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TOOLS FOR PRACTICE

NEXT-GENERATION SEQUENCING OF A PANEL OF GENES FOR SOMATIC GENETIC ANALYSIS

/ Method Validation

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VALIDATION OF METHOD: NEXT-GENERATION SEQUENCING OF A PANEL OF GENES FOR SOMATIC GENETIC ANALYSIS

As part of medical biology reform, all molecular genetics centres laboratories must be partly accredited by the French Accreditation Board (COFRAC) according to ISO15189 by 2016. The objective of this procedure is to guarantee the reliability of biomedical testing performed and the quality of the medical service offered by a biomedical laboratory. This process requires the creation of method validation files for each type of analysis conducted by the laboratories.

In order to support the laboratories through the process, working groups were constituted for the most up to date analytical techniques, under the aegis of INCa, from spring 2013. Each working group was made up of representatives of the molecular genetics centres using the relevant technique in routine diagnosis, and with expertise in the area. Participants included clinical pathologists, biologists, engineers and quality specialists. This work made it possible to write a document to assist in the validation of methods for somatic genetics[1].

Currently, most of the molecular genetics centres laboratories are in the process of implementing new sequencing techniques (NGS). These technologies present numerous challenges. In order to facilitate the sharing of experience and support the laboratories, a dedicated working group was constituted by INCa in 2015. This group enabled the preparation of this document, which has been submitted for review to all those involved. It is available to staff in molecular biology laboratories to guide them in the establishment of a validation file for methods to detect somatic mutations within the COFRAC accreditation framework for biomedical laboratories. According to the COFRAC classification (SH-INF 50), these analyses constitute a subfamily of tests within the scope of accreditation for genetic analyses. This document highlights the critical control points when using NGS techniques.

[1] *Validation de méthodes pour la recherche de mutations en génétique somatique* (Validation of methods for the detection of mutations in somatic genetics), INCa, 2013

Contents

SCOPE OF ACCREDITATION

Methods used in somatic genetics generally provide information on the presence or absence of a mutation. In this sense, they can be considered qualitative. Since in certain cases the determination of mutational status is dependent on quantitative values, it may be useful to include validation of criteria of repeatability, reproducibility, and limit of detection in the file, in order to estimate the analytical sensitivity of the method.

The method validation file should thus contain the parameters for quantitative validation:

- it is possible to offer an approach for the overall validation of the technique and;
- additional verification files to specifically validate the different gene panels used.

In the extended flexible scope (B), it is possible to create a general file to validate the method as a whole. This file can be created on the basis of one or more parameters, e.g. target genes or loci. Specific files also need to be created for each locus or gene studied. In these specific files, it will be necessary to verify parameters such as primer specificity or enrichment design. This verification can be done by validating the coverage of the regions to be studied. The parameters can be validated according to form SH-FORM 43, currently in force.

The next generation sequencing method (NGS) applied to somatic genetics is a complex method comprising multiple processes. Sequencing can be divided into several steps: enrichment, sequencing and bioinformatics analysis. The validation file should include all the different processes requiring specific validation.

DEFINITIONS

❖ GENERAL DEFINITIONS:

Test blank: this is an analysis containing all reagents without DNA. DNA is replaced by water.

Extraction blank: This is the extraction product obtained with the reagents alone, with no tumour sample.

Internal quality control (IQC): the IQC is performed within the laboratory, using control samples during the measurement of patient biological samples, to verify control of the analytical process. Interpretation will depend on pre-established tolerance limits.

Limit of detection (LOD):

1) Quantitative techniques: the limit of detection corresponds to the lowest signal that can be distinguished from a reaction blank performed under the same conditions.

2) Qualitative techniques: the limit of detection corresponds to the detection threshold of the test, which defines the analytical sensitivity of the test. In practice, in molecular biology, it corresponds to the lowest value (% of tumour cells) that allows a mutant signal to be distinguished from a non-mutant signal.

Reference materials: these are samples that can be used either during method validation, or for monitoring quality. They should be stable over time to allow comparison. They can be used as an internal quality control.

Robustness: ability of a technique to not be affected by small but deliberate variations in method parameters. Robustness provides an indication of the reliability of measurements under the normal conditions of use of a test. Robustness can be assessed based on results from analyses of risk and reproducibility.

Diagnostic sensitivity: probability that a system gives a positive result in the presence of the target marker. It is calculated according to the following formula:

$$\textbf{diagnostic sensitivity} = \frac{\textbf{No.}_{\textbf{true positives}}}{\textbf{No.}_{\textbf{true positives}} + \textbf{No.}_{\textbf{false negatives}}}$$

Diagnostic specificity: probability that a system returns a negative result in the absence of a target marker. It is calculated using the following formula:

$$\textbf{diagnostic specificity} = \frac{\textbf{No.}_{\textbf{true negatives}}}{\textbf{No.}_{\textbf{true negatives}} + \textbf{No.}_{\textbf{false positives}}}$$

❖ DEFINITIONS SPECIFIC TO NEXT GENERATION SEQUENCING:

NGS: Next Generation Sequencing

Read Succession of bases read by the sequencer in each cluster or well, depending on the technology used. The sequencer generates as many reads as there are functional clusters/wells. Each read can be aligned and positioned on the human genome. A minimum number of reads may be required in order to reduce the background noise inherent to the technique. For a given base, the number of reads carrying a variant can be determined. The number of reads is also used to calculate the frequency of mutant alleles.

Depth: For a given base, the sequencing depth corresponds to the number of times that base is sequenced. Sequencing depth is a function of the number of reads for each position. One often defines a "mean depth," which corresponds to the mean depth observed over the entire gene panel. One can also set a "minimum depth" to be achieved, as a quality threshold for validating the result for a given base.

Coverage: Coverage represents the percentage of the panel for which the sequencing depth exceeds a predefined minimum value.

Gene panel: All genes/exons sequenced during an NGS analysis.

Pipeline: Bioinformatics analysis of data generated by NGS requires the use of a set of complementary analytical tools. This includes tools for sequence alignment, for the detection and annotation of variants, etc. The entire set of tools used constitutes the analysis pipeline.

Run: A run corresponds to a pass through an NGS sequencer. It therefore comprises all samples sequenced on the same occasion.

SNP: Single Nucleotide Polymorphism: Variation of a single base pair in the genome, between individuals of one species, in a population.

DESCRIPTION OF THE METHOD

❖ ENRICHMENT METHODS

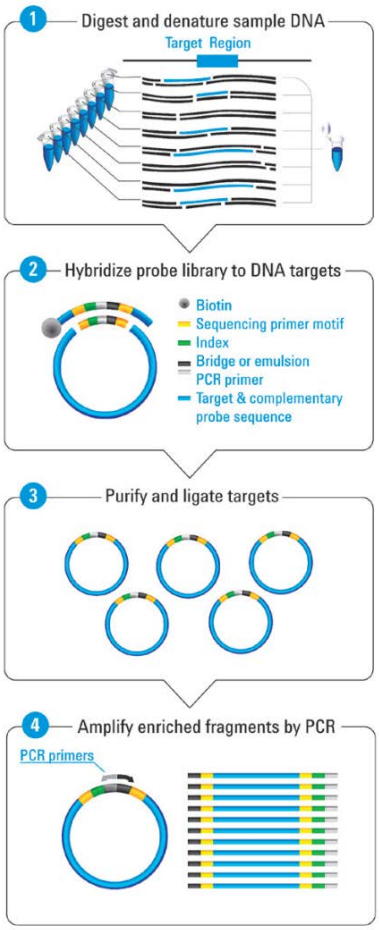
Capture technique

Capture enrichment involves using nucleotide probes (RNA or DNA, depending on the supplier) of about a hundred complementary bases in the regions of interest. These probes are often designed to overlap so that a base in the region of interest is captured by several different probes ("tailing"). The design of probes takes into account regions of low complexity and repeat regions in the target genome in order to ensure specific hybridisation to the regions of interest. It is also possible when designing probes to duplicate some groups of specific probes complementary to regions known to be difficult to cover by sequencing (e.g. "balancing" of GC- and AT-rich regions, and orphan probes). The probes synthesised are generally linked to a biotinylated residue, and hybridise to the DNA to be sequenced during the production of libraries. Since the probes are long, they can hybridise while tolerating many mismatches. As a result, variant genomic regions are also captured. The probes hybridised to the regions of interest are then captured by a magnetic system. After purification of the enriched DNA fragments, PCR is generally performed in order to increase the quantities of enriched libraries.

The enriched libraries are indexed using oligonucleotide barcodes (unique to each patient within the same sequencing) prior to sequencing. The amplification products are then mixed in order to perform sequencing.

HaloPlex technique

HaloPlex (Agilent Technologies) is a technique for enriching target genes (a panel of genes) using capture probes on a defined region of the genome. This method makes it possible to target a few genes, and up to 5 Mb (or 200,000 amplicons) from 200 ng of DNA. The technical protocol involves five steps and takes approximately 8 h. The first step involves fragmentation of total genomic DNA from each patient, in 8 different tubes, by double enzyme digestion (8 pairs of enzymes defined by the supplier depending on the design). The genes of interest are then captured using biotinylated probes specific for the 5' and 3' ends of the predigested fragments of interest, which leads to circularisation of the target DNA fragments. The central part of the probes is complementary to the specific adapters for the sequencer and the oligonucleotide barcodes (according to the user's sequencer). The barcodes/indexes are added at this hybridisation step. Purification is performed by capturing the DNA fragments with streptavidin-coated magnetic beads. There follows an amplification of the fragments by universal PCR. The banks thus prepared for each sample have to be quantified, qualified and normalised prior to sequencing.



This technique offers, for a given locus, coverage by different amplicons, and hence makes it possible to eliminate bias introduced by PCR and mutations associated with sequencing artefacts. The limitations of this strategy are the initial quantity of DNA required. Although it can be limited to 100 ng of DNA, this may be incompatible with tumour samples. The number of steps remains larger than for other amplicon-based techniques, based solely on amplification by PCR.

Amplicon

The amplicon approach proceeds by an initial multiplex PCR for each sample. The number of multiplexes depends on the size of the loci studied and the overlap of the primers. The amplification products are then barcoded. The amplification products are purified and combined for sequencing. This approach raises a problem of evenness of depth, which depends on the efficacy of PCR and multiplex PCR. It can be done using smaller quantities of DNA (10 ng). It is subject to allele dropout (technical bias due to the amplification of a single DNA strand) if there is a variant present close to the 3' end of the primers. It is limiting in the design of primers because any modifications require revalidation of the overall positioning of the primers.

REMARK. Each enrichment method must be submitted to risk analysis, and its efficacy cannot be determined until the end of the process during analysis of results. The parameters to be studied can be coverage, depth, and detection of the expected mutations.

❖ SEQUENCING TECHNIQUES:

Sequencing approaches will have to be included in the risk analysis. The specific features of the methods influence the quality of sequencing, background noise and limit of detection.

❖ ANALYSIS PIPELINE:

Validation of analysis pipelines used in NGS is mandatory. Given the complexity of the pipelines used, it seems appropriate to perform a similar exercise specific for the analysis pipeline. The analysis pipeline used in NGS should be part of the method validation file, since it is an integral part of the process (production, sequencing and analysis). **Indeed, the pipeline is what contributes to assessment of sensitivity and specificity.**

RISK MANAGEMENT

All elements in which variation may influence the result given to the patient must be considered **critical** if there is no means of control. Control samples can enable this control.

> REMARK. Any critical element must be traceable, to allow *a postiori* identification of any analysis performed with this element, in order to make it possible to return to the relevant patients if an abnormality is detected. If these points cause strong variability in the results, a test for robustness should be considered for these elements.

As in any analytical technique, particular attention should be paid to the following points:

- Freezers: Since the stability of reagents considered to be critical depends on proper storage conditions, it is essential to verify and monitor the freezers used to store them, using certified sensors. Other freezers are less sensitive, and do not necessarily have to be considered critical.
- Thermocyclers: Thermocyclers are an essential element in the steps involved in high-throughput sequencing. The presence of a positive control on each block can help in validating its correct operation. However, a positive control does not guarantee an even temperature throughout the block. Additionally, regular validation and monitoring of evenness by means of an annual verification are advised.
- Pipettes: Since the results of analysis are qualitative, the precision of volumes pipetted is not systematically critical. A verification of robustness of the test in relation to variation in pipetting volumes makes it possible to check whether deviation in pipetting is critical or not. In any event, regular calibration or verification of the laboratory's stock of pipettes is recommended.
- Sequencers: These should undergo preventive maintenance as indicated by the supplier. All malfunctions should be traced.

Sample risk management file (see SH-FORM 43)

Input data	Critical control points	Control procedures
Primary sample (urine, blood, etc.) or secondary samples **Type of recipient (tubes, etc.)**	1-Reversal of tubes 2. Sample contamination by amplicons: -risk of false negatives if there is contamination by non-mutant amplicons -risk of false positives if there is contamination by mutant amplicons	1-Sample traceability 2-Molecular genetics centre divided into pre- and post-PCR areas with controlled access 3-Secure technical work areas under hoods equipped with UV lamps. 4-Filter pipettor tips at all steps of analysis
Sample pretreatment (centrifugation, dilution, etc.)	Dilution error	1-Secure preformatted sheets for calculating dilutions 2-Robustness of the test: a test that is relatively insensitive to variations in DNA concentration (Dilution tests using DNA from mutant cell lines).
Competence (staff enablement): clarify references for procedures and record-keeping.	Lack of specific theoretical and practical training of laboratory technicians performing the analyses.	1-Theoretical training: provided by the biologist in charge 2-Practical training: provided by the technicians 3-Accurate traceability of training (initial manipulations performed under the supervision of a trainer, and initial tests performed independently must be tracked) 4-Access to equipment tracked by staff identification code
Ambient conditions required (e.g. temperature, arrangement of rooms, lighting, etc.)	1- Contamination of work areas 2- Air conditioning 3- Observance of storage conditions	1- Maintenance of rooms and observance of the users' charter for the molecular genetics centre 2- Maintenance of air conditioning 3- Surveillance of temperatures by certified sensors 4- Map of +4°C and -20°C refrigerated chambers
Reagent reference (supplier reference, version):	Storage condition and validation of sensor lots	1- Freezer (-20°C), aliquoting and storage according to ad hoc procedure 2- Refrigerator (+4°C), aliquoting and storage according to ad hoc procedure. Validation of sensor lots according to ad hoc procedure 3- Management of reagent drift and traceability
Reference materials (controls):	1-Cell lines 2-DNA sourced commercially 3-Mutant samples	Points 1 and 2: consistent material, gold standard/positive controls Point 3: validation of typing on two independent amplifications (+/- sequencing)
Equipment: **Metrological requirements (define the critical parameters)** **Specific computational requirements**	1-Metrology 2-Computer files	Point 1: verification of metrology by the supplier and general verification procedure 2- Access tracked by staff identification code - Procedure for management and storage of data - Validation of analytical software - Verification of data from automatic results export with visual reading of traces for techniques using qPCR -Secure storage of raw and analysed data.

Other than the usual criteria, there are specific identified points for NGS that must be taken into account in this analysis:

- risks related to poor sample quality: including the risk of false negatives and false positives
- risk of inter-run contamination;
- risk of inter-sample (intra-run) contamination associated with sample multiplexing and the many purification steps;
- Risk of error in analysis algorithms.

SAMPLE QUALIFICATION

Sample qualification is performed in both histology and molecular biology. At histological level, the preanalytical specifics (fixation, preservation) are verified, and the percentage of tumour cells on the HES slide estimated, before or after cutting sections for extraction. These data are collected for the interpretation of results. Ideally, the tumour cell content should be higher than the limit of detection of the test.

The laboratory should define the qualification criteria for the samples analysed (cellularity below the limit of detection of the method, small quantity of DNA, low amplification level). The limit values can vary depending on the techniques used and the limit of detection for the laboratory, and cannot therefore be standardised. These criteria will be used in writing the report. It is recommended that an "uncontrolled" result be considered non-contributory where these criteria are negative.

For specimens taken outside the laboratory, the latter cannot normalise the quality of the specimens (cellularity, fixation time, variable cold ischaemia, etc.). As a result, the laboratory should qualify specimens according to objective criteria, and should set decision-making rules that are compatible with the type of specimen generally received. The laboratory should therefore use a technique with an appropriate detection limit for the samples to be analysed (see section "Limit of detection").

Quality criteria for samples being assessed

- Tumour cellularity
- Exclusion of some fixatives (Bouin, etc.)
- Molecular quality: quantity and quality of double-stranded DNA (measured by intercalating fluorescent dye / qPCR), ligation yield, for instance. The quantification method used should be described in detail.

REMARK. Generally speaking, because of the scarcity of samples and the difficulty of obtaining a repeat specimen from a patient, it is recommended to accept samples that do not fulfil qualification criteria. In this case, the final report should indicate the nature of the problem and the resulting reservations regarding the interpretation of results. Thus, in the event of a negative finding, the result will be considered as non-contributory.

REFERENCE MATERIAL / INTERNAL QUALITY CONTROL

The reference samples used in these assessments may come from several sources. It is advised that they be qualified independently, either by a supplier or by another laboratory. Qualification should include both the type of mutation and the allelic frequency of the mutations. These samples may be:

- patient DNA where the mutant/wild-type status of the nucleotides has been previously verified by another technique;
- DNA extracted from cell lines with/without mutations in the nucleotides being assessed;
- Commercial calibrated DNA with/without mutations in the nucleotides being assessed.

Materials from cell lines or commercial DNA are preferable, since the DNA is of constant quality and the presence of the mutation has been externally validated. The use of a patient tumour sample should be limited if possible. The reference material may be used both for validation and internal quality control (IQC). It is vital to ensure that the material used for IQC is stable over time. It also makes it possible to monitor the impact of changes. Given the technology and the broad genomic coverage, it is not mandatory to use a wild-type sample with no mutations ("negative control").

It is recommended that at least one internal control and one blank be used. The use of a PCR blank is necessary. However, it is not essential to sequence this control or complete the analysis for it.

REMARK. **Internal quality control:**

Monitoring variations in the allelic frequency of the IQC is a good quality indicator, which allows the stability of the process to be monitored. However, to date there is no numerical limit deviation that should not be exceeded.

For an IQC, especially when performing trend analysis, a mixture of previously genotyped tumour DNA, prepared in large quantity, may be sufficient, provided that, between every change in "lot of controls," the latest lot is qualified in a series bookended by the previous lot. IQCs make it possible to monitor contamination (appearance of unexpected mutations in the IQC). IQCs cannot be used alone to determine the limit of detection. On the other hand, they should allow assessment of the background noise of a run.

The strategy to be adopted in the event of invalidation of IQC results should be predefined within an operating procedure. It is a matter of specifying whether the specimens from all patients from the same run must be reanalysed, or whether it is possible to limit verification to certain samples (samples with low cellularity, samples giving a negative result, etc.).

VALIDATION OF PRIMERS AND CAPTURE BIAS

The laboratory should ensure that the primer panel used allows coverage of all the regions of interest being explored. This validation can be performed on the assessment of coverage following analysis of the first results, and can be performed *in silico* on the primers used.

The validation criteria for the primers are the same as those used in traditional PCR, including analysis of sequencing results:

- absence of polymorphisms (at a frequency > 1% of the population) in the last 3 nucleotides. The recommendations of ANPGM[2], for activities of constitutional genetics, are for a maximum tolerance of 1% of polymorphisms in the last 5 bases of primers. This verification should be repeated at regular intervals (every 2 years), since the databases evolve regularly;
- assessment of coverage on the results produced; ensure that the design aligns well with the target regions;
- with respect to commercial libraries, the primer positions are not always exactly known. It is therefore advisable to ask the supplier to provide the SNPCheck for the primers used;
- check the suppliers' tolerances in the design of libraries. Suppliers seem to be generally more tolerant in relation to SNPs for NGS panels than for targeted techniques. Primer design software allows tolerance limits to be set, but often with a rather high lower limit (5% of SNP).

It is essential to prove that one has control of one's design and that one knows its limits. For capture techniques, a verification of the areas covered by the designs, to include 100% of the regions of interest, is sufficient. Control of coverage and depth in relation to the targets should be documented.

WHICH VARIANTS SHOULD BE VALIDATED DURING METHOD VALIDATION?

The laboratory should perform method validation on a list of mutations representative of its activity. These mutations should be distributed among different genes or exons, and should take into account the different types of variants one wants to identify (point mutations, indels, etc.).

It is advisable to ensure that there is correct detection of those variants that can present particular problems with the technique employed (GC-rich areas, mutations located near the ends of the amplicon, insertions/deletions, etc.). It may be useful to have a list of DNA samples to be used for these validations.

[2] French National Association of Practitioners of Molecular Genetics

REMARK If the panel is being changed, it is necessary to perform at least one validation run (see section "Management of flexible scope"), and to revalidate all parameters.

PARAMETERS TO BE VERIFIED

Detection of mutations using NGS provides information on the presence or otherwise of a mutation.

The following parameters should be validated:

- diagnostic specificity;
- diagnostic sensitivity;
- contamination;
- reagent stability;
- robustness;
- comparison with a reference method and/or a method already used in the laboratory.

Furthermore, given the specific features of existing methods for detecting mutations (use of quantitative measurements), the **limits of detection (wildtype allele/mutated allele), repeatability** and **reproducibility** may be subject to assessment.

The parameters of **trueness** and **accuracy** cannot be assessed, given the scarceness and very high cost of measurement standards.

REMARK Tests performed for method validation should allow the limits of the technique to be assessed before it enters routine use. These tests should be performed after the laboratory has mastered the technique well, and are not therefore sufficient to allow implementation of the technique. The many tests related to staff training and taking ownership of the technique should be performed beforehand. It is important that these tests be performed on a completely stabilised process (protocols, size of series and bioinformatics analyses).

❖ MEASUREMENT INTERVAL – LIMIT OF DETECTION

The minimum limit of detection to be achieved in somatic genetics is set at 10% mutant alleles for targets of validated clinical interest. This limit should, however, be defined by each laboratory, based on the quality of the samples to be analysed (see section "Sample qualification").

A laboratory that receives many specimens with low numbers of tumour cells must consequently adapt its analytical technique to allow detection of mutations at a frequency below 10%. The object is to reduce the number of non-contributory results. An acceptable level of non-contributory results is approximately 10%.

Minimum sequencing depth:

This cannot be predetermined, as it depends on the analysis strategy and upstream processes such as:

- limit of detection defined by the laboratory;
- the number of genome copies present on the chip;
- percentage of tumour cells in the sample;
- background noise. Background noise needs to be assessed. In practice, its impact is decisive when the limit of detection set by the laboratory is below 10%.

In practice, sequencing depth is decisive for negative results (risk of false negatives if the depth is insufficient) or for positive results with a very low allelic frequency.

The sequencing depth required depends on the protocols and on the level of background noise, but for a limit of detection of 10%, a depth of 300X seems appropriate for PCR-based protocols, and 200X for capture protocols.

❖ DIAGNOSTIC SPECIFICITY AND SENSITIVITY

According to COFRAC[3], analytical specificity and sensitivity should be calculated for at least 15 non-mutant samples and 15 mutant samples representative of the laboratory's activity. In the context of NGS analyses, where several mutations can be identified for one patient, the concept of true or false positives used for calculations is defined as the number of mutations and not as the number of patients. All mutations included in the calculation must be verified. These parameters can be determined from the IQCs. The number of samples can be reduced depending on the number of mutations contained in these samples.

$$\textbf{diagnostic sensitivity} = \frac{\textbf{No.}_{\text{ true positive mutations}}}{\textbf{No.}_{\text{ true positive mutations}} + \textbf{No.}_{\text{ false negative mutations}}}$$

$$\textbf{diagnostic specificity} = \frac{\textbf{No.}_{\text{ true negative mutations}}}{\textbf{No.}_{\text{ true negative mutations}} + \textbf{No.}_{\text{ false positive mutations}}}$$

After stabilisation of the analysis protocol, validation needs to be performed on external data and on real samples. The number of samples assessed should be from at least 30 patients. It is important to define the minimum background noise of the method in order to have objective decision criteria regarding the final estimate of variants. The laboratory should ensure that the sensitivity of its technique is at least equal to that mentioned in the report.

❖ INTER-RUN AND INTER-SAMPLE CONTAMINATION

A test for contamination should be performed for any accreditation in scope B, in order to ensure that no inter-sample or inter-reagent contamination can occur under the normal conditions in which the analysis is performed. However, this test does not guarantee the absence of external contamination, which can only be controlled by good traceability of routinely performed analyses.

<u>Particular points to watch for NGS:</u>

- Inter-run contamination: at sequencer level, or during preparation of libraries.
- Intra-run contamination. This criterion is decisive insofar as multiplexing of patient samples is uncommon. It is most especially a matter of taking measures to ensure that there is no mixing of samples from different patients in the same run.

[3] SH GTA 04 – *Guide technique d'accréditation de vérification (portée A) / Validation (portée B) des méthodes en biologie médicale* (Technical guide for accreditation of verification (scope A) / Validation (scope B) of methods in medical biology). COFRAC, April 2011

REMARK **Monitoring of contamination:**

- Changing the set of barcodes between each run to control inter-run contamination;
- It is important to monitor mutations on the IQC, and to ensure that there are no new mutations in the positive controls. It is advisable to use an extraction and PCR blank. If this is not sequenced, DNA quantification can be performed to check for contamination in the pre-analytical steps. If a blank is sequenced, a limit must be defined for the number of reads tolerated for blanks / contaminating barcodes;
- During run validation, it is necessary to check that a rare mutation is not found among several patients in the same run. It may be useful to compare SNPs between patients. This comparison is, however, likely to be difficult in somatic genetics, since the panels used do not generally contain many polymorphisms.

Test for specific contamination:

For instance, during the validation phase, wells containing water could be included, alternating with wells containing samples, to assess this contamination.

❖ COMPARISON OF METHODS

Comparison of methods can use the results produced for determining sensitivity and specificity. Comparison of methods should be performed for at least 15 non-mutant samples and 15 mutant samples representative of the laboratory's activity.

The Sanger sequencing technique has been regarded as a reference method. It can be useful for variant qualification over 10%. For variants with an allelic frequency below 10%, comparison should be made with another method routinely used in the laboratory. The technique used for comparison of methods should be adapted to the target limit of detection. Previous data can be repeated. Data from external quality controls can consolidate the comparison of methods.

❖ STABILITY OF REAGENTS AND OF LIBRARIES

Procedures should be put in place in the laboratory to avoid deterioration in the quality of results obtained due to problems of stability in reagents or PCR products:

- reagents should be stored according to the supplier's recommendations;
- reagents should not be used after expiry date;
- the number of times reagents are thawed must be limited. The reagents should be aliquoted to limit this number.
- the period of storage of amplification products (purified or not) should be defined and stated in the operating procedures. It can be verified during routine use.
- for reasons of cost, each new lot of reagents cannot be tested prior to routine use. An analysis of IQCs and a retrospective comparison can be done prior to validation of the run.

REMARK The quality of sequencing should be monitored for the IQC over time, in order to detect deterioration of reagents.

❖ DETERMINATION OF BACKGROUND NOISE

Determination of background noise is an important step in the validation of the NGS method. It allows determination of the minimum number of reads in considering a variant. Background noise can be determined by assessing the runs used for determination of sensitivity/specificity. It can also use the data on repeatability/reproducibility produced using reference samples.

❖ REPEATABILITY

This parameter is not mandatory for validation of a qualitative technique. It can, however, be assessed for a qualitative procedure (mutant/non-mutant).

A test can be performed by comparing, at a minimum, a sample containing representative mutations in triplicate in the same run. Assessment of repeatability is performed on quality parameters identified for each method (quantifiable value or mutant/non-mutant).

❖ REPRODUCIBILITY

Assessment of reproducibility involves analysing the same sample under different conditions over time (operator, reagent lot, etc.). According to COFRAC documents[4], it is established over at least 15 days with 30 determinations and a minimum of two levels. For reasons of reagent cost, the number of tests may be limited. The samples used for repeatability may be used for reproducibility at a minimum of two mutation levels (one sample with a low percentage of a mutant allele and one highly mutated sample). These data can also be obtained by longitudinal monitoring of internal quality controls (IQC).

The reproducibility test can be complemented by data generated while performing the analyses concurrently with techniques routinely performed in the laboratory.

❖ ROBUSTNESS

In qualitative methods, robustness is approached by risk management. Specific tests for robustness should be performed if critical elements have been identified (see section "Risk management"). Comparison of results from IQCs can be used to verify robustness, provided that all parameters to be assessed can be monitored during performance of the tests (traceability of parameters being verified, pipettes, lots, thermocyclers, etc.). Data from the literature can be used to support these data.

REMARK It is advisable to assess robustness retrospectively by comparing the stability of results in time via a retrospective comparison of the results from internal quality controls. Specific tests can be performed at a later time if a particular risk is detected.

❖ INTERFERENCES

Interferences can exist at PCR level (Wilson et al, 1997; Wong et al, 2014). Common types of interference for the samples studied should be listed, and measures taken to prevent PCR inhibition should be described in detail in the file.

The most common consequences of interference for analyses are either an inability to provide a result, or the presence of an artefact. The most common types of interference are the following:

- conditions of fixation;
- quality of fixative;
- necrosis;
- presence of mucin or melanin.

These interferences have an impact on DNA quality and amplifiability. The presence of these factors must not prevent the performance of these analyses. The final result should take these factors into account, particularly for situations where no mutations are detected, or conversely, where too many mutations are detected.

REMARK In the absence of precise information on the quality of fixation of specimens, the risk of interference cannot be subsequently controlled by the laboratory. Interferences can appear as follows:

- The presence of a large number of mutations for a patient should be an alert to the risk of observing artefacts. Artefactual mutations are most often present at low allelic frequency, between 0 and 10%. Depending on the positivity threshold defined by the laboratory, there could therefore be a risk of considering an artefactual mutation as a genuine variant. The analysis strategy established by the

[4] SH GTA 04 – *Guide technique d'accréditation de vérification (portée A) / Validation (portée B) des méthodes en biologie médicale* (Technical guide for accreditation of verification (scope A) / Validation (scope B) of methods in medical biology). COFRAC, April 2011

laboratory should take background noise into account, e.g. by eliminating variants present at low frequency, by eliminating recurrent artefacts identified or by using dual-index sequencing approaches.
- an insufficient sequencing depth or too many poorly sequenced regions.

ROUTINE ANALYSIS OF RESULTS

❖ VALIDATION OF A RUN

- Verification of mutations found in the positive control (IQC);
- Verification of the frequency of mutant alleles in the IQC(s);
- Verification of blanks used, where sequenced, and absence of contamination;
- Verification of absence of systematic lack of coverage for all samples for a given amplicon. All regions covered in the report should meet the quality criteria set by the laboratory in terms of sequencing depth (minimum of 200X by capture methods, and 300X by amplicon-based methods);
- Verification of overall depth for all samples in order to detect potential aberrant results (outliers).

❖ VALIDATION OF LIBRARIES

- Verification of overall sequencing depth for all samples
- Verification of overall quality (Q30, Qmapping) of the data generated for each sample

❖ VALIDATION OF ROUTINELY IDENTIFIED VARIANTS

- For negative results, it is important to verify the minimum depth for the locus being studied (at least 300X for PCR enrichment, 200X for capture-based techniques).
- For variants, a minimum number of mutant reads should be defined for validating a mutation. This should be higher than the background noise, and can be expressed as an absolute value or as a percentage of the total number of reads at one position. If the threshold is defined as a percentage of number of reads, the total number of reads at this position needs to be at least equal to the minimum depth defined for the negatives.
- It is important to identify any samples with a large number of mutations, which could be a warning of a risk of observing artefactual mutations. In general, an increase in background noise is associated with a quality defect, and creates the risk of false positives/negatives.

REMARK In the event of invalidation, there should be a predefined fallback strategy for variants found at hotspots.

REMARK Confirmation of variants by an alternative technique can be limited to the validation phase. Once the technique is validated and in routine use, it is not indispensable to continue to verify all variants by another technique. *In silico* verifications (BAM) may be acceptable.

CHANGES TO THE PROTOCOL (SCOPE B)

The adoption of flexible type scopes is accompanied by a procedure known as “management of flexible scope” (or “management of technical changes”), listing all operations to be performed in order to control the process when a method or reagent is changed without the involvement of a new skill. The laboratory should establish a specific procedure to correspond to scope B and control changes in methods. This procedure describes all steps, from the laboratory’s initial needs (new robot, method development, etc.), to updating the detailed list of tests and reporting to COFRAC[5], with the responsibilities associated with each step.

For NGS techniques, rapid changes in protocols are currently being witnessed. This is especially true of changes to libraries and modifications in analysis algorithms. As a result, it is necessary to predefine procedures for verifying the performances of the method for these changes in protocol. Thus, when changing the library, it is necessary to perform at least one validation run, for which the experimental design should be adapted to the modification of the protocol (to be documented when updating the validation file or an appendix, depending on internal procedures).

REMARK It is not necessary to repeat a complete validation when the protocol is changed, but the performances of the method should be verified at every change. It is also imperative to provide traceability and monitoring for versions of the protocols in routine use.

[5] SH REF 08 – *Expression et évaluation des portées d’accréditation* (Expression and assessment of scopes of accreditation). COFRAC, June 2010

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