The Belgian next generation sequencing guidelines for haematological and solid tumours

A. Hébrant, Ir, PhD[1], G. Froyen, PhD[2], B. Maes, MD, PhD[2], R. Salgado, MD[3], M. Le Mercier, PhD[4], N. D'Haene, MD, PhD[4], S. De Keersmaecker, PhD[5], K. Claes, PhD[6], J. Van der Meulen, PhD[6], P. Aftimos, MD[7], J. Van Houdt, PhD[8], K. Cuppens, MD[9], K. Vanneste, PhD[5], E. Dequeker, PhD[8], S. Van Dooren, PhD[10], J. Van Huysse, MD[11], F. Nollet, PhD[12], S. van Laere, PhD[13], B. Denys, MD[12], V. Ghislain[14], C. Van Campenhout, PhD[14], M. Van den Bulcke, PhD[1]

SUMMARY

Targeted next generation sequencing is a complex procedure including the 'wet bench' and 'dry bench' parts. Both parts are composed of many steps for which optimal assay conditions and settings must be determined.

The aim of these guidelines is to provide generic, platform independent, recommendations for targeted next generation sequencing tests to detect acquired somatic mutations in DNA, in (haemato)-oncology that are complementary to the ISO 15189 norm (medical laboratories) in order to:

1) facilitate the implementation of the required quality metrics for the detection of somatic variants by next generation sequencing in oncology and haemato-oncology in the Belgian laboratories,
2) harmonise test validation and verification,
3) harmonise clinical interpretation and reporting of variants and,
4) assure and maintain optimal test performance by establishing procedures and modalities for internal quality control and external quality assessments.

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[1]Cancer Centre, Scientific Institute of Public Health (WIV-ISP), Brussels, Belgium, [2]Department of Clinical Biology, Jessa Hospital, Hasselt, Belgium, [3]Breast Cancer Translational Research Laboratory, Institut Jules Bordet, Brussels, Belgium and Department of Pathology, GZA Antwerp, [4]Department of Pathology, Erasme Hospital, Université Libre de Bruxelles, Brussels, Belgium, [5]Platform Biotechnology and Molecular Biology, Scientific Institute of Public Health (WIV-ISP), Brussels, Belgium, [6]Molecular Diagnostics Ghent University Hospital (MDG), Ghent University Hospital, Ghent, Belgium, [7]Medical Oncology Clinic, Institut Jules Bordet – Université Libre de Bruxelles, Brussels, Belgium, [8]Biomedical Quality Assurance Research Unit, Department of Public Health and Primary Care, KU Leuven-University of Leuven, Leuven, Belgium, [9]Respiratory Medicine, Thoracic Oncology, Jessa Hospital, Hasselt, Belgium, [10]Brussels Interuniversity Genomics High Throughput core (BRIGHTcore) of Vrije Universiteit Brussel (VUB) & Université Libre de Bruxelles (ULB) & Centre for Medical Genetics, Reproduction and Genetics, Reproduction Genetics and Regenerative Medicine, Vrije Universiteit Brussel (VUB), UZ Brussel, Brussels, Belgium, [11]Department of Pathology, AZ Sint-Jan Brugge-Oostende AV, Bruges, Belgium, [12]Department of Laboratory Medicine, AZ Sint-Jan Brugge-Oostende AV, Bruges, Belgium, [13]Translational Cancer Research Unit, Centre for Oncological Research, Faculty of Medicine and Health Sciences, University of Antwerp, Antwerp, Belgium, [14]Department Quality of Medical Laboratories, Scientific Institute of Public Health, Brussels, Belgium.

Please send all correspondence to: A. Hébrant, Ir, PhD, Juliette Wytsman 14, 1050 Brussels, Belgium, tel: +32 2 642 57 32, email: aline.hebrant@wiv-isp.be.

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DEFINITIONS

Accreditation: procedure by which an authoritative body gives formal recognition that an organization is competent to carry out specific tasks.[1]

Allelic read percentage or allelic frequency: is the percentage of variant reads in a background of normal reads.

Analytical accuracy: measurement of the discrepancy between the measured value and the true value. Can be established by analysing well-characterised reference samples with known confirmed sequence variants.[2-4]

Analytical sensitivity: likelihood that the assay will detect the targeted sequence variations if present (true positive rate).5

Analytical specificity: probability that the assay will not detect a sequence variation when none are present (true negative rate).[5]

DNA library: collection of DNA fragments.

FASTQ format: is a text-based format for storing nucleotide sequence and its corresponding quality scores (encoded with a single ASCII character).

Limit of detection: is the lowest actual percentage of variants that can be consistently detected.

Log file: is a file that records events that occur in an operating system or other software runs, or messages between different users of communication software.

Pipeline: is a bioinformatics workflow management system which executes a series of computational or data manipulation steps that relate to bioinformatics and is organized so that the output of one is the input of the following.

Precision: degree of agreement between replicate measurements of the same material that can be determined by assessing the reproducibility (between-run precision, the consistency of results from the same sample under different conditions) and repeatability (within-run precision, the consistency of results from the same sample under the same condition).[5]

Referral laboratory: 'external laboratory to which a sample is submitted for examination'.[1]

Reference Materials: are well-characterised, homogeneous, stable samples with certified properties for their intended purpose.[6]

Reportable range: region of the genome for which the sequence derived by the NGS test meets the quality determined during the validation process.[5]

Region of interest (ROI): region of the genome that the NGS test claims to assess.

Targeted NGS: the sequencing of a subset of genes or regions of the genome.

Template: is the DNA strand that serves as a pattern for the generation of other molecule.

Turnaround time: 'elapsed time between two specified points through pre-examination, examination and post-examination processes'.[1]

Validation: 'confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled'.[1]

Variant: a called nucleotide that differs from the reference sequence.

Verification: 'confirmation, through provision of objective evidence, that specified requirements have been fulfilled'.[1]

INTRODUCTION

Targeted next generation sequencing (NGS) analysis is a complex procedure including two main parts, the 'wet bench' part or lab experimental test and the 'dry bench' or bioinformatics part. Both parts are composed of many steps and for each of these steps optimal assay conditions and analysis settings must be determined.[7]

The aim of this document is to provide guidelines to facilitate and harmonise implementation, verification and validation of targeted NGS tests to detect acquired somatic mutations in DNA, in (haemato)-oncology. These guidelines aim to harmonise clinical conclusions if a sample is processed through different pipelines by different operators at different times and at different sites.[8] Actual platforms are based on different chemistries and each of them has specific parameters and test requirements. These guidelines aim to provide generic recommendations to all stakeholders (laboratories, BELAC-auditors, experts, etc.) valid independently of the platform used. They are to be considered as complementary to the International Organization for Standardization (ISO)15189 standard (medical laboratories) as well as to other international NGS guidelines, for example those from EuroGentest, the Association for Clinical Genetic Science (ACGS), the Dutch Society for Clinical Genetic Laboratory Diagnostics (VKGL), the College of American Pathologists (CAP), IQN Path ASBL and the Centers for Disease Control and Prevention (CDC).[3,5,7-10]

Validation or verification, and internal quality control (IQC) procedures at the different steps in the workflow (by defining quality parameters and by measuring quality metrics) and on the whole process (by determining performance characteristics), must be performed to assure and maintain accurate test results. Moreover, periodic external quality assessment (EQA) should be performed in order to ensure that performance complies

with (inter)nationally accepted performance criteria.

GENERAL REQUIREMENTS FOR TARGETED NGS TESTS

TRACEABILITY

GENERAL DOCUMENTATION

Laboratories should have Standard Operating Procedures (SOPs) for all steps involved in sample preparation, wet and dry bench parts of the NGS process, as well as in the review and reporting of results according to the requirements of ISO 15189.[2,4,8] Accurate systems for tracking the software version used should be available.

LOGISTICAL DOCUMENTATION

As required by the ISO 15189 standard, for each test, information regarding the operation of instruments (e.g. calibration and maintenance records, log files, etc.) as well as any essential information on critical reagents (e.g. lot number, expiration data, etc.) should be recorded.

TARGETED GENE PANEL DESCRIPTION DOCUMENTATION

During the test development, a precise description (at the genome and transcript level) of all specific gene hotspots that can be analysed should be available. The minimally required variants to be analysed for a specific tumour type are those which have a proven clinical utility (to define diagnosis and/or to predict response or resistance to specific cancer therapies (therapeutic) and/or to determine prognosis (patient outcome)) for that specific tumour type. These variants have been established within the expert group of the 'Commission of Personalized Medicine' (ComPerMed).

In addition, the NGS gene panel may also contain a limited number of gene targets with hotspots that are expected to have clinical relevance in the future, in a specific or in another tumour type.

All variants which are part of the validation should be listed with the transcript accession (NM-reference) number of the gene, all exons and the specific, delineated regions that are targeted.

Targeted NGS gene panels should generally contain only single nucleotide variants (SNVs) and small indels (Insertion or Deletion of bases in the DNA). At present other types of alterations (large indels, amplifications, translocations) are preferentially detected by other techniques.[8] The size up to which indels can be detected should be carefully determined and reported in the validation dossier. If alterations other than SNVs and small indels are included, a thorough validation and quality assurance should be established for each type of these reported molecular alterations.

VALIDATION/VERIFICATION REPORT

The experimental approach, results, conclusions and any other relevant details of the validation or verification process (validation and verification chapters) should be recorded in a validation/verification report. The validation report should contain the empirically determined performance characteristics of the test (e.g. sensitivity, specificity, precision, accuracy and limit of detection) as well as critically relevant quality metrics (test development and validation chapters). The validation/verification criteria can be reported in peer-reviewed publications.[11]

Any deviation from the developed and validated test should be recorded and documented according to ISO 15189 standards and revalidation/reverification (validation chapter) should be considered.[2,3,5,12]

TEST RUN DOCUMENTATION

For each run, a test report should contain the values of relevant quality metrics in order to demonstrate that the reported sequence meets the quality criteria set in the validation report.

A test report should also contain the sequences which are in the reportable ranges.

DATA STORAGE

The FASTQ (in a compressed manner) or BAM files (Binary version of a SAM file), the Variant Call Format (VCF) (>v4.0) and the final clinical report which interprets the clinically relevant somatic variants should be stored in the laboratory for a time period as legally required.[2,7-9,12,13] These files should be stored together with the traceability documents, as in some circumstances it may help in explaining the results.[7,9,14]

DATA TRANSFER

All data transfers should use secure network connections that allow verification of the data transfers. An external hard disk between the various components of the computing hardware i.e. from sequencer to the analytical computer and/or to storage location can also be used.[2] The policy and procedure should be adequately documented.

Appropriate and validated measures should be taken to avoid data corruption during transfer (e.g. by using checksum generation during file transfer, management of data permissions, secured backup of copies of FASTQ files maintained elsewhere).[2] Appropriate er-

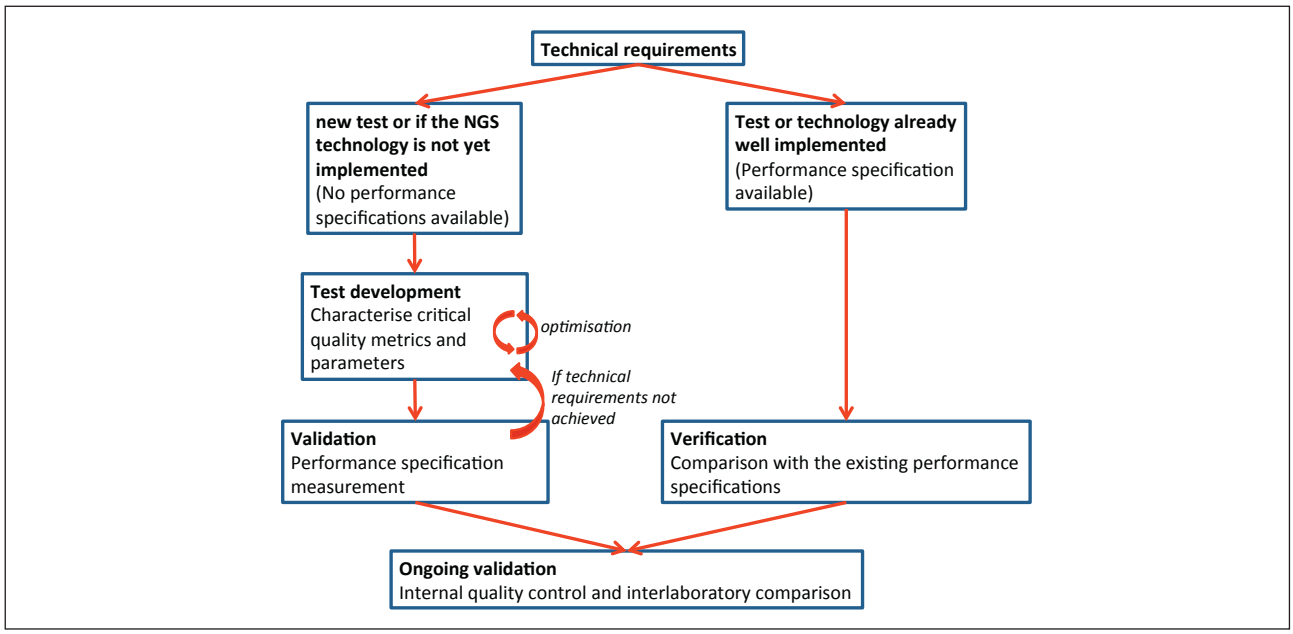

FIGURE 1 Workflow for the implementation of a NGS test.

ror messages should be generated where case corrupted files are detected.

REFERENCE MATERIAL

Reference materials can exist or variant-engineered human cell lines (Reference sample) as well as informatics data files (Reference informatics data file). Reference materials should be used for test validation/verification prior to implementation and for continued quality assessment of the validated NGS pipeline (validation, verification and quality control chapters).[5]

Reference samples should have well-documented sequencing data, should ideally be available on a continuous basis and should resemble as much as possible the patient specimens in order to accurately reflect the testing conditions.[2,5,12]

Reference informatics data files are files created by computational methods simulating patient sample sequences or by sequencing biological samples with well-documented variants (SNVs and indels) and allelic frequency close to real data.[2,5,12] They should be compatible with the sequencing platform's output taking into account the used platform characteristics such as read length, read number, etc. These reference informatics data files can be used for the validation of the dry bench part.[5]

A combination of reference informatics data files and reference samples is recommended to provide a robust framework for test validation/verification (validation, verification and quality control chapters).[5]

RISK ANALYSIS

The ISO 15189 standard requires that the laboratory evaluate the impact of work processes and potential failures on examination results as they affect patient safety, and that the laboratory modify processes to reduce or eliminate the identified risks, and document decisions and actions taken.[1] As NGS workflows are complex and consist of many different steps (from pre-analytical step to tertiary interpretation), risk analysis is particularly appropriate to reduce potential erroneous results and should be performed prior to implementation, e.g. as part of the validation process. Any identified risk should be included in the validation report (e.g. in a Fishbone diagram), and addressed appropriately within the validation of the test.

OUTSOURCING

If tests are outsourced, the ISO 15189 requires that the referring laboratory has a procedure for the selection and evaluation of the referral laboratory. The referral laboratory should be accredited according to ISO 15189 for the NGS test in (haemato)-oncology and licensed by the Minister of Public Health. In addition, the quality of the referral laboratory should be continuously monitored by the referring laboratory. Responsibilities towards the interpretation and reporting of the results stay with the referring laboratory.[1]

Specifically for NGS tests, there is a tendency to outsource only parts of the tests that may not necessarily comply with present license requirements. In any case,

however, in agreement with the ISO 15189 standard, for any outsourced parts of the activity, the referring laboratory should be able to monitor the quality of the subcontractor, and demonstrate that outsourcing does not negatively influence the reliability of the final results. The outsourcing of parts of the NGS pipeline should be subject to the required risk analysis.

TECHNICAL REQUIREMENTS AND PERFORMANCE SPECIFICATION

It is necessary to prove that the test performs to the highest achievable level of performance required for answering to a particular clinical question and that this level of performance is maintained in all routine analyses.[11] Desired performance characteristics for performing any NGS in (haemato)-oncology should be defined in advance and integrated in the validation plan.

The level of validation/verification depends on the availability of acceptable performance specifications (*Figure 1*):

- in case of a new in-house or modified IVD CE-marked test or technology, the entire process should be validated (validation chapter) for meeting the a priori defined performance specifications.
- in case of the implementation of a IVD-CE marked test or technology with documented performance specifications or of a validated test with minor modification of the experimental protocol or of the composition of the gene panel (e.g. when adding a new gene), only a verification (verification chapter) is required.

SAMPLE ACCEPTANCE CRITERIA AND SAMPLE PREPARATION

SAMPLING AND FIXATION

Sample preparation is a crucial step for all high-quality molecular analyses. Samples of poor quality (e.g. due to fixation artefacts) or with insufficient quantity of amplifiable DNA can significantly affect the sensitivity and specificity of the test (validation chapter) and can lead to false negative or false positive results.[2]

For tumour tissue, the sample fixation after surgical removal should be validated for the purpose of NGS sequence analysis. It is recommended to proceed to the fixation within 1h after surgical removal preferably in 10% neutral buffered formalin during a specific time-lapse (generally between 6-72h). Delayed or suboptimal fixation may result in DNA degradation due to apoptosis and/or necrosis.[15] For blood and bone marrow samples, recipients with common anticoagulants such as EDTA should be used.[16] NGS on decalcified bone samples is possible if a weak acid- or EDTA-based decalcification protocol has been properly administered, though it may affect the success rate of the NGS test.[17,18]

SPECIMEN SELECTION QUALITY

The sample (tissue from primary tumour or metastasis, blood, bone marrow, etc.) should contain sufficient neoplastic cells. The minimally required percentage of neoplastic cells in a background of normal cells should be defined during the validation process, taking into account that the assessment of the tumour cell content by different pathologists can be imprecise and inaccurate.[19] The latter will be estimated on Haematoxylin and Eosin (HE) stained adjacent slides for biopsies and by cytological and/or flow cytometric examination for blood/bone marrow by adequately trained staff with documented competence. For formalin fixed paraffin embedded (FFPE) material, macrodissection or (manual) microdissection may be performed to enrich the neoplastic cell proportion.[1,7] The estimated neoplastic cell content of the material used for the DNA extraction should be reported and taken into account in the technical validation of the (negative) results.

In case neoplastic cell content is below the minimally required percentage as determined in the validation report, laboratories should state in the clinical report that negative results (e.g. the absence of any mutation) may not be reliable and that repeated analysis on material with a sufficiently high neoplastic cell content is advised. The treating physician should receive this information as soon as possible in order to allow for repeated sampling with as little delay as possible. A registration of transfer/receipt of this information should be kept.

DNA QUALITY AND QUANTITY

After DNA extraction, DNA quality (e.g. purity, degradation) and/or quantity can be assessed by fluorescence, by optical density or by qPCR amplification. The laboratories should determine a measurement method for DNA quality and/or quantity and should evaluate how DNA quantity and/or quality influence the reliability of the results during the validation process (validation chapter). The minimal amount of specimen required and the success rate of the pipeline considering the DNA quantity/quality of the representative clinical samples should be evaluated during the validation process.

TEST DEVELOPMENT

GENERAL

For each NGS test or technology not yet implemented, usually no performance specifications are available and

a protocol (SOP) should be established. Desired performance characteristics should be adequately defined in advance and integrated in the development and validation plan. A formally validated NGS-assay should be finalised before the implementation in routine practice.

The laboratories should determine the optimal conditions for the test in order to meet the predefined minimal performance requirements (validation chapter). Iterative cycles should be performed until all assay conditions and analysis settings (test development chapter) meet the minimal predefined performance requirements. In case the latter cannot be reached, the lab should re-determine the minimal performance requirements and restart the procedure.

At each step of the test, critical parameters, quality metrics, their thresholds and their acceptable ranges should be defined. This development step will allow:

1) gaining the necessary experience with the test by identifying any critical step, parameter and quality metrics that may affect performance of the test,
2) interrupting the run prematurely before completion if significant deviations from the acceptance criteria are detected or if quality metric thresholds are not achieved, and
3) ensuring reliable NGS test results.[5,11] As quality metrics may vary between or even within laboratories, depending on the different platforms applied, each laboratory should establish its own quality metric thresholds.[2]

WET BENCH PART OF THE NGS PROCESS: DNA LIBRARY PREPARATION

There are different methods to prepare the DNA library for a targeted NGS analysis.[20] Each of them is a succession of a number of the following critical steps, depending on the assay used:

• Fragmentation

At first, DNA will be fragmented into DNA fragments of an optimal length determined by the downstream platform. Input mass of DNA and fragmentation conditions should be determined.

At the end of the fragmentation, two main quality metrics have to be measured and documented: (a) the size distribution of fragmented DNA samples and (b) the amount of fragmented DNA sample.[2,3] Both can be assessed by fragment analyser, spectrophotometric readings, gel image or real-time qPCR or similar instruments

• Target enrichment

a. Hybridization target capture allows the enrichment of the library with targeted regions. Biased capture can occur (e.g. in poor DNA quality sample) which is especially critical for equal representation of the different barcoded samples if pooled during DNA library preparation.[2,12]
b. Enrichment can also be achieved by amplification-based methods. Fragments are generated by PCR with primers targeting specific regions.

In general, hybridisation-based target capture is generally less sensitive but generates less false positives than PCR-based enrichments.[21]

• Adapter and barcoding ligation

Platform-specific adapter sequences and sequencing primers will be ligated to both ends of the DNA.

If different DNA samples are pooled, barcodes are added to enable individual sample identification and to extract sequences obtained from a particular patient sample from the total data set. These barcodes consist of a unique DNA sequence having at least 1 bp difference between each other, typically three or more.[9] If every run contains the same targets, different barcode indexes should be used between consecutive runs in order to avoid sample leakage.[2] The number of samples that can be pooled should be determined during validation (validation chapter) and depends on the desired coverage read depth of the regions to be sequenced.[5,12]

• Tagmentation

The tagmentation method prepares genomic DNA libraries by using a transposase enzyme to simultaneously fragment and tag DNA by adding specific adapters to both ends of the fragments. These adapter sequences will amplify the insert DNA by PCR which adds index barcodes sequences.

Different parameters are critical in the tagmentation method:

- The amount of DNA input: ratio of transposase complexes to sample DNA is critical in order to obtain transposition events separated by the appropriate distances.
- All reaction parameters, such as temperatures and reaction time, must be tightly controlled as the fragment size depends on the reaction efficiency.[20]

NORMALISATION

If DNA samples are pooled, normalisation should be performed in order to have equal representation of each sample. This can be achieved by diluting the DNA libraries to equal molarities based on fluorescence analy-

sis, optical density measurement or qPCR. Alternatively, normalisation can be performed directly on beads.

CLONAL AMPLIFICATION

Before the clonal amplification step, an optimal quantity of DNA should be determined during the test development. An accurate estimation of the purified library quantity (e.g. DNA fragments with proper ligated adaptors and indexes) is crucial to obtain the optimal clonal amplification. Depending on the method used, optional quality and quantity controls can be performed at the end of the clonal amplification.

SEQUENCING

During this step, the clonal amplicons or the DNA fragments are sequenced in a flow cell by generation of luminescence or fluorescence images, which are then converted into sequences. Actual commercial platforms are based on different chemistries and each of them have specific parameters and test requirements.

DRY BENCH PART OF THE NGS PROCESS OR BIOINFORMATICS

The bioinformatics pipeline can be divided into three analytical steps: Primary analysis, secondary analysis and tertiary analysis. Tertiary analysis is discussed in chapter tertiary analysis.

Several bioinformatics pipelines may be evaluated during the development plan, separately and/or combined. Combination of two or more pipelines may result in a higher sensitivity and specificity than with the use of just one. This analysis should adequately be documented in the validation report.

PRIMARY ANALYSIS

Primary analysis consists of different steps but not all of those are mandatory in this phase, since they can also be done later in the process; the selection of those should be defined during the validation process together with their corresponding quality metrics and thresholds:

- Base calling: the raw electronic information from the sequencer is converted into nucleotide positions, and quality scores are assigned to each base. This is performed by the instrument's algorithms. The laboratory has relatively limited control in this phase.
- Demultiplexing: when samples are pooled before the sequencing, the data must be assigned in silico to the sample of origin by comparing the index barcodes and the reference index set.
- Primer and adapter trimming: primers and adapters have to be trimmed from the obtained sequences in order to align them properly to the reference sequence and call variants.
- Low-quality base trimming: a base quality score (Q-score or Phred score) is assigned to each base that estimates the error probability for each base.[3,4,9] This is platform dependent and should be monitored during the run.[3]
- Read quality control: allows for checking whether the sequencing data is of sufficiently good quality to ensure variant calling analysis. Reads containing bases with many too low Q-scores should be removed by informatics filters before aligning to the reference sequence. If only the 3rd end of the read has low Q-scores, only this part of the read can be trimmed before alignment.[5] Too short reads after trimming should also be removed as it might cause problems during the mapping.

The outputs of the primary analysis phase are FASTQ files, which contain the succession of nucleotides corresponding to all the reads produced by the sequencer.[8,12]

SECONDARY ANALYSIS

This phase contains different steps. Again, not all of them are mandatory; the selection should be defined during the validation process together with their corresponding quality metrics and thresholds:

- Reads contained in the FASTQ files (often 50–400 base pairs) are aligned to the reference sequence (read mapping), with software of choice which depends on local preferences and platforms. Mapping can be performed onto the target sequences or onto the full reference genome. Mapping to the whole reference sequence is preferred as it considerably reduces false-positive variant calls despite the fact that more computation time and space are required.[3]
- When a fragmentation or tagmentation step or amplicon-based technology is performed, duplicate reads resulting from clonal amplification should be removed by using informatics filters, as their inclusion generates a risk of skewing the allelic fractions. Keeping only the one with the highest quality score is recommended.[5]
- Indels should be evaluated on sufficient samples with (large > 15 bp) insertions/deletions and a local realignment should be evaluated to check if this additional step can improve the detection of indels.
- Base quality recalibration algorithms might be used to generate more accurate Q-scores.[5]

After these different steps, the output is the SAM (Sequence Alignment/Map) file, which is a tab-delimit-

ed text file that contains sequence alignment data, or the BAM file. It includes several types of information such as the mapped read sequences, base quality scores, mapping quality scores, and the position of insertions/deletions/matches in the alignment.[8]

- Variant calling: once the reads are mapped, differences with the reference sequence are identified as SNVs or indels.

The output of this last part of the secondary analysis is the VCF file, which contains for each variant the chromosomal position, type of variant, coverage, allelic frequency, gene name and the quality scores.

These secondary analysis steps can be performed on or off the NGS instrument.[12]

During the test development, settings and quality metrics are determined and optimised in order to increase performance characteristics (validation chapter). Acceptable ranges and thresholds for each of these quality metrics should be defined and documented.[2,3,8,9]

Settings are:

- Alignment settings (seed length, mismatch tolerance, mismatch penalties, gap penalties and gap extension penalties).
- Informatics filter settings which allow ignoring any read that map to non-targeted regions and analysing only reads mapping to the specific regions targeted.[5]

They are determined and optimised in order to increase the specificity (validation chapter) of the test. For example, by sequencing at the minimal coverage read depth (validation subchapter) a sufficient number of normal samples in which no variants are present, including low quality samples, and then adjusting the settings in order that no false-positives are detected.

Quality metrics are:

- Mapping quality scores which measure the uncertainty that a read is mapped properly to the reference sequence / genome.
- Proportion of duplicated reads (if appropriate).
- Coverage read depth of the region(s) of interest, which is the number of independent overlapping base calls. Coverage read depth threshold should be established during the validation to ensure adequate sensitivity (validation chapter) in the region(s) of interest.[2,3,5,9,12] Reference materials (general requirements for targeted NGS tests chapter) are preferably used to define the minimum coverage read depth for which additional coverage does not significantly improve the accuracy of the sequence (e.g. plotting the number of false positive and negative results as a function of coverage).[5] Variants not meeting the minimum coverage read depth should be tested by other methods or reported as not informative. Table or graph (e.g. histogram or box-and-whisker plot) of coverage read depths for each target area should be provided.[4]
- Average read coverage depth which is the average number of overlapping reads within the total sequenced area.[4]
- Allelic read percentage (also called variant allelic frequency (VAF)) which defines the percentage of variant reads in a background of normal reads. A minimum allelic read percentage should be equal to or higher than the limit of detection, which is determined during the validation process (validation chapter).[4,5]

In order to assess that the sequencing run has performed correctly, some quality metrics can also be measured and their corresponding threshold should be determined during the validation plan. These metrics are platform specific and should be determined during the validation process.

- For example, for the Illumina platform, the optimal range of the cluster density (CD), which is the number of clones (or clusters) per mm^2 can be defined. Cluster density mostly depends on the concentration of the DNA library pool and should reach a minimal threshold in order to obtain a sufficient number of reads.
- If applicable, a threshold for the error rate, which is based on the sequence of a known spiked-in control (e.g. PhiX), can be determined. The error rate is directly related to the Q-scores.

VALIDATION

GENERAL

Test validation is necessary to ensure that a new test is performing properly as intended for its clinical use. Desired performance characteristics should be defined in advance and integrated in the validation plan. The level of validation is function of the type of test.

Following the ISO 15189 norm, a validation is required for any new in-house (NGS) technology. The validation should apply to all intermediate steps of the entire (NGS) process and should include a deep investigation of the critical parameters defined in the test development, in order to detect any source of variation and interference and to verify that the desired performance criteria and requirements for process control are met.

PERFORMANCE CHARACTERISTICS DETERMINATION

Performance characteristics include limit of detection, analytical sensitivity, specificity, precision and accura-

cy (see definitions). These characteristics should be empirically established and validated separately for each type of variant.[4,5]

These performance characteristics depend on several quality metrics described in the chapter on test development such as coverage read depth, allelic read percentage and base quality scores (Q-score) and on pipeline settings. The influence of the sample types on the performance characteristics should be considered.

LIMIT OF DETECTION

A crucial step in every validation plan is the establishment of the limit of detection (LOD). LOD can be assessed, for example, by dilution series of well-characterised DNA samples with known mutations in wild-type DNA, or by using reference samples (for example HorizonDx samples in which different mutation types, at various VAF's ranging from 30% to less than 2%, in various genes are engineered).[12] This way of testing has the advantage that it may incorporate several confounding factors that may impede the LOD in daily practice. The dilution should be performed to the point that the variant of interest can no longer be detected. From these dilution series, the minimal required coverage read depth to detect a variant at desired VAF can be determined.

The limit of detection is usually around 5% of allelic frequency. Minimum five variants (SNVs and indels) should be analysed.

ANALYTICAL SENSITIVITY AND SPECIFICITY

Analytical sensitivity and specificity are related to different quality metrics described in chapter 6. For example, the desired sensitivity and specificity may not be achieved when coverage read depth and base quality scores (Q-score) are below the threshold.[5]

Analytical sensitivity and specificity should be empirically established separately for each type of variant (SNVs and indels), using samples that are representative for the intended clinical sample type. Well-characterised reference materials (such as HorizonDx references) or clinical samples already analysed by another independent method such as Sanger sequencing, qPCR, or NGS by another lab may be used.[4,5,8]

A sufficiently high number of variants and samples, adequately defined and representative for the clinical purpose, should be investigated in the validation process.[6]

The numbers of tested variants will be smaller for smaller gene panels and higher for bigger ones.[22,23]

A sensitivity of at least 95% and a specificity of at least 99% should be pursued.

ANALYTICAL PRECISION

Repeatability can be established by sequencing the same sample (minimum three different samples) using different barcodes in triplicate at least under the same conditions in the same run.[4,5,7,8]

Reproducibility can be established by sequencing the same sample (minimum two different samples per variant type (indels and SNVs)), in three different runs on the same instrument, or on different instruments if applicable (instrument variability), and by different technicians (inter operator variability).[3,4,7,8,12] The inter-operator reproducibility for the classification of variants should also be assessed.

A repeatability and a reproducibility of at least 95% should be pursued.[12]

ANALYTICAL ACCURACY

Analytical accuracy should be established by sequencing well-characterised reference materials with multiple variants (that are representative for the intended clinical sample type) including those with allelic frequencies close to the established detection limits. In addition, the data obtained from the analytical sensitivity and specificity assays should be included. Analytical accuracy should be ≥99%.

VALIDATION OF CHANGES IN THE BIOINFORMATICS PART ONLY

Change in a part of the process, for example software updates or software changes, requires a validation of the particular bioinformatics part. In-house available data files or files from other NGS-accredited labs may be used.[2,4]

The validation can be achieved by using existing data, which are representative of the analysed tumour samples from at least 50 variants and 30 previously analysed samples (depending on the size of the panel, see above) with known mutations that include SNVs and indels to verify that all the variants are still detected with the same analytical sensitivity, preferentially across a wide range of coverage levels.

Assessment of the quality metrics should be done to ensure that no significant differences exist between the different software versions to enable the detection of all relevant variants.[2-4,9,12]

The software update release notes describing the modifications should be logged.

VERIFICATION

If performance specifications are available (for CE-marked IVD-compliant kits), the NGS test should be

verified in their own laboratory in order to establish that specifications are met, in other words that the test is performing correctly as stated by the manufacturer. Moreover, critical quality metrics and parameters (test development chapter) should be measured.
The verification procedure is also applicable when minor modifications to the experimental protocol or to the composition of the gene panel (e.g. when adding a new gene) of a validated workflow are performed.
For verification, at least 10 retrospective samples with known variants (including SNV and indels) should be tested and at least 10 variants detected in prospective samples should be confirmed by an independent reference method, which may be the original NGS method.[4]

QUALITY CONTROL

GENERAL

To ensure and maintain accurate test results, quality controls should be performed periodically at different levels:

- Internal Quality Control (IQC) should ensure that the process (instrument-reagents-operators) is working properly each time samples are processed. Procedures for IQC should cover checkpoints at different critical steps (by controlling quality metrics and quality parameters) and on the whole process (by determining performance characteristics).
- External Quality Assessment (EQA), with unknown material provided by a third party, should ensure that the performance of the laboratory itself and of the method used, complies with (inter)nationally accepted performance criteria.

INTERNAL QUALITY CONTROL

Procedures on internal quality control should be implemented by the laboratory to monitor the performance of the entire analytical process for each NGS test and its reproducibility over time. This should also allow for detecting errors or nonconformities during the process and eventually will indicate the need to interrupt the process if necessary.[5] The performance specifications and quality metric thresholds derived from the validation/verification process or from the manufacturer will be used to assess the validity of each test run.

QUALITY CONTROL MATERIALS

Positive controls

A positive control, such as an engineered DNA reference material, should be included to assess the NGS test on a regular basis and at critical steps (for example when starting a new lot of critical reagents) and should contain multiple known somatic variants of different types, preferably near the limit of detection of the assay in order to assess that low percentage variants can reproducibly be identified.[2,4,5]
The frequency of analysing positive controls should be based on the stability of the procedure and the risk of harm to the patient from an erroneous result.[1] Particularly for sequencing, evaluating predefined run quality metrics may be adequate to assure the validity of each single run, making the analysis of a positive control in each run superfluous.
Laboratories should document the use of positive controls and monitor the results over time.

Negative controls

It is advised to include a no-template control during the PCR steps within the template preparation to check for sample contamination.[4,9]
Moreover, data analysis can be performed to check if reads are generated from a barcode used in the previous run and not in the current run and if reads are generated for targets not included in the current run.
Different mutational profile for each sample is a strong indication that there is no sample contamination, making the use of negative control superfluous.

Performance monitoring

The performance measures determined in the validation process (validation chapter) should be recorded in the validation/verification report (validation/verification report subchapter) and in subsequent routine diagnostic runs. Comparison to those of an optimal validated run can be used to monitor the reproducibility and the overall quality.[2]

Quality metrics monitoring

Quality metrics should be monitored at each run and routinely collected and compared to those of an optimal validated run.[2]
Any significant deviations should be investigated and may require repeating the test.[2,5] It can also help in defining the source of the problem in an underperforming test.[2,4]

External quality control

Proficiency testing (PT) and EQA should be performed periodically at least once a year to monitor the test performance, by analysing well-defined materials provid-

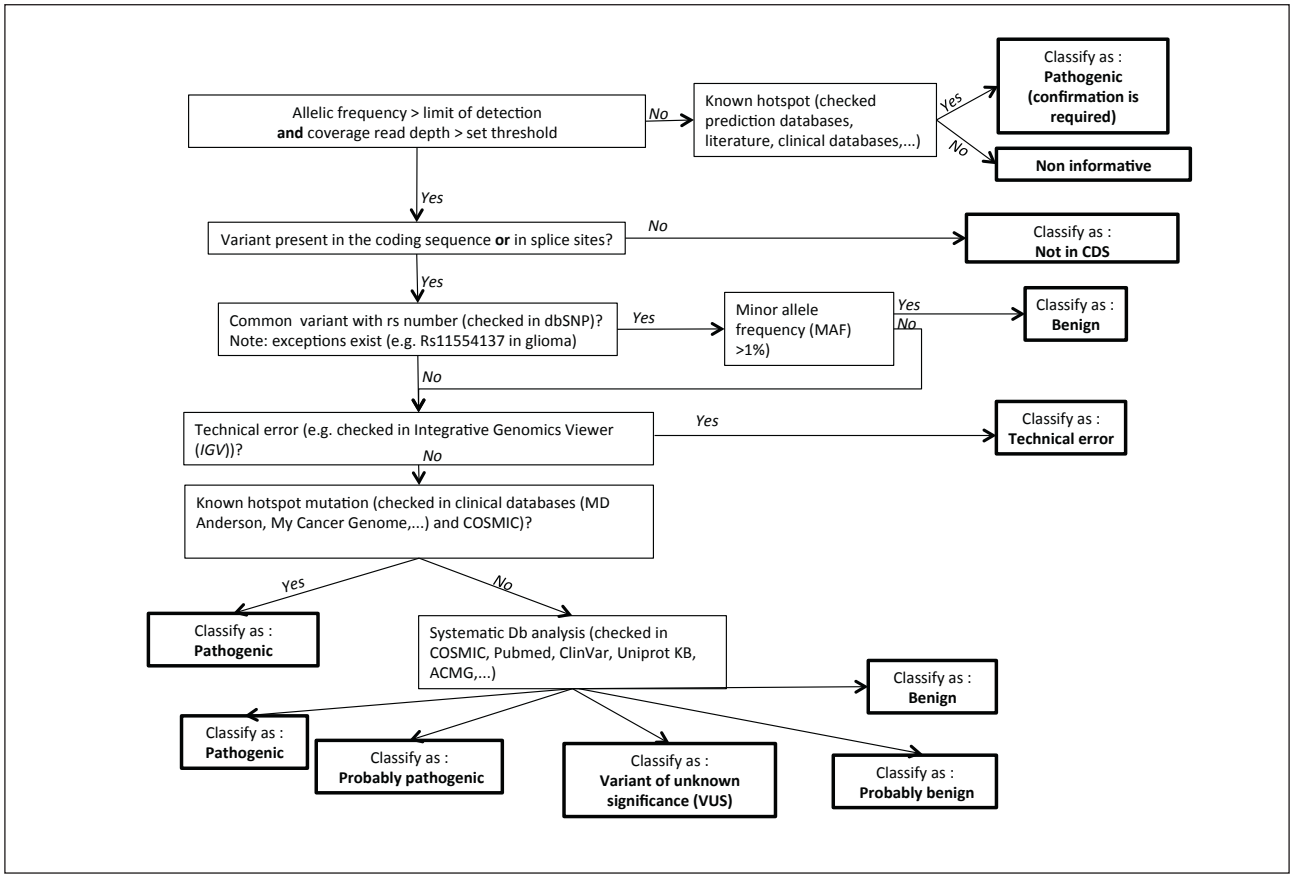

FIGURE 2 Biological classification of variants. Modified with permission from Froyen et al., 2016.

ed by an independent third party but unknown to the laboratory.[2] Laboratories should share with each other well-characterised samples and data files to collaboratively improve and standardise NGS testing.[8]

TERTIARY ANALYSIS

Based upon secondary analysis data, tertiary analysis is mainly composed of two different steps: (1) the annotation and the biologic classification of the identified sequence variants and (2) their clinical classification and their clinical utilities annotations. This part is performed off-instrument.

1) Each variant should be annotated with dedicated software that annotates each variant in relation to its position in the gene (exonic, coding, amino acid change, etc.), classified into biologic classes (for example, pathogenic, likely pathogenic, unknown significance, likely benign and benign), following a systematic and documented procedure which should be described in the traceability documents and be part of the quality system.
2) Secondly, each variant should be classified into clinical classes (for example, known clinical impact, potential clinical impact, unknown clinical impact) and annotated with their clinical utilities (diagnostic, prognostic or therapeutic). Clinical classification and clinical utilities annotations are based on literature search and screening into different database (such as Cosmic, dbSNP, My Cancer Genome, ClinVar, Civic, MD Anderson) and both should be discussed within a post-analytical discussion forum, also called Molecular Advisory Board (MAB) (national or local) (composed of clinicians, pathologists, molecular geneticists, etc.). The functionality of the MAB should be adequately documented.

Since there are a variety of classification systems available, each type of classification should be discussed and convened with the requesting clinicians. *Figure 2* describes an example of a systematic variant classification procedure recommended by the Belgian Commission of Personalised Medicine.

REPORTING

Reporting allows for conveying genomic data into accurate, interpretable, succinct and relevant information for patient treatment. The clinical report is sent to the

oncologist and should include all known and potential clinical impacts of the somatic variants that are convened within the MAB, with their clinical class, clinical utility, allelic frequency, any limitations in the test and analysis performance of a specific sample (e.g. which targets lack sufficient coverage to confidently determine variant status).[3,4] Cellularity of the tumour sample should always be taken into consideration, especially for negative results. For pathogenic variants that could be potentiay germline variants, a notification such as 'genetic counselling is advised' should be included.
Variants with unknown clinical impact can also be included but should not be misleading for oncologist interpretation and therefore be listed separately from the known and potential clinical impact variants.
Gene variants should be reported using a standardised nomenclature at cDNA and protein level such as the Human Genome Variation Society (HGVS) system, to increase consistency between laboratories. However, at least for known clinical impact variants, the HGVS designation should be presented together with a more common, locally acceptable nomenclature (e.g. V660E). The transcript accession number used for variant numbering should be provided in the report to avoid confusion due to the lack of consensus for some genes. The layout of the variants section of the report should allow clear visualisation. A tabulated format is recommended.

TURNAROUND TIME

The turnaround time for the entire NGS analysis from biopsy to reporting should be appropriate for the intended clinical purpose and in agreement with the tumour specific guidelines, if available, though in general a turnaround time of ≤15 working days is highly recommended.

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