EBE-EFPIA Position Paper on

Next Generation Sequencing (NGS)

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Executive summary

Next generation sequencing (NGS), also known as high-throughput sequencing, is a DNA and RNA sequencing technology which has revolutionized genomic research. Today, an entire human genome can be sequenced within a single day using NGS. In contrast, the previous Sanger sequencing technology, used to decode the human genome and enable the discovery of novel genetic alterations causing diseases, required over a decade to deliver the final result.

The joint Personalised Medicine Working Group of the European Biopharmaceutical Enterprises (EBE) and the European Federation of Pharmaceutical Industries and Associations (EFPIA) has developed this position paper to explain, in a first part, the complex technical specifications of testing methodologies based on NGS (p3 to p8). The paper describes the steps performed in NGS (i.e. sample preparation, library generation, sequencing, data analysis and assay validation), providing in one test what was previously available only through multiple analysis, which often lead to the depletion of valuable patient specimens.

The following part of the position paper highlights the regulatory challenges associated with this complex technology and provides concrete recommendations for a way forward (p8 to p13).
Finally, the last part of the paper emphasizes the importance of personal data provided by NGS and the need for special measures to protect the individual's privacy, as well as special storage (p14 to p15).

NGS technology has transformed medicine through the assessment of patients' genetic makeup. This technology has the potential to accelerate the early detection of disorders and the identification of pharmacogenetics and pharmacogenomics markers to customize treatments based on patients' needs. Sequencing analyses is already being used clinically throughout the EU, particularly for guiding oncology treatment selection, and will likely become a central part of the routine diagnostic testing in the near future. However, a number of important issues have to be addressed to improve the development of innovative therapies guided by NGS in Europe. The regulatory challenges highlighted in this position paper have to be considered and discussed at Member States and European levels to ensure that a broader number of patients can access such promising technologies.

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List of abbreviations

ACMG	American College of Medical Genetics
ATCC	American Type Culture Collection
cDNA	Complementary deoxyribonucleic acid
CE mark	European Conformity mark
cfDNA	Cell-free DNA
ctDNA	Circulating tumour DNA
CNV	Copy-number variation
DNA	Deoxyribonucleic acid
EBE	European Biopharmaceutical Enterprises
EFPIA	European Federation of Pharmaceutical Industries and Associations
FASTQ	FAST alignment with quality scores
FFPE	Formalin-fixed paraffin-embedded
FiSH	Fluorescence in situ hybridization
GC content	Guanine-cytosine content
GC rich	Guanine-cytosine rich
ICH	International Council for Harmonisation
IHC	Immunohistochemistry
LncRNA	Long non-coding ribonucleic acid
mRNA	Messenger ribonucleic acid
NGS	Next Generation Sequencing
NIST	National Institute of Standards and Technology
PCR	Polymerase chain reaction
PGx	Pharmacogenomics
pH	Power of hydrogen
Poly(A)	Polyadenylation
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RNA-Seq	Ribonucleic acid sequencing
SNP	Single nucleotide polymorphisms
SNV	Single nucleotide variants
VAF	Variant allele frequency
WGS	Whole genome sequencing



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1) Overview of technical aspects

This section describes the different processes that are needed for NGS: sample preparation, library generation, sequencing, data analysis (interpretation and report generation) and assay validation.

a) Sample preparation

Generally speaking, any sample from which nucleic acids can be isolated could be suitable for NGS. Common NGS protocols examine saliva, sputum, urine, plasma (including cell free DNA), serum, tissue, tumors or isolated cells that may be fresh, frozen or preserved (formalin-fixed paraffin-embedded (FFPE)). The specific sample requirements are dependent on the assay being run and should be established by investigators or manufacturers during validation. Samples may require additional separate analyses to determine whether they meet the appropriate criteria for the assay. For instance, tumor sections may require pathological examination to determine whether sufficient viable tumor cells relative to stroma is present in the sample to allow for identification of mutations above the detection limit.

Specimen collection guidelines will be based on the assay criteria as described above and may include the volume or mass of sample, the thresholds for the acceptance or rejection of mixed samples containing both target and normal cells, and the collection protocol, including the collection method and conditions required to minimize sample degradation. Following DNA or RNA extraction from the sample, library preparation guidelines may include the quantity of DNA and RNA required for the assay, the length and/or fragmentation of the DNA and RNA isolated from the sample and the purity of the DNA and RNA samples (for instance, removal of PCR inhibitors from blood samples).

Given the sensitivity of NGS assays, especially those that feature multiple PCR amplification steps, rigorous steps should be taken to limit cross-contamination. Workspaces should be regularly decontaminated and items used for sample preparation should be either single use or thoroughly decontaminated between samples. If the protocol targets sample types with limited nucleic acid content, optimization of the nucleic acid extraction steps may be required to maximize yield and minimize contamination.

The addition of sample-specific quality control steps may be necessary in some cases. For instance, deamination of DNA extracted from FFPE blocks can generate apparent C->T mutations that were not present in the source tissue. These errors can be identified computationally if the region is sequenced bi-directionally; however, this limits assay sensitivity. Alternatively, the samples can be enzymatically treated to remove deaminated bases, though this requires higher DNA input.

b) Library generation and sequencing

Library generation produces DNA fragments of a size appropriate for the target sequencing platform with platform-specific adapter sequences at both ends. The three most common types of NGS libraries are derived from genomic DNA, exome DNA or a specific panel of target genes.



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Whole genome sequencing (WGS) is the most technically straightforward of the common library generation approaches. Briefly, extracted DNA is fragmented through enzymatic digestion or manual disruption. Adapters containing platform-specific sequences are ligated to the fragments and the entire pool is sequenced. This approach captures all regions of the genome and allows for the examination of coding regions as well as their regulatory areas, along with structural variants and rearrangements that may not be detected with more focused sequencing approaches. While the cost of whole genome sequencing has been decreasing with increased automation and higher throughput sequencers, WGS offers the lowest overall depth at the greatest cost and highest computational analysis requirements. The limited depth hinders the ability to characterize mixed samples; for instance, mutations within a tumor sample that are present at low frequency may not be reliably detected using WGS with a standard 30x average read depth.

In comparison to WGS, **whole exome sequencing** focuses on the coding regions of the genome. In addition to the fragmentation and adapter ligation used for WGS, a hybridization step isolates exome-specific DNA fragments using a pool of oligonucleotides that correspond to the exome. These exome-specific DNA fragments are sequenced to higher depth with lower cost and computational requirements than the WGS equivalent, though information in non-coding regions is lost. Coverage may be uneven as hybridization can be dependent on the base composition of the exome regions.

The final library preparation method, **gene panels**, targets specific genes, regions of genes or rearrangements, looking at regions of known or suspected importance for the target indication. Gene panels can be generated through a variety of methods including multiplex PCR, gap filling extension and ligation, or hybrid capture. Multiplex PCR and gap fill methods are susceptible to the fundamental issues that affect PCR design, including cross-reactivity, sensitivity to single base mutations in the primer binding region and limitations on the GC content to ensure binding; however, the protocols are generally faster with lower operator time requirements than hybridization methods.

By focusing on specific regions rather than the broader genome or exome, it is possible for gene panels to achieve higher depth with fewer reads, allowing for multiple samples to be run together on smaller desktop sequencers. As the panel would target areas with known disease drivers or indicators of clinical relevance, interpretation of results can be more computationally efficient. Additionally, the greater read depth allows for increased detection and confidence when calling minor variants in mixed samples.

Library preparation for the sequencing of RNA molecules (RNA-Seq) involves similar steps as DNA sequencing, with a few alterations to accommodate the different nucleic acid input. Since ribosomal RNA is the most prevalent RNA species present in samples, it must be removed from the sample prior to library generation. Typically, this is done with poly-A selection, ribosomal depletion, or exome-capture (akin to exome sequencing in DNA). In poly-A selection, biotinylated oligonucleotides that bind to the poly-A tail found in eukaryotic mRNAs are hybridized to the isolated RNA. The hybridized complex is then isolated. In the most common protocol, the bound mRNA is eluted, fragmented and primed with random oligonucleotides for cDNA generation. In ribosomal depletion, biotinylated oligonucleotides corresponding to the ribosome of the species being sequenced are hybridized to the isolated RNA. The hybridized complex is then removed and the remaining RNA (including mRNA, lncRNA and other RNA species) is fragmented and primed for cDNA generation.



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The cDNA and library preparation protocol can be either stranded or non-stranded. The most commonly used approach in stranded applications implies that the first cDNA strand is generated with a standard nucleotide mix and the second strand is "marked" with a nucleotide mix containing dU. After adapter ligation, the second strand can be digested or otherwise neutralized and only the parts of the library corresponding to the first strand will be amplified. The modification allows for specific determination of which DNA strand the original RNA molecule corresponded to, allowing for easier identification and mapping of sequences to reference genomes.

Similar to gene panels, targeted RNA expression analysis allows for multiplex evaluation of specific genes. This process generally uses the same chemistry as DNA gene panels with cDNA used as the input material in the place of genomic DNA.

As increasing capacity of NGS platforms has allowed for combining multiple individual samples in single sequencing run, one common step amongst library preparation methods is the incorporation of index sequences for sample identification. Index (or barcode) sequences are unique identifying sequences added to each library either during the adapter ligation or in a subsequent PCR step. Multiple uniquely barcoded samples are pooled for sequencing. After sequencing has completed, the software associated with the sequencing platform will identify the barcode and assign each read to the sample that particular barcode was associated with.

As with sample preparation, quality control metrics should be included at each step of library generation. The generation of a high quality library is essential for reliable, consistent NGS assay performance. For approaches that use hybridization (e.g., whole exome sequencing, SureSelect gene panels), care should be taken during design and optimization to ensure that the stringency level is optimized and all regions are covered efficiently. For difficult to target regions, using gentler fragmentation methods for larger input DNA fragments and targeting adjacent regions may generate better coverage at the cost of read depth from the additional flanking regions being sequenced.

For amplicon approaches, increased cycles of amplification can result in PCR errors introduced at low levels, adding noise to the sequencing and reducing the ability to reliably determine low copy variants. Hybridization methods generate random start and stop coordinates that can be used to determine PCR duplicates; this approach is not possible in amplicon approaches where the primers are fixed in the sequence. As such, proper primer design and validation must ensure consistent and reliable efficiency to avoid significant statistical sampling, especially in samples with low input copy numbers.

For all approaches, libraries should be monitored for indications of potential library generation failures including incorrect library size, the presence of excessive adapter dimers or low yield. Sample tracking methods should include the barcode assigned to a particular sample.

c) Choice of sequencing platform

The choice of specific sequencing platforms requires consideration of the intended purpose of the analysis (clinical or research), turnaround time (WGS (slow) vs small gene panels (faster)), economic, technical, and practical differences between currently available platforms. As individual platforms each



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have specific strengths, weaknesses, and limitations, knowledge of the operation and specifications for each individual system is important during selection of a sequencing platform for a specific assay.

Instrument and individual run costs, number of reads per run, read length, instrument run time, operator time, scalability, and the required computational resources can differ significantly between platforms. These factors should be considered in the context of the assay being developed, as the number of targets being examined, the required sensitivity and the characteristics of the region of interest can alter the feasibility of specific platforms. For example, more sequencing reads will be required to cover targets in larger sequencing panels at sufficient depth and increased sensitivity requirements will increase the number of reads required to capture rare variants that are present. These increases in required reads per sample will limit the number of samples that can be sequenced simultaneously, potentially making sequencing platforms not viable for economic (high per-sample sequencing cost) or technical (number of reads required is greater than the platform specifications) reasons.

Additionally, each sequencing platform manufacturer uses unique chemistry that will generate platform-specific error profiles. The examination of homopolymer regions, required accuracy at the end of long reads or utilization of paired-end sequencing are a few specific examples of platform-specific technical considerations that could exclude sequencing platforms from consideration.

d) Data analysis

NGS assays can generate large amounts of raw and processed data, requiring the development of comprehensive data handling and software analysis pipelines. Base calling, mapping, alignment, variant calling, variant filtering and variant annotation compose the standard data analysis pipeline. Clinical interpretation and generation of a report may be incorporated with the inclusion of appropriate support tools.

Base calling is the process through which the detection of fluorescent reporters or pH changes associated with nucleotide incorporation are converted to the corresponding nucleotide with an associated base-calling accuracy measurement in a FASTQ file. The accuracy measurement is typically recorded as Phred Q scores, the log-scaled probability that a single base will be called incorrectly. For example, a Q score of 30 indicates a probability of one erroneous call per one thousand bases, while a Q score of 10 indicates one error every ten base calls. Base calling is typically performed by the on-board sequencing software for each individual platform.

After base calling, the generated sequence file is mapped onto a reference sequence, generating alignment files (.sam, .bam). The reference used could represent the entire human genome or a smaller reference representing regions of interest for a faster, less computationally intensive approach. Given the differences between alignment software, the optimal alignment algorithm should be determined empirically using real and simulated data. Following optimization, the software version and any deviations from the default configuration in any parameter, cut-off or value should be recorded. In addition, the reference sequence version number and assembly details of the reference need to be retained. For RNA sequencing, the counts of individual genes and transcripts are calculated based on the number of individual reads that map to that gene.



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The aligned sequences are aggregated at each position in the genome to report variations from the reference sequence. Single nucleotide changes, insertions, deletions or structural variations can be reported as variants within a .vcf (variant call format) file. Called variants are dependent on the read depth, quality scores and percent of variant reads (variant allele frequency or VAF). Thresholds for each should be determined. For germline sequencing, VAF is generally 0% for homozygous normal, 50% for heterozygous and 100% for homozygous mutant. Deviations from those values may be due to PCR error, sequencing error, sampling error (especially for heterozygous loci with low read depth) or chromosomal abnormalities (copy number variations). For somatic sequencing, the VAF may vary if the sample is heterogeneous. Since sampling of DNA within the individual sample is presumed to be random, the VAF can be used as a measure of prevalence of a mutation within the mixed sample. The limit of detection of variants that are present in a minority of cells is highly dependent on the read depth of the sample.

Finally, variant annotation and filtering connect the called variants to functional information that can indicate biological and clinical relevance. Variant annotation can determine whether variants are likely to affect protein expression, alter protein sequences and occur in conserved loci. Variant filtering prioritizes variants that are likely to have biological or clinical relevance. Incorporation of public databases and literature can eliminate common or known benign variants in order to streamline later analysis steps. For whole genome and whole exome sequencing, filters may focus on particular candidate genes, predictions of effect on protein function, predictions of effect on biologic pathways or prevalence in control populations. The specific filters will need to be generated based on the specific biological question being raised and specific to the input matrix. For targeted disease panels, variants that are known to be relevant based on established databases and literature can be identified. Similarly, variants that alter essential protein residues can be prioritized over mutations that don't result in alterations to the primary structure of the protein or its predicted expression.

Recommendations from ACMG regarding germline variants are to include sequence reference (as described above) in clinical reports and to use the term "variant" with the following modifiers: (i) pathogenic, (ii) likely pathogenic, (iii) uncertain significance, (iv) likely benign, or (v) benign. In addition, findings should be reported with respect to a condition (e.g. c.1521_1523delCTT (p.Phe508del), pathogenic, cystic fibrosis). Also, results should be interpreted by a board-certified clinical molecular geneticist or molecular genetic pathologist or the equivalent.

For each of the listed steps, there are a number of commercial and open-source programs that perform the specific required functions. The assumptions and parameters for each program can vary, giving different results for the same input sample file. Additionally, detection of SNPs, insertions and deletions, base pair substitutions, copy number alterations and structural variants may require separate analysis pipelines for optimal accuracy. As a result, the pipeline should be validated as part of the full assay and version control measures should be implemented to ensure consistent results across operators and institutions. Of significant note, the pipeline is expected to undergo continuous improvement and validation with each sequencing run performed and as more data become available in databases that serve as a source of data for the pipeline.

During assay development, the requirements of the data analysis pipeline should be considered. The quality of NGS reads decays over the course of the read, especially for very long reads, so the confidence of variants will be greatest closer to the start of the read. The required read depth, which influences the



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choice of sequencing platform, can be approximated using the required sensitivity, the minimum detection threshold, the expected quality of the sequencing reads in that region and the tolerance for false reports.

e) Assay validation

All components of the assay (sample preparation, library generation, data analysis) should be validated using approaches according to international standards when possible and appropriate. In some cases, clinical specimens should be used to validate the performance of an assay, while in other cases, well-characterized reference samples should be used to determine whether the test is able to capture and identify regions of interest, even if the reference samples do not contain pathogenic variants. These reference samples should be renewable (e.g., cell lines) so that future modifications of the protocol can be assessed relative to the same baseline standard. For assays that will be quantifying the mutational burden within a sample, reference samples containing specific mutations can be mixed at varying ratios to generate the appropriate reference standard. Additionally, the assay should be validated for all accepted sample sources (blood, tissue, tumor, FFPE, etc.).

The level of validation and approach taken will vary depending on the intended use of the NGS assay. For an assay intended to aid in the selection of patients for treatment, validation could require extensive characterization of single variants as compared to an orthogonal method using clinical specimens linked to clinical outcomes. In contrast, validation may be achieved via a variant class approach using contrived samples for an assay designated for research use only. Interactions with global health authorities and reimbursement bodies will continue to shape the definition of validation for different intended uses and it will be important to ensure that the level of validation is determined on a risk-based approach.

2) Regulatory challenges associated with NGS

Following the description of NGS processes above, this section highlights in a pragmatic way the regulatory challenges associated with this complex technology. Each challenge is supported by a concrete recommendation for a way forward.

a) One size does not fit all: Differences in performance consistent with the intended use

***Problem statement*:**
Just as for other technologies, the application of uniform performance standards is not appropriate for testing methodologies based on NGS.

***Key considerations*:**

- NGS test results can be used for many different clinical purposes. As a consequence, the clinical impact of differences in device performance varies greatly:
  - Detection of mutations via NGS can have disparate impact on clinical decision-making ranging from aid in diagnosis in the context of multiple other clinical indicators, or as a stand-alone decision-making tool.

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- The performance of a NGS test should also be evaluated in the context of the clinical performance of the corresponding therapeutic product. Variability in testing performance can have very different impact on the safe and effective use of the corresponding medicinal product.

- Technical performance can vary for different molecular alterations (e.g. SNVs, CNV, Indels) and sample types.
- Disease biology can limit technical performance and dictate that differences in performance will be observed, e.g. through elements such as tumour tissue heterogeneity.

Recommendations:
Performance metrics should therefore not be uniformly applied. There is a need for recognition that differences in clinical application of the test mandates different performance standards. EBE-EFPIA therefore call for "fit- for purpose" performance specifications instead of standardized, fixed performance criteria.

b) <u>Differentiation between somatic versus germline mutation testing</u>

Problem statement:
Performance criteria for somatic mutation testing are more difficult to define than for germline mutation testing.

Key considerations:

- Germline cells have a uniform genetic makeup. A uniform sample allows for definition of uniform performance criteria. Somatic mutations, on the other hand, can result in significant genetic heterogeneity in the sample, which in turn leads to variability in testing.
- For example, reproducibility for testing for a sample with a uniform genetic make-up will be higher than in the case of a sample of a very heterogeneous genetic make-up. Therefore, it would be inappropriate to apply the sample reproducibility acceptance criteria. The same consideration may be applied to all other aspects of technical performance evaluation (accuracy, reproducibility, sensitivity and stability).

***Recommendation*:**
In point a, EBE-EFPIA points out that performance metrics should not be uniformly applied, but instead we call for fit-for-purpose performance goals defined based on the context of use. This is especially important when considering NGS tests for somatic mutation testing, where overall more variability is to be expected than germline mutation testing uses.

c) <u>Reference Materials and Performance Standards</u>

***Problem statement*:**
NGS technologies are developed and characterized using many different samples and materials without standardized assay performance measures. To ensure a clear understanding of the performance of different assays, there is a need for the development and broad availability of appropriate reference materials and performance metrics to facilitate consistent performance evaluation of analytical validation of NGS-based tests.



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Key considerations:

- Application (somatic vs germline, disease area/intended use), specimen medium (tissue vs saliva vs blood), and analyte (RNA vs DNA) will influence what is appropriate for use in different cases.
- There is a need for an independent centralized repository (possibly a commercial provider, e.g., Coriell Institute, ATCC, Horizon, SeraCare or an independent agency, e.g., 1000 Genome Project, NIST) that manufactures and performs Quality Control on the reference materials.
- Utilization of multiple orthogonal methods that can detect the entirety of variant types (SNVs, CNVs, structural variants, epigenetic, etc.) can be used to determine ground truth. Note that assessment of structural variants frequently requires evaluation by more than one NGS method.
- Reference materials must be globalized and globally accepted – they should be designed to account for genomic variations based on ethnicities.
- A diversity of reference materials will be required for germline testing (including hereditary oncology)
    - Reference materials should include diversity of variant types (e.g., in/del, CNV, substitutions, etc.) as well as "challenging sequences" (e.g., homopolymeric tracts, GC rich, etc.)
- Associated standardized Quality Control metrics for reference material (specified expected calls at specified read depths, etc.)
    - Could be implemented in the form of product specifications/package insert
    - Could aid in the broader adoption of proficiency testing.

Recommendation:
There is a need for global standard reference materials that are specific to the application of the test and specimen type being tested with defined performance metrics based on the intended use/risk of the test (For example, MDIC SRS).

d) Transparency in NGS labelling

Problem statement:
Different tests and/or test providers that run NGS testing for the detection of the same variant may derive different/conflicting results (due to lack of analytic harmonization (technical performance) or because of different tests having different cut-offs for what is called positive related to clinical validity of the test which is in turn related to the intended use), which can ultimately impact the treatment plan for a patient (clinical utility). There is therefore a strong need to ensure the availability of accurate, truthful information about all available tests.

Key considerations:

- Need to develop a patient- and provider-friendly label templated with flexible yet comparable fields/sections defined for NGS biomarker tests to facilitate the transparency of test performance characteristics (e.g. sensitivity and specificity), improve the level of evidence generated for the intended use of the test, and highlight the limitations of the test.
    - A requirement to demonstrate proficiency via internal validation with a standardized reference material and orthogonal method could help to resolve this challenge.



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- Not all NGS-based IVD tests will have equivalent performance, therefore results may not be comparable from one test to another. There is a need to standardize multi-stakeholder understanding of test cut-offs, how cut-offs are achieved, the quality and depth of the sequencing reads, and the data relied upon to support the test being offered (e.g. validation sample choice of either more challenging clinical specimen or cell line derived contrived specimen). Until standardization is achieved, the cut-off and associated testing platform should be identified to increase transparency.

Recommendation:
There is a need for template, comparable labelling to impart greater transparency on limitations and performance characteristics.

e) <u>Flexibility: Certain changes to NGS workflow components or claims should be able to be incorporated quickly</u>

Problem statement:
NGS technologies can rapidly evolve with new methods and chemistry generating continual improvement in accuracy and sensitivity. Additionally, as the use of NGS in the clinic continues to increase, progressively larger amounts of clinically relevant data will be generated that could lead to the identification or validation of clinically relevant markers. The regulatory framework for next generation tests should be able to accommodate future improvements, both predicted and unanticipated, to support using the most up-to-date (scientific knowledge) while ensuring safe and effective diagnostic tests.

Key considerations:
- Establishment of pre-specification plan for anticipated changes to the variants, specimens or claims is recommended to allow for rapid improvement.
  - Expected claims or test modifications can be demonstrated with protocols and acceptance criteria for performance metrics
  - The pre-specification plan should include requirements for documentation and communication with the relevant regulatory bodies
  - In the event of the modification leading to a label change, the pre-specification plan should include agreement on the labelling update
  - Criteria for accepting software and database updates within the current approval should be included in the pre-specification plan.
- In case of post-market expansion outside of pre-specification plan
  - Expansion of the assay to new targets outside of the pre-specification plan should build upon the previous validation
  - If additional genes or targets are added to a previously validated assay – new regions will require separate validation of performance, and depending on completion of risk analyses, validation of previous regions could be limited to well-characterized samples to establish previous performance.

Recommendation:
There is a need to develop best practices for change management. There is also a need for guidance on which changes would prompt additional regulatory interactions.



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f) Levels of evidence required for a diagnostic test used in investigational settings

Problem statement:

Recently, several pharmaceutical companies have been requested by National Competent Authorities and Ethic Committees to CE mark the diagnostic used in clinical development programs, a requirement which would greatly hinder clinical development (further details in EBE-EFPIA position on "risk-based approach for biomarker assay development in clinical trials as an alternative to CE marking").

Key considerations:

- Explorative test procedures should be acceptable in early investigational settings prior to development of a diagnostic test. Basic requirements concerning validation should be defined and completed prior to use in clinical trials.
- A stepwise validation approach is suggested and should be aligned with clinical development.
    - For early exploratory clinical trials: respective validation for specificity/sensitivity should be required. Specificity/sensitivity should be sufficiently defined (e.g. variant allele frequency > 5%, maybe further confirmed or narrowed in later clinical development phase), ensure test robustness. Confirmatory/registrational trials should require more robust assessment.
    - Accuracy testing: proposed to be conducted with samples collected in clinical program. Problem: available standard reference method or orthogonal test (Example for (1) DNA-based NGS: sanger sequencing is currently the 'standard reference' method, which may not be sensitive enough for e.g. low variant allele frequency; (2) RNA-based NGS: qRT-PCR or digital PCR as standard reference method are suitable for analysis of individual genes but does not allow for high-throughput profiling of multiple genes; (3) gene-fusions: no established standard methodology available, RNA-sequencing possible, comparison versus protein based assays (e.g. IHC) is not always concordant, availability of standardized reference materials is limited). It is suggested for later clinical development once clinical data are available to support the assessment of concordance on a technical level:
        - Consider a standardized repository of reference material (e.g. Coriell Institute)
        - Consider standard methodologies vs. case-by-case approach (dependent on degree of established clinical utility of biomarker)
        - Generalization across different types of variants with respect to assay validation requirements: orthogonal testing based on selected representative methods, selection of representative genes.

Other Suggestions:

- Development/generation of a common laboratory certification for laboratories specialized and qualified to perform clinical NGS testing that help develop companion diagnostic tests.
- Initiative for development/testing of standards for NGS data generation (e.g. sequencing platforms), processing and bioinformatics analysis (e.g. analysis pipeline, variant calling). (For example FDA initiative at https://precision.fda.gov).

Recommendation:



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Regulatory guidance in conjunction with technical and clinical expertise is needed on the appropriate validation requirements for investigational use of NGS tests during drug/diagnostic development considering mutual dependency of clinical and assay development.

g) Variant calling database/algorithm

Problem statement:

The rapid evolution of next generation sequencing tests in the clinic has led to the identification of an increasing number of genetic variants. The clinical potential of these variants holds great promise, though this requires the aggregation of the data in accessible databases using common standards to allow for analysis and determination of clinical significance.

Key considerations

- To ensure reproducibility for the individual algorithms used, the software documentation should include:
  - Documentation of NGS software to be used, including origin, modifications, versions, traceability, reference sequence assembly, components needed to assemble the analysis pipeline, criteria for annotation and filtering, and whether the data will be run locally or remotely
  - Documentation of all database(s) the algorithm is dependent on, including origin of databases (internal/third-party), the content of the database (full genome, exome, target sequences, etc.), record of the version of the database, description of how new version of existing databases will be incorporated and re-validated
  - Public databases (if applicable)
  - Data preservation - methods for incorporating changes and updates, along with version control of the database
  - Validation - Inclusion of indicated populations in the dataset.
- To ensure reproducibility and data integrity for any internal databases used, the database protocols should adhere to the following guidelines:
  - Data preservation – administrators should have processes in place for establishing database stability and for ensuring that any dependencies on secondary databases are maintained
  - File formats – databases should utilize commonly accepted formats and nomenclature with regards to gene names, symbols, coordinates, variants, classifications, etc., to minimize ambiguity and allow for assessment of performance
  - Metadata – identified variant classifications and interpretations should be accompanied by all relevant information including studies reporting the classification, the detection method and the variant characteristics
  - Curation – databases should be curated to ensure individual data points are unique and represented once in the database. Assertions within the database should generally be descriptive (benign, likely pathogenic, etc.) with source of the evidence clearly identified. Definitive assertions should not be made unless supported by multiple lines of evidence.



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- To ensure that all of the aforementioned points are in proper and compliant working order, there is a need for validation of database infrastructure (i.e. inclusive of local SOPs dictating operation of all of the above).

Recommendation:
There is a need for guidance on best practices related to variant calling database/algorithm.

3) Data protection and data storage issues

As an individual stretch of genomic sequence in isolation does not reveal personal data of an unknown donor, generally genomic data produced by NGS technologies should not be regarded automatically as personal information. Nevertheless, the data require protection as under certain circumstances, in particular in combination with data from other sources, there might be a risk of reidentification and consequently a risk for the rights and personal freedoms of the individual. For this reason, in clinical research, genomic data follows the same high standards of confidentiality as other clinical datasets. Typically genomic datasets are coded using schemes as described e.g. in ICH E15. Pseudonymisation techniques such as single and double coding of the data thereby support traceability of genomic data, which is a key prerequisite for:

- Maintaining a high level of data quality which facilitates Good (Clinical) Research Practice
- Enabling transparency and accountability for data usage by maintaining the chain of custody
- Safeguarding confidentiality while maintaining subject rights
- Supporting active donor consent management
- Advancing research in Precision Medicine to the benefit of the patients by allowing data to be aggregated thereby connecting previously unlinked genomic data to phenotypic data.

a) Coding schemes to protect privacy

Principles of data minimization help to protect donor's privacy. There are a number of ways by which the protection of individual privacy can be ensured, depending on the intended result of the analysis performed and the degree of risk that the kind of performed analysis represents to the affected individual. Anonymisation is one way to accomplish this. The use of anonymised datasets yield the highest level of data privacy but may limit the re-use of these datasets. For example, as future developments in biomedical research cannot be foreseen there might be a high need to revisit NGS based datasets particularly in disease areas where NGS represents an enabling technology such as cancer research. Here, anonymised NGS datasets might be of limited use for meta-studies and data aggregation. Hence, in cases where large scale genomic data integration is required, data aggregation based on pseudonymised datasets could leverage concepts of differential privacy to maintain a certain level of data privacy while still facilitating traceability.

b) Informed consent

Generally, patient consent in clinical research follows ICH E6. For PGx (Pharmacogenomics / genetics)

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research as described in ICH E15, local regulations, policies and / or other country specific legislation may apply. The heterogeneity of different regularities poses a challenge for operational implementation of consent management.

c) Transparency in communication of NGS results

Whole genome scale NGS datasets exhibit multiple genomic features potentially resulting in incidental findings. Communication of these findings should be planned in accordance with country specific legislation, local regulations and local ethics, e.g. respecting the patient's right to know or not to know about these findings. Other factors to be considered in making incidental findings available are whether it is ethically-appropriate to inform subjects of results from studies which have not been validated and whether the researcher has the competence to assess findings that may lie outside the scope of the study. In addition, the availability of NGS assays which would qualify for clinical decision making (e.g. cleared or approved NGS tests) is limited. As a result, clinical reporting of incidental findings should also be transparent with respect to the NGS assay's capabilities and predictive power for clinical practice.

d) Storage of and access to NGS data and samples

NGS based techniques typically are producing large datasets. In the interest of high quality research and reproducibility of the results, reconstruction of or access to raw data should be provided for a reasonable period of time. Lossless DNA compression techniques may help to reduce the data volume for archival purposes while maintaining the ability to reconstruct the full raw results. Access should be documented. It should be restricted to authorized and qualified personnel only.
At the same time, access to high quality clinical samples is critical as more sensitive NGS methods may become available such as single cell sequencing, sequencing of cfDNA from liquid biopsies etc. particularly to support development of treatment accompanying diagnostics (called companion diagnostics (CDx)). Here, Biobanks play a pivotal role to ensure access to suitable high quality samples allowing broader assessment of stratifying biomarkers across diseases.

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SUMMARY of key regulatory recommendations to consider

2) a – One size does not fit all: Differences in performance consistent with the intended use
Performance metrics should not be uniformly applied. There is a need for recognition that differences in clinical application of the test mandates different performance standards. EBE-EFPIA therefore call for "fit- for purpose" performance specifications instead of standardized, fixed performance criteria.

2) b - Differentiation between somatic versus germline mutation testing
EBE-EFPIA point out that performance metrics should not be uniformly applied, but instead we call for fit-for-purpose performance goals defined based on the context of use. This is especially important when considering NGS tests for somatic mutation testing, where overall more variability is to be expected than germline mutation testing uses.

2) c - Reference Materials and Performance Standards
There is a need for global standard reference materials that are specific to the application of the test and specimen type being tested with defined performance metrics based on the intended use/risk of the test.

2) d - Transparency in NGS labelling
There is a need for template, comparable labelling to impart greater transparency on limitations and performance characteristics.

2) e - Flexibility: Certain changes to NGS workflow components or claims should be able to be incorporated quickly
There is a need to develop best practices for change management. There is also a need for guidance on which changes would prompt additional regulatory interactions.

2) f - Levels of evidence required for a diagnostic test used in investigational settings
Regulatory guidance in conjunction with technical and clinical expertise is needed on the appropriate validation requirements for investigational use of NGS tests during drug/diagnostic development considering mutual dependency of clinical and assay development.

2) g - Variant calling database/algorithm
There is a need for guidance on best practices related to variant calling database/algorithm.



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