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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 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:

- 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.
- Must include a step-by-step description of the entire testing process, from sample receipt through library preparation, sequencing, data analysis and interpretation.
- Must include the specific procedure(s) used for confirmation testing, including criteria for when confirmation must be performed.

QC:

- Quality control metrics and acceptance criteria must be clearly described in the SOP.
- 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 (genes). A minimum average of 500 reads or greater is **strongly** recommended.
- Define the minimum coverage required for a target area below which you cannot confidently define the area's mutation status; the minimum target area coverage required may be different for confidently calling a variant detected vs. having adequate depth of coverage to confidently call the target area free of detectable variants.
- Define the minimum percentage and/or number of variant reads in a background of normal reads required to call a variant 'detected' at your established level of confidence and sensitivity.

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- 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 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 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 analytical accuracy and specificity.
- A **positive/sensitivity control** should be included in each run. We suggest this control be a low positive sample (near the sensitivity of the assay) containing multiple known variants, of each kind to be detected by 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 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).

  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"*.
- Variants can only be reported if they are confirmed or if the assay has been fully validated and confirmation is no longer necessary (see below).
- 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, with a recommendation that the variants need to be confirmed by a laboratory permitted to perform germline genetic testing.

Reports must include statement(s) that identify the limitations of the assay, including for which target area(s) the assay lacked adequate coverage to confidently determine mutational status, maximum indel length, sensitivity for all variant types targeted by the assay, etc.

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), insertions, deletions, copy number variants (CNVs), structural variants, as well as MSI status and tumor mutation burden.
- Performance characteristics for each sample type (e.g. FFPE, FF, WB, BM, FNA, ctDNA) 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.
- **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):
    - **Analytical accuracy**: Sequence a minimum of 3 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 50 patient samples comprising specimens of all intended sample and tumor types. However, if FFPE is included then ≥ 75% of samples should be derived from FFPE. This is to ensure that the assay is robust when utilizing the most degraded source of sample input. These samples must contain a representative distribution of reportable variants across all target areas (including GC-rich 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.
        - ctDNA based assays must meet all validation requirements independently.
    - **Full validation**: 10 positive samples for each type of intended variant in each target area must be sequenced and confirmed. For reported variants with clinical significance identified during clinical runs that have not yet been fully validated an ongoing validation, i.e. confirmation by an independent reference method, must be performed for each distinct target area until such a time that at least 10 positive samples per type of intended variant have been verified using the independent reference method.
        - SNVs: Confirmation can be ceased once a **minimum of 20 target areas** have been fully validated/confirmed with accuracy greater than or equal to your established specificity. This requirement is independent of the size of the panel; panels smaller than 20 genes cannot meet this 20 target area requirement and thus all genes must be fully validated. Incorporation of additional target areas to a panel will require reassessment of specificity within those additional target areas. There should be particular attention paid to regions of the target areas with high probability of error (e.g. GC rich regions).

- Insertions and deletions: Confirmation can be ceased once a **minimum of 20 target areas** have been fully validated/confirmed with accuracy greater than or equal to your established specificity. This requirement is independent of the size of the panel; panels smaller than 20 genes cannot meet this 20 target area requirement and thus all genes must be fully validated. Incorporation of additional target areas to a panel will require reassessment of specificity within those additional target areas. There should be particular attention paid to regions of the target areas with high probability of error (e.g. homopolymer or repetitive regions).
- CNVs: All target areas you intend to report CNVs in must be confirmed a minimum of 10 times.
- Translocations will require a minimum of 3 confirmations per gene fusion partner **targeted** by the assay. For example, if the assay targets ALK then you need only confirm 3 positive ALK rearrangements to fully validate **any** rearrangements that contain the ALK gene as one of the fusion partners.
- MSI: Confirm 10 MSS & 10 MSI-H samples.
- Tumor mutation burden

- **Analytical sensitivity**: Establish the analytical sensitivity of the assay for each type of variant detected by the assay at the lower limit of nucleic acid input. This can initially be established with defined mixtures of cell line DNAs (not plasmids), but needs to be verified with 3-5 patient samples. You need to establish both the lowest input amount of DNA/RNA that still gives reliable results and the lowest variant allele fraction (VAF) that can reliably be detected at that amount of input DNA/RNA.

All precision and reproducibility studies should be performed for all intended variant types at the lower limit of nucleic acid input and VAF.

- **Precision** (within run)**:** for each type of variant a minimum of 3 positive patient samples must be analyzed in triplicate in the same run.
- **Reproducibility** (between run): for each type of variant 4-5 positive patient samples must be analyzed in 4-5 separate runs using different barcodes (from the original DNA/RNA through sequencing and data analysis) on different days by 2 different technologists and sequencers (if possible).
  - For SNVs, insertions, and deletions, a minimum of 20 replicates each must be analyzed with 95% overall reproducibility.
  - For structural variants a minimum of 3 positive patient samples (each targeting different fusion partners) must be analyzed in three separate runs on different days by 2 different technologist and sequencers (if possible).
  - For CNVs a minimum of 3 positive patient samples for gains and 3 positive patient samples for losses (all in different genes) must be analyzed in three separate runs on different days by 2 different technologists and sequencers (if possible).
  - For MSI, a minimum of 3 MSS and 3 MSI-H patient samples must be analyzed in three separate runs on different days by 2 different technologists and sequencers (if possible).

- For tumor mutation burden, a minimum of 3 patient samples in each class (e.g. low, indeterminate, high) must be analyzed in three separate 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.

- Demonstrate the accuracy, reproducibility, and sensitivity of the bioinformatics process, both for the detection of all types of variants and 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.

FDA 3rd party review

The third party review process is only available to laboratories with a current NYS permit in Oncology-Molecular and Cellular Tumor Markers. If you are interested in using the third party review process to submit your NGS oncology panel to the FDA through NYS, you must, in addition to the materials requested by NYS as described above, also ensure that all special controls listed in the MSK-IMPACT decision summary (section U.(b)) are addressed. These must be clearly identified in the submitted documents. Furthermore, you must also include with your submission a draft decision summary modelled after that for the MSK-IMPACT assay. The MSK-IMPACT decision summary can be found at https://www.accessdata.fda.gov/cdrh_docs/reviews/DEN170058.pdf.
In addition to the information as in the MSK-IMPACT decision summary, you must include a section comparing your assay to the predicate device (assay) that includes the following Substantial Equivalence Information:

1. Predicate device (assay) name(s):
2. Predicate 510(k) number(s)
3. Comparison with predicate (use as many rows as necessary)

	Similarities	
Item	**Your device (assay)** Name	**Predicate Device (assay)** Name
	Differences	
Item	**Your device (assay)** Name	**Predicate Device (assay)** Name

Requests for third party review of your assay must be made at the time of the original LDT submission to NYS and will be accepted at our discretion. If accepted, we will only act on your request once all requirements for NYS approval and the additional FDA requirements have been met. Note: NYS approval does not guarantee FDA clearance; the FDA will make the final decision whether your assay is substantially equivalent to the respective predicate device.

Please contact us regarding requests for LDTs that have already been reviewed and approved by NYS.

Further information about the 3rd party review process in general can be found on the FDA website at

https://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/HowtoMarketYourDevice/PremarketSubmissions/ThirdParyReview/ucm124005.htm

http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/HowtoMarketYourDevice/PremarketSubmissions/ThirdParyReview/ucm123993.htm

A slide deck describing the third party review process is available by request from erasmus.schneider@health.ny.gov