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UPDATED and REVISED

Genetic Testing – Molecular

Next Generation Sequencing (NGS) guidelines for germline genetic variant detection

The following describes requirements for the development of procedures and the establishment of performance (validation) of assays for the detection of genetic germline variants by Next Generation/massively parallel sequencing (NGS) technologies. These requirements should be used in conjunction with and not in lieu of any other applicable checklist at https://www.wadsworth.org/regulatory/clep/clinical-labs/obtain-permit/test-approval. Overall, clinical validation of NGS assays follows the same basic principles for validating most other complex molecular diagnostic procedures. It is anticipated that these guidelines will evolve as the field matures. Please make sure you use the most up-to-date version of these guidelines.

Items that must specifically be addressed in the validation submission include:

SOP

- Summarize the purpose of the test and the indications for testing (intended use).
- Describe the region(s) of the genome targeted by the assay as well as the type(s) of variants the assay is intended to detect, with any associated limitations. For targeted NGS panels, which target a limited number of genes, provide a list of the genes on the panel. Indicate whether subpanels of genes or a single gene within the panel can be requested. Provide justification for the selection of genes citing key references demonstrating clinical relevance.
- Provide a step-by-step description of the entire testing process, from sample receipt through library preparation, sequencing, data analysis and interpretation, including detailed steps of all automated processes. If a third party performs any function, including data analysis and/or interpretation, then the protocols must detail their specific involvement in the overall testing process.
- Include the criteria for when confirmatory testing is performed and indicate the specific methods used.
- Indicate if targeted familial testing is available.
- Describe the use of patient clinical information used to guide analysis of NGS data and facilitate interpretation of genetic variants causative of the observed phenotype.
- State the acceptability criteria for all variant types as defined by your validation studies.

QC

- Quality control metrics and acceptance criteria must be clearly defined in the SOP.
- Quality of base scoring must meet a minimum of Q20 or equivalent per base for all single nucleotide polymorphism (SNP) and insertion deletion (indel) calls. Quality of base scoring for copy number variants (CNVs) and other structural variants must be clearly defined based on validation studies.
- Establish minimum criteria for depth and uniformity of coverage. A minimum average of 30 unique reads or greater is **strongly** recommended.
- Define the minimum coverage required to call a variant and minimum percent of variant reads to determine both heterozygous and homozygous calls.

- Define maximum allowable strand bias (if applicable).
- New reagent lots require verification/confirmation of the analytical sensitivity and specificity to ensure that variants will not be missed by new lots of reagents. This applies to all critical reagents and includes depth and uniformity of coverage to detect possible target area drop out.
- All software updates that affect key parameters, such as base calling, alignment, etc., must be revalidated using data from at least 3-5 previously analyzed runs to verify that all variants are still detected with the same analytical sensitivity and specificity as previously determined. The revalidation process must be clearly described in the SOP.
- If analysis of a subpanel of genes within a larger panel can be requested (e.g., analysis of dilated cardiomyopathy only within a comprehensive cardiomyopathy panel), provide your procedure for masking data for unrequested targets.
- Data retention: All FASTQ files (or equivalent) should be maintained for a minimum of 2 years per *Document and Specimen Retention Standard of Practice 8 (DSR S8): Analytic System Records Retention.*
- All QC metrics must be followed and documented over time to verify that there is no decrease (drift) in performance, e.g., Levy-Jennings plots, tracking allele fractions (AFs) of positive controls.

Controls

- A **No Template Control** (NTC) must be included and taken through the entire testing process (including sequencing) to verify that there is no contamination across samples and reagents.
- A **positive control** must be included in each run. We suggest this control contain multiple known variants of each type to be detected by the assay to verify that all variant types and zygosities can be detected. The control input should be at the validated limit of detection for the assay. A defined rotation schedule should be employed if not all variant types can be incorporated in a single control sample and/or if multiple positive controls are used.

Reports

- Reports should include all detected variants, whether of known or unknown clinical significance, in a manner clearly identifying each variant's significance with supporting level of evidence as described in the AMP guidelines (http://jmd.amjpathol.org/article/S1525- 1578(16)30223-9/pdf). However, we would not object to the inclusion of a statement such as *"This test is designed to detect x, y and z…in genes a, b and c… However, variants other than the ones listed above may also have been detected. If interested, these can be released upon request"*.
- Variants can only be reported if they are confirmed by an independent reference method or if the assay has been fully validated and confirmation is no longer necessary (see Validation section below for more information on confirmation testing and full validation requirements).
- Reports must clearly define the laboratory's policy for reporting incidental findings (see the most recent list published by the American College of Medical Genetics and Genomics). We suggest you include these on your report separately and alert the treating physician to their potential clinical relevance.
- Reports must include a statement(s) that identify the limitations of the assay.
- Reports must include a statement indicating when a target area(s) of the assay failed to meet the lab's acceptability criteria.
- Reports must indicate any limitations in the detection of specific types of variants (e.g., CNV, maximum length of indels).
- If the full list of targeted genes is not included on the report, then reference to where this list can be found must be included on the report.
- Reports should contain a statement regarding the laboratory's policy for variant reanalysis.

Validation

- Provide a detailed description of all validation studies. All data must include, at minimum, gene/variant nomenclature, allele fractions, coverage, and Q score.
- Performance characteristics must be **established and validated separately** for **each type** of variant the assay is intended to detect, e.g., single nucleotide polymorphisms (SNPs), insertions, deletions, and copy number gains & losses.
- Performance characteristics for each sample type (e.g., blood, buccal, cultured cells) must be established and validated along with demonstration of quality sequences for all target areas without sample type bias. Areas that consistently fail to meet minimum quality metrics must clearly be defined. **Note:** cell-free DNA (cfDNA) requires separate full validation from all other specimen types (see Full Validation below).
- **Analytical accuracy**: Sequence a minimum of 2 well-characterized reference samples (e.g., HapMap DNA NA12878, NA19240, or Genome in a Bottle) to determine a robust laboratory specific error rate across all areas targeted by your assay (specificity). This error rate is expected to be < 2%.
- **Limit of Detection (LOD)**: Establish for each type of variant detected by the assay the lower limit of nucleic acid input (e.g., ng/µl) using a minimum of 3-5 patient specimens.
- **Initial validation**: Must include a minimum of 25 unique patient samples representing all specimen types accepted, with a representative distribution of variant types across all target areas (including GC-rich and homopolymer sequences) and must be confirmed by an independent reference method. The independent reference method cannot utilize the same technology as the NGS platform unless it is performed in a different lab. Provide a summary table of variants detected in each sample, as well as independent reference method results as obtained from orthogonal testing.

  NOTE: RNA transcript sequencing requires their own validation. Contact clepval@health.ny.gov to discuss requirements.

  Full validation: For reportable variants that have not yet been fully validated, an ongoing validation, i.e., confirmation by an independent reference method, must be performed until such a time that the criteria outlined below have been met.

  - Full validation for SNPs will be considered in 3 separate categories:
    1) Standard sequence (not GC rich or homopolymer)
    2) GC rich (60% or greater)
    3) Homopolymers (≥ 3 bases)

    Confirmation is required within each category until the following number of cases have been reached:

    For Category 1, full validation requires confirmation of 50 SNPs with no more than 5 cases in a particular gene. Once an individual gene under category 1 has been confirmed 5 times that gene no longer requires confirmation before reporting.

    For categories 2 **and** 3 (they are treated separately), full validation requires confirmation of 75 SNPs for each category with no more than 5 confirmations in a particular gene. Once an individual gene under categories 2 and 3 has been confirmed 5 times that area no longer requires confirmation before reporting.

  - Full validation for indels will be considered in 3 separate categories:
    1) Standard sequence (not GC rich or homopolymer)
    2) GC rich (60% or greater)
    3) Homopolymers (≥ 3 bases)

    Confirmation is required within each category until the following number of cases have been reached:

    For Category 1, full validation requires confirmation of 50 indels with no more than 5 cases in a particular gene. Once an individual gene under category 1 has been confirmed 5 times that gene no longer requires confirmation before reporting.

For categories 2 **and** 3 (they are treated separately), full validation requires confirmation of 75 indels for each category with no more than 5 confirmations in a particular gene. Once an individual gene under categories 2 and 3 has been confirmed 5 times that area no longer requires confirmation before reporting.

- Full validation for CNVs will be considered in 3 separate categories:
  1) Single gene gains and losses
  2) Large gains and losses that cover entire chromosomal arms
  3) Moderate gains and losses that fall in size between the above two categories, typically megabase in size.

  Confirmation is required within each category until the following number of cases have been reached:

  For category 1, full validation requires confirmation of 100 gains/losses with no more than 10 cases at a particular locus. Once an individual locus under category 1 has been confirmed 10 times that area no longer requires confirmation before reporting.

  For categories 2 **and** 3 (they are treated separately), full validation requires confirmation of 50 gains/losses for each category with no more than 5 confirmations at a particular locus. Once an individual locus under categories 2 and 3 has been confirmed 5 times that area no longer requires confirmation before reporting.

- **Reproducibility:** For each type of variant (i.e., SNP, indel, CNV), a minimum of 3 unique positive patient samples must be analyzed in 3 independent runs on different days. If possible, perform these studies by multiple technologists and sequencers. Reproducibility studies for all intended variant types should be performed at the lower limit of nucleic acid input.
- The initial validation studies should be done with a single version of all software utilized.