JA, Klein RD, Leonard DG, Lubin IM, Mao R, Nagan N, Pratt VM, Sobel ME, Voelkerding KV, Gibson JS: Opportunities and challenges associated with clinical diagnostic genome sequencing: a report of the Association for Molecular Pathology. J Mol Diagn 2012, 14:525–540
4. Rehm HL, Bale SJ, Bayrak-Toydemir P, Berg JS, Brown KK, Deignan JL, Friez MJ, Funke BH, Hegde MR, Lyon E; Working Group of the American College of Medical Genetics and Genomics Laboratory Quality Assurance Committee: ACMG clinical laboratory standards for next-generation sequencing. Genet Med 2013, 15: 733–747
5. CLSI: Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine; Approved Guideline – Second Edition. CLSI document MM09-A2. Wayne, PA, Clinical and Laboratory Standards Institute, 2014
6. Schrijver I, Aziz N, Jennings L, Richards CS, Voelkerding KV, Weck KE: Methods-based proficiency testing in molecular genetic pathology. J Mol Diagn 2014, 16:283–287
7. Gargis AS, Kalman L, Bick DP, da Silva C, Dimmock DP, Funke BH, et al: Good laboratory practice for clinical next-generation sequencing informatics pipelines. Nat Biotechnol 2015, 33:689–693
8. Aziz N, Zhao Q, Bry L, Driscoll DK, Funke B, Gibson JS, Grody WW, Hegde MR, Hoeltge GA, Leonard DG, Merker JD, Nagarajan R, Palicki LA, Robetorye RS, Schrijver I, Weck KE, Voelkerding KV: College of American Pathologists' laboratory standards for next-generation sequencing clinical tests. Arch Pathol Lab Med 2015, 139:481–493
9. Matthijs G, Souche E, Alders M, Corveleyn A, Eck S, Feenstra I, Race V, Sistermans E, Sturm M, Weiss M, Yntema H, Bakker E, Scheffer H, Bauer P: Guidelines for diagnostic next-generation sequencing. Eur J Hum Genet 2016, 24:2–5
10. Santani A, Murrell J, Funke B, Yu Z, Hegde M, Mao R, Ferreira-Gonzalez A, Voelkerding KV, Weck KE: Development and validation of targeted next-generation sequencing panels for detection of germline variants in inherited diseases. Arch Pathol Lab Med 2017, 141:787–797
11. Hegde M, Santani A, Mao R, Ferreira-Gonzalez A, Weck KE, Voelkerding KV: Development and validation of clinical whole-exome and whole-genome sequencing for detection of germline variants in inherited disease. Arch Pathol Lab Med 2017, 141:798–805
12. Roy S, Coldren C, Karunamurthy A, Kip NS, Klee EW, Lincoln SE, Leon A, Pullambhatla M, Temple-Smolkin RL, Voelkerding KV, Wang C, Carter AB: Standards and guidelines for validating next-generation sequencing bioinformatics pipelines: a joint recommendation of the Association for Molecular Pathology and the College of American Pathologists. J Mol Diagn 2018, 20:4–27
13. College of American Pathologists, Commission on Laboratory Accreditation: Molecular Pathology Checklist. August 21, 2017 edition: Northfield, Illinois, College of American Pathologists, 2017