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Oncology – Molecular and Cellular Tumor Markers

"Next Generation" Sequencing (NGS) guidelines for somatic genetic variant detection

The following describes requirements for the development of procedures and the establishment of performance (validation) of assays for the detection of somatic genetic variants by Next Generation/massively parallel sequencing (NGS) technologies. These requirements should be used in conjunction with and not in lieu of the existing molecular oncology guidelines (http://www.wadsworth.org/labcert/TestApproval/forms/Oncology_Molecular_Checklist.pdf). 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:
- must include step-by-step description of all steps involved, from template to library preparation to data analysis and interpretation
- must include indication for the specific procedure(s) used for confirmation testing, including criteria for when confirmation must be performed

QC:
- Quality of the base scoring must meet a minimum of Q20 or equivalent per base.
- Establish minimum criteria for depth and uniformity of coverage, i.e. number of reads, across all target areas. A minimum average of 500 reads or greater is **strongly** recommended.
- Define the minimum coverage required for a target area (such as amplicon, exon) below which you cannot confidently define the area's mutation status; the minimum coverage required may be different for mutated vs. unmutated calls.
- Define the minimum percentage of variant reads in a background of normal reads required to call a variant 'detected' at your established confidence and sensitivity.
- Define maximal allowable strand bias (if applicable).
- New reagent lots require verification/confirmation of the analytical sensitivity to ensure that low positives will not be missed by new lots of reagents. This also includes depth and uniformity of coverage to detect possible target area drop out. This applies to all critical reagents.
- All QC metrics must be followed and documented over time to verify that there is no decrease 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 genetic variants are still detected with the same analytical sensitivity as previously determined. This validation must also

verify that coverage depth and variant read prevalence is not significantly different between the two software versions. The revalidation process must be clearly described in the SOP.

Controls:

- A **No Template Control** (NTC) must be included in all amplification steps to verify that there is no contamination across samples and reagents. This control can be analyzed by any suitable method before proceeding to the actual sequencing.
- During the initial validation and periodically thereafter, a **negative control,** such as DNA from a HapMap cell line, e.g. NA12878 and/or NA19240, should be included to verify analytic accuracy and specificity for the detection of variants.
- A **positive/sensitivity control** should be included in each run. We suggest this control be an individually barcoded low positive DNA sample containing multiple known variants of each kind to be detected near the sensitivity of the assay, to verify that low percentage variants can be identified consistently. A defined rotation schedule should be employed if not all variants in all target areas can be incorporated in a single control sample.

Reports:

- Reports should include all detected somatic variants, whether targeted or incidental, and whether of known or unknown clinical significance, in a manner clearly identifying each variant's significance.

  Our intent in including all detected somatic variants on the report is to absolve the lab from having to potentially report these out at a later time. If the oncologist has all the NGS data then he/she would not have to contact the performing laboratory further in case new information from a clinical trial emerges. 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"*.
- Incidental findings of possible germline variants: we suggest you include these on your report separately and alert the treating physician to their potential clinical relevance.
- Reports must include statement(s) that identify the limitations of the assay, including for which target area(s) the assay lacked sufficient coverage to confidently determine mutational status.

Validation:

- 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), indels, copy number variants (CNVs) and structural variants.
- Performance characteristics for each sample type (e.g. FFPE, FF, WB, BM, FNA) must be established and validated, along with demonstration of quality sequences for all target areas without sample type bias.
- **Minimum** data required to establish key performance characteristics (please include the prevalence, i.e. number of total, normal and variant reads of each detected variant for all studies; a table and graph (e.g. histogram, or box-and-whisker plot) of read depths for each target area is recommended):
  - **Accuracy**: Sequence a well-characterized reference sample (e.g. HapMap DNA NA12878) to determine error rate across all target areas (specificity).
  - **Initial validation**: Must include a minimum of 50 patient samples (comprising specimens of all intended sample and tumor types; however, if FFPE is included then ≥ 75% of samples must be derived from FFPE) with a representative distribution of reportable variants across all target areas

(including GC-rich sequences), and 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.

- **Full validation**: 10 positive samples for each type of intended variant in each target area must be confirmed. For variants with clinical significance that have not yet been fully validated confirmation by an independent reference method must be performed on an ongoing basis until at least 10 positive samples per type of intended variant and target area have been verified using the independent reference method.
- Incidental findings of unknown significance for which there is no established confirmatory assay can be reported provided a disclaimer is included that clearly states that this variant has not been confirmed.
- Each reportable variant does not require confirmation every time it is encountered ONCE the type of variant and target area (gene) containing it was fully validated (10 confirmed positives for each variant type intended to be detected in that gene).
- **Precision** (within run)**:** for each type of variant a minimum of 3 positive patient samples containing variants preferably near the stated sensitivity of the assay must be analyzed in triplicate in the same run using different barcodes.
- **Reproducibility** (between run): for each type of variant a minimum of 3 positive patient samples containing variants near the stated sensitivity of the assay must be analyzed in three separate runs using different barcodes (from the original DNA through sequencing and data analysis) on different days by 2 different technologists (if possible).
- **Analytical sensitivity**: Establish the analytical sensitivity of the assay for each type of variant detected by the assay. This can initially be established with defined mixtures of cell line DNAs (not plasmids), but needs to be verified with 3-5 patient samples.

- Demonstrate the accuracy and reproducibility of the bioinformatics process, both for the detection of all types of variants and (if applicable) the identification and sorting of individually barcoded and multiplexed patient samples.
- The initial validation studies should be done with a single version of all analyses software.