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Validation of Next Generation Sequencing (NGS)-Based Methods for Identification and/or Characterization of Infectious Agents (Isolates only)

The following guidelines are applicable to whole-genome sequencing (WGS) using NGS-based methods for identification and/or characterization of infectious agent isolates. These guidelines should be used in conjunction with and not in lieu of the existing microbiology molecular guidelines: (http://www.wadsworth.org/labcert/TestApproval/forms/Microbiology_NAAT_Checklist.pdf).

Overall, clinical validation of NGS assays follows the same basic principles that have been established for validating most other complex molecular diagnostic procedures. It is anticipated that these guidelines will evolve as the field matures and more experience is gained. Please make sure you use the most up-to-date version of these guidelines (http://www.wadsworth.org/labcert/TestApproval/index.htm).

General Requirements:

- The detailed standard operating procedure manual (SOPM) must include all relevant quality assurance and proficiency testing details for this test.
- The SOPM must include a step-by-step description of all steps involved, from template to library preparation to data analysis and interpretation of results.
- The SOPM must include all expected reporting and reflex testing scenarios. The SOPM must clearly define what will be reported from the NGS results and what will not be reported. It must include or refer to the procedure(s) for confirmation testing, including clear criteria for when confirmation is required.
- The SOPM must include statements that identify the limitations of the assay.

Reporting

- In addition to the actual results of the NGS analysis, test reports must include a clinically relevant interpretation of the findings. Representative examples of test reports must be included with the submission.
- Incidental findings of unknown significance should not be reported (*e.g.* if a mutation is detected that has an unknown impact on drug resistance it should not be included on the test report).

- Reports should include any appropriate disclaimer(s) for the assay, including technical and clinical limitations.

QC Guidance:

- Quality of base calling and read mapping should be included in the SOP and meet a minimum of Q20 or equivalent per base and Q20 or equivalent per mapped read.

- The SOP should describe proposed parameters for genomic variant reporting which are supported by validation data. For example, when testing bacterial isolates, a variant is expected to be detected in at least 95% of the reads for single nucleotide polymorphisms (SNPs) and at least 80% for insertions/deletions to be reported. These same parameters would not apply to viral isolates that may contain mixed populations.

- Minimum criteria should be established for depth and uniformity of depth of coverage (*i.e.* number of reads, across all target areas). A minimum average of 40X depth of coverage is recommended. For regions used for strain identification or mutation detection, a minimum depth of coverage of 20X is recommended.

- All QC metrics must be documented and monitored over time to verify that there is no decrease in performance.

- New reagent lots/shipments require positive and negative control testing, prior to or concurrent with, initial clinical use. If performed concurrently with patient testing, all QC results (as described in the SOPM) must be reviewed and meet the acceptance criteria prior to release of patient results. This applies to all critical reagents.

- All software updates that affect key processes such as base calling, alignment, etc., should be revalidated using data from at least 3 previously analyzed runs to verify that all data are generated with at least the same run parameters as previously determined. This validation should also verify that coverage depth and variant read prevalence is not significantly different between the two software versions. The revalidation procedure should be clearly described in the SOPM.

Controls:

- A Reagent Contamination Control (*i.e.* no-template control) must be included in all polymerase/amplification steps involved in NGS (including the library preparation) to verify that there is no contamination across samples and reagents. This control should be analyzed by any suitable method (*e.g.* agarose gel electrophoresis or dsDNA mass measurements with a Qubit assay kit or similar measurement) before proceeding to sequencing of samples.

- There should be a control procedure with clearly specified parameters that ensures that the quality of the DNA starting material for NGS is adequate. For example, on certain instruments, a control such as PhiX that can verify base calling with >90% Q30 should be included according to manufacturer's recommendations. This control can serve as the positive sequencing control for the run.

Validation:

Analytical performance must be established for each extraction procedure, library preparation procedure, and data analysis or result interpretation procedure. The validation must include demonstration of the accuracy and reproducibility of the bioinformatics process. The initial validation studies should be performed with a single version of all analysis software.

- Specificity: At least 5 organisms must be tested in the specificity panel including those genetically related, organisms that could be isolated from the original specimen, and organisms that can produce similar symptomology or illness. If there is any misidentification, a procedure must be developed to show the results will be resolved or interpreted. All organisms tested and the results of their testing with the assay for the specificity study must be clearly documented.

- Inter-assay Reproducibility: At least three clinical isolates should be analyzed on three different days. These samples should be processed through the entire procedure from template to library preparation to data analysis and interpretation. Also, if different instruments, platforms, models or technicians will be used to perform the assay this variation should be included to demonstrate assay consistency.

- Intra-assay Reproducibility: At least three clinical isolates should be analyzed in triplicate. These samples should be processed through the entire procedure from template to library preparation to data analysis and interpretation.

- Accuracy Verification: At least 30 isolates that the assay is designed to detect and 10 negative isolates should be tested in a blinded validation study. The 30 isolates should include a representative range of currently circulating strains (including multiple genotypes or subtypes if applicable). When available, the results should be compared to a generally accepted method or FDA or CLEP approved assay.