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

The following guidelines are applicable to whole genome sequencing (WGS) and other Next Generation Sequencing (NGS) methods for identification and/or characterization of infectious agents, including viruses, bacteria, fungi, and parasites. These guidelines should be used in conjunction with and not in lieu of the existing microbiology molecular guidelines available at: https://www.wadsworth.org/regulatory/clep/clinical-labs/obtain-permit/test-approval.

The clinical validation of NGS assays should apply the same basic principles that have been established for validating most other complex molecular diagnostic procedures. These guidelines include considerations for both the NGS sequencing methods as well as the bioinformatic analysis. It is anticipated that these guidelines will evolve as the field matures and more experience is gained. Please utilize the most up-to-date version of these guidelines. (https://www.wadsworth.org/regulatory/clep/clinical-labs/obtain-permit/test-approval)

Standard Operating Procedure Manual (SOPM)

A Standard Operating Procedure Manual (SOPM) for NGS/WGS methods must include**:**

- The purpose or intended application of the assay.
  -Include all intended applications of the assay (*e.g.,* identification, detection, quantification, typing, subtyping, drug resistance, strain analysis/differentiation) and if samples tested will be pre-characterized by another method.
- Statements describing any limitations of the assay.
- Acceptable specimen type(s) and specimen collection and storage requirements.
- All quality controls used in the assay including controls to monitor reagent contamination, library preparation, sequencing, and analysis as well as all relevant quality assurance metrics.
- Instrumentation utilized in the assay, including the specific model used, as well as all reagents, consumables, and sources.
- Step-by-step procedures for the entire testing process including, as applicable:
  -Specimen processing, nucleic acid extraction, amplification, fragmentation and size selection, cDNA synthesis (for RNA templates), library preparation,

all post-processing steps.

-All laboratory-based QC steps and criteria for assessing nucleic acid quantity and purity.

-All laboratory-based QC steps and criteria for assessing sequence quality.

-A detailed description of the bioinformatic analysis including documentation of programs and software versions utilized, as well as data quality metrics and quality control criteria for base calling, filtering, and trimming parameters, read mapping, assembly, and depth and uniformity of coverage and final interpretation.

-A detailed description of the interpretation algorithm, including criteria that will be used for determining confidence of the interpretation, if applicable.

-A detailed description of any database utilized for data analysis and procedures for either updating a local database or assuring external databases are current and updating them as applicable.

General Guidance on QC metrics for NGS/WGS methods

Note: Depending on the NGS methods, other QC criteria may apply.

- Parameters for genomic variant reporting which are supported by validation data should be defined (*e.g.*, allele frequencies, quality scores or any other statistical parameters supporting variant or invariant positions, indels or structural variations). Describe how contaminated samples or samples with mixed populations are reported as well as allele or target dropouts.
- Minimum criteria should be established to determine when a sample has been successfully sequenced and results are considered reliable and acceptable. These may include minimum depth of coverage, selection of the reference meeting minimum coverage, uniformity of coverage, percent of reads mapped, and/or other applicable criteria.
- Criteria for appropriate depth and coverage may vary depending on target agents, method, and purpose. The following criteria are given as general guides.

  -For bacterial WGS, a minimum average of 50X-100X depth of coverage is recommended depending on the purpose of the assay. For genomic regions used for strain identification or mutation detection, a minimum depth of coverage of 50X is recommended. If less than 50X coverage is used, justification must be provided.

  -For diagnostic assays relying on low allele frequencies, single nucleotide polymorphism (SNP) positions, rare subpopulations or quasispecies should use minimum genome coverages of 100X or higher.

  -For targeted NGS and targeted WGS applications, appropriate coverage may range from 40X-1000X depth depending on the agent, method, and purpose.
- It is also advisable to test the robustness of the analytic bioinformatic pipeline

under different conditions using simulated *in silico* or well characterized sets of data. For example, a pipeline's lower limit for accuracy can be assessed with *in-silico* serial down-sampling of sequencing reads. Simulation can also be used to test intermixture of populations of species in the data at different levels of contamination.

- All QC metrics must be documented and monitored over time to verify that there is no decrease in performance.
- New reagent lots and 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.
- The SOPM must include a description of how all software updates will be managed.
- For sequencing instrument updates or new instruments, verifications can include resequencing previously sequenced samples or concurrent side by side testing with a new and existing instrument.
- For analysis software/pipeline updates, parameters that affect key processes such as base calling, alignment, phenotypic interpretation etc., must be revalidated using data from at least 3 previously analyzed runs to verify that all data are generated with at least the equivalent analytical sensitivity and specificity as previously determined. For variant detection pipelines, the revalidation process must include procedures to verify that variant read prevalence is not different between the two software versions.

Controls for NGS methods

The assay must include controls that allow all steps of the procedure to be assessed. There should be a control procedure with clearly specified parameters that includes negative, positive, and inhibition controls depending on the test, sample type, and whether pre-characterization was performed:

- A negative/ 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, instruments that assess DNA and RNA quality and quantity, or dsDNA mass measurements) before proceeding to sequencing of samples.
- A positive control sample should be included that assesses the entire sequencing assay including nucleic acid extraction, library preparation and sequencing. One or more positive controls may be utilized to cover the entire process as applicable. For example, one positive control could be utilized through extraction and library prep and a second control such as PhiX can be utilized as the positive control for the sequencing step of the assay. A PhiX control can be

used to verify base calling accuracy according to manufacturer's recommendations.

- An internal/ inhibition control must be utilized when sequencing directly from primary clinical specimens unless previous molecular testing/pre-characterization and inhibition assessment is performed.

Reporting for WGS/NGS methods

- Reports should include the method utilized such as next generation sequencing (NGS), targeted NGS or whole genome sequencing (WGS).
- Test reports should include clinically relevant interpretations of the findings supported by peer reviewed publications, clinical guidelines, or recognized standards of practice. Representative examples of test reports must be included with the submission.
- Incidental findings not supported by peer reviewed clinical studies, guidelines or recommendations should either not be listed on the report or clearly listed as incidental.
- Reporting mixed populations or heteroresistance must be detailed in the SOP and validated for reporting.
- Reports must include any appropriate disclaimer(s) for the assay, including technical and clinical limitations.

Validation Guidelines for NGS/WGS methods

Analytical performance must be established for aspects of the assay including extraction procedures, library preparation, data analysis and result interpretation in the validation. The validation must include demonstration of the accuracy and reproducibility of the bioinformatic process. The initial validation studies should be performed with a single, locked-down version of the data analysis software or pipeline.

Validation Guidelines for WGS of isolates/culture

- **Specificity**: Depending on the application, a small number of organisms (5-10) should be tested in an exclusivity specificity panel including those genetically related organisms that could be isolated from the original specimen, and organisms that can produce similar symptoms or illness. A larger number of organisms should be included to demonstrate inclusivity of the assay for the intended use. If there are any misidentifications, a procedure must be developed to show how the results will be resolved or interpreted. All organisms tested and their results must be clearly documented.
- **Inter-assay Reproducibility**: At least three clinical isolates should be analyzed on each of three different days. These samples should be processed through the entire procedure from template & library preparation to data analysis and interpretation. If different instruments, platforms, models, or technicians will be used to perform the assay these variables should be included to demonstrate

assay consistency.

- **Intra-assay Reproducibility**: At least three clinical isolates should be analyzed in triplicate on the same day. These samples should be processed through the entire procedure from template & library preparation to data analysis and interpretation.
- **Accuracy**: At least 30 isolates that the assay is designed to detect and representing a wide range of potentially reportable results must be tested in a blinded and randomized 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 generally accepted methods (*e.g.*, susceptibility testing) or FDA or CLEP approved assays.

Validation Guidelines for NGS methods using primary clinical specimens

- **Sensitivity**: The limit of detection (LOD) for targeted, amplification-based NGS assays must be established. However, depending on the number of potential targets, the approach for conducting the LOD study may vary. The LOD must be established for all assays regardless of whether the assay is intended for pathogen detection (*i.e.*, aiding in the diagnosis of infection) or for characterizing infectious agents in specimens known to be positive for the agent (*e.g.,* drug resistance testing, genotyping/subtyping). An exception may be made in some cases where NGS is only performed as a reflex test on specimens that are first tested using a CLEP-approved or FDA-approved detection assay and specific criteria for reflexing to the NGS assay have been established.

  -For highly multiplexed assays, determining an LOD for every possible target may not be feasible. For these assays, dilution series should be prepared by spiking specimen matrix with representatives from each major category of pathogen for the which the assay is designed to detect (*e.g.*, sequencing applications designed to detect or identify multiple viruses should include representative viruses from each relevant major virus family in the LOD study).

  -The LOD study should be performed by using whole organisms or whole viruses, when available and consist of six to eight 10-fold dilutions in specimen matrix with at least one dilution that goes to extinction.

  -Data from LOD studies performed in triplicate on 3 different days may be used to satisfy validation requirements for intra- and inter-assay reproducibility.

  -Separate LOD estimates are required for each sample type or specimen source. For example, separate LOD estimates would be required for respiratory swabs and respiratory washes (*e.g.,* BAL specimens), serum, CSF, tissues, stool, etc.

  -Data should be submitted to validate the detection of co-infections demonstrating the absence of adverse effect on the LOD by the presence and detection of multiple agents in a sample. Mock samples including up to 4 agents or targets with a mix of high and low concentrations should be included.

-For metagenomic sequencing assays, a LOD should be determined for representative members of each category of pathogen that may be reported for the assay (*e.g.,* DNA virus, RNA virus, gram-positive bacteria, gram-negative bacteria, mold, yeast, parasite).

- **Specificity:** Targeted NGS applications for primary clinical specimens should be performed.

  -Specificity testing should include a small number of organisms (5-10) tested in an exclusivity specificity panel that includes genetically related organisms that could be isolated from the original specimen, and organisms that can produce similar symptoms or illness.

  -A larger number of organisms should be included to demonstrate inclusivity of the assay when the assay is intended to detect or genotype a large number or a variety of organisms.

  -If there are any misidentifications, a procedure must be developed to show how the results will be resolved or interpreted. All organisms tested and their results must be clearly documented.

  -Specificity testing is not needed for non-targeted NGS applications intended to detect all DNA or RNA in a sample.

- **Inter-assay Reproducibility:** At least three clinical specimens should be analyzed on each of three different days. These samples should be processed through the entire procedure from template & library preparation to data analysis and interpretation. If different instruments, platforms, or models will be used, and different operators will be performing the assay, the inter-assay reproducibility study should be designed to evaluate the impact of these variables on assay consistency.

- **Intra-assay Reproducibility**: At least three clinical specimens should be analyzed in triplicate under fixed conditions (same day, same operator) to assess intrinsic variability of the assay. These samples should be processed through the entire procedure from template & library preparation to data analysis and interpretation.

- **Accuracy Verification**- Thirty clinical specimens should be tested using the assay and an appropriate comparator method. Specimens should include a representative range of currently circulating strains, genotypes, or subtypes, if applicable. The expected results should be blinded to individuals performing laboratory and data analysis components of the assay. If a comparator assay is not available, specimens should be spiked with different concentrations of infectious agents including a representative range of currently circulating strains, genotypes, or subtypes if applicable. Ten of these specimens should be spiked with concentrations of infectious agent near the limit of detection of the assay.