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 the existing Genetic Testing - Molecular checklist available

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 and gains experience. Please make sure you use the most up-to-date version of these guidelines. Issues that must specifically be addressed include:

SOP
➢ Summarize the purpose of the test and the indications for testing (intended use).

➢ Must include a description of 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.

➢ Must include a step-by-step description of the entire testing process, from sample receipt through library preparation, sequencing, data analysis and interpretation. 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.

➢ Must include the specific procedure(s) used for confirmation testing, including criteria for when confirmation must be performed.

➢ Indicate if targeted familial testing is available.

➢ Describe your process for collection of patient clinical information used to guide analysis of NGS data and facilitate interpretation of genetic variants causative of the observed phenotype.

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.

➢ Establish minimum criteria for depth and uniformity of coverage.

➢ Define the minimum coverage required to call a variant and minimum percent of variant reads to determine a heterozygous call.

- Define maximal allowable strand bias (if applicable).

- New reagent lots require verification/confirmation of the analytical sensitivity 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 QC metrics must be followed and documented over time to verify that there is no decrease (drift) in performance.

- 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 & 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.

- If mitochondrial DNA sequencing is performed, provide the required quality metrics including depth of coverage and percent of variant reads to report heteroplasmy.

- 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.*

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.

Reports
- Reports must include a statement(s) that identify the limitations of the assay, including which target area(s) the assay lacked adequate coverage to confidently determine variant status

- Reports must indicate any limitations in the detection of specific types of variants (e.g. copy number variants (CNV), repeat expansions, maximum length of indels)

- Reports must indicate any variants identified but not included on the report (e.g. benign polymorphisms)

- 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.

- Performance characteristics must be **established and validated separately** for **each type** of variant the assay is intended to detect, e.g. single nucleotide variants (SNVs), insertions, deletions, copy number gains & losses, repeat expansions.

- 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.

- **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%.

- ➤ **Initial validation**: Must include a minimum of 25 patient samples representing all specimen types accepted, with a representative distribution of variant types across all target areas. The number of samples multiplexed in a single run should be representative of the number anticipated for routine testing. Provide a summary table of variants detected in each sample.

- ➤ We expect all reported variants to be confirmed by an independent reference method, however, for targeted gene panels, we will consider waiving the requirement for confirmation of single nucleotide variants (SNVs) under the following conditions:
  - a. depth of coverage is ≥100X
  - b. at least 10 variants have previously been confirmed for that gene
  - • At this time, all other types of variants (i.e. indels, large deletions, repeat expansions, copy number gains and losses) must always be confirmed by an independent reference method.

- ➤ **Reproducibility:** For each type of variant (i.e. SNP, indel, CNV), a minimum of 3 positive patient samples must be analyzed in 3 independent runs on different days by 2 different technologists and sequencers (if possible).

- ➤ If multiplexing samples with distinct barcodes, it must be verified that there is no cross talk between samples and barcodes and that the combinations of patients/barcodes in a run provides reproducible results for all target areas and types of variants independent of which patient/barcode combination is used.

- ➤ Please provide sample data sets for three anonymized cases including the initial variant call file and an explanation of the process for filtering the variant call file down to those variants that are reported. Include the clinical information obtained for that patient, data from additional testing that was performed (i.e. Sanger sequencing confirmation, copy number variant testing, etc.), and the final report that was generated from each case. These cases should represent the different types of variants detected by the assay.

- ➤ The initial validation studies should be done with a single version of all software utilized.