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Guidelines

Part II - Analytical phase

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Summary
Next-generation sequencing (NGS) has become a cornerstone of precision oncology, requiring standardized workflows in molecular pathology laboratories. The analytical phase, which includes all procedures from nucleic acid quantification to variant interpretation, plays a central role in ensuring the accuracy and clinical utility of molecular results. This document aims at supporting pathology teams – comprising pathologists, technicians, and molecular biologists – during the implementation and execution of the analytical phase of NGS testing. Key topics include clinical indications, platform and gene panel selection, bioinformatics pipelines, quality assurance strategies, and organizational considerations. The goal is to promote standardized, high-quality molecular diagnostics to advance precision pathology.

Key words: Pathology, recommendations, analytics, precision medicine, standardization

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Introduction

Next-generation sequencing (NGS) is a powerful tool for simultaneously detecting multiple genetic alterations across diverse samples, significantly advancing molecular tumor characterization to inform treatment decisions and guide patient management [1,2]. Recognizing the growing importance of NGS, national and international scientific societies and regulatory agencies now recommend using this method in clinical practice [2-4]. However, the implementation of NGS technologies within routine pathology workflows presents considerable challenges. Furthermore, the standardization of NGS procedures is essential for harmonizing predictive molecular testing and ensuring inter-laboratory consistency [5,6].
The analytical phase in predictive molecular pathology encompasses all

procedures involved in the generation, processing, and analysis of molecular data, starting from nucleic acid quantification to the identification of clinically relevant variants [7,8]. This document presents recommendations for the analytical aspects of NGS, offering comprehensive best practices for: (i) current clinical indications for NGS testing; (ii) available NGS platforms; (iii) gene panel design; (iv) bioinformatic tools; (v) quality assurance measures; and (vi) key organizational factors essential for successful implementation of the NGS workflow.

Overview of the current recommendations for NGS testing in molecular diagnostics

The integration of NGS into molecular diagnostics has enabled the sequencing of extensive genomic regions, facilitating the simultaneous identification of multiple clinically relevant molecular alterations [9]. However, the application of NGS across all cancer patients and the clinical utility of Comprehensive Genomic Profiling (CGP) remain topics of ongoing debate [10]. To promote appropriate patient selection for NGS testing, national and international scientific societies, alongside regulatory agencies, have established specific recommendations [11,12] (Tab. I). These guidelines consider not only the biological actionability of molecular alterations but also several key regulatory factors, including:

- The availability of approved targeted therapies for specific molecular alterations within distinct cancer types and clinical stages;
- The list of biomarkers and alteration types

Table I. Actionable genes recommended for testing by tumor type, based on national (Italian) and international guidelines. For each tumor type, molecular testing indications vary depending on the clinical context. In this table, the clinical setting has been intentionally omitted to focus on the general association between tumor types and actionable genes.

Tumor type	Italian health system	ESMO	NCCN
NSCLC	*EGFR, ALK, ROS1, BRAF, KRAS, ERBB2, RET, MET, NTRK1-3*	*ESCAT I: EGFR, ALK, KRAS, RET, ROS1, BRAF, MET, NTRK1-3* *ESCAT II: ERBB2, NRG1*	*EGFR, ALK, KRAS, RET, ROS1, BRAF, MET, HER2, NTRK1-3*
Cholangiocarcinoma	*IDH1, ERBB2, FGFR2, NTRK1-3, BRAF, MSI*	*ESCAT I: IDH1, FGFR2, BRAF, KRAS, ERBB2*	*FGFR2, IDH1, HER2* overexpression/ amplification, *RET, BRAF, KRAS^{G12C}, NTRK1-3*, MSI, TMB
Colorectal cancer	*KRAS, NRAS, BRAF, MSI, ERBB2, NTRK1-3*	*ESCAT I: KRAS, NRAS, BRAF, MSI* *ESCAT II: ERBB2, POLE*	*KRAS, NRAS, BRAF*, MSI, *HER2* amplification
Breast cancer	*ERBB2 amplification, PIK3CA, ESR1, PTEN, AKT* *Solid tumors without available therapeutic options*	*ESCAT I: ERBB2 amplification, PIK3CA, ESR1, PTEN, AKT1, BRCA1/2 (germline)* *ESCAT II: PALB2, BRCA1/2 (somatic), ERBB2 hotspot mutations*	*HER2, PIK3CA, AKT1, PTEN, ESR1, BRCA1/2* (germline), *NTRK1-3*, MSI, *RET*
Prostate cancer	*BRCA1-2, NTRK1-3*	*ESCAT I: BRCA1/2 (germline/somatic)* *ESCAT II: PTEN, ATM, PALB2*	*BRCA1-2* (germline/somatic), *ATM, PALB2, FANCA, RAD51D, CHEK2, CDK12*, MSI, TMB
Pancreatic adenocarcinoma	Not reported	*ESCAT I: BRCA1/2 (germline), KRASG12C, NTRK1-3 fusion, MSI* *ESCAT II: PTEN, ATM, PALB2*	*ALK, NRG1, NTRK1-3, ROS1, FGFR2, RET, BRAF, KRAS, BRCA1-2, PALB2, HER2* amplification, MSI, TMB
Ovarian cancer	*BRCA1-2, HRD status*	*ESCAT I: BRCA1/2 (germline/somatic), HRD$^{\$}$*	BRCA1-2, HRD status, MSI, TMB, BRAF, FOLR1, RET, NTRK1-3
GIST	Not reported	*ESCAT I: KIT, PDGFRa*	*BRAF, NF1, NTRK1-3, FGFR2** **NGS recommended in KIT-PDGFRA wildtype cases*
Thyroid cancer	*BRAF, RET, NTRK1-3*	*ESCAT I: BRAFV600E°, RET£^, NTRK1-3 fusion*°*	*ALK, BRAF, NTRK1-3, RET*, MSI, TMB
Urothelial cancer	*FGFR3* mutations/fusions, NTRK1-3	*ESCAT I: FGFR1/2/3* mutations/ fusions, NTRK	*FGFR2, FGFR3*
Melanoma	Not reported	*ESCAT I: BRAF, NTRK1-3 fusion*	*BRAF, NRAS, KIT, ALK, ROS1, NTRK1-3*
Endometrial cancer	*POLE, TP53, MSI, NTRK1-3*	*ESCAT I: MSI*	*POLE, TP53, NTRK1-3, MSI, TMB*
Carcinoma of unknown primary origin	Comprehensive genomic testing	*ESCAT I: TMB* *ESCAT II: ALK*	Comprehensive genomic analysis, including gene expression profiling and mutational testing

(e.g., mutations, copy number variations, gene rearrangements) to be tested;
- The likelihood of detecting clinically relevant alterations in specific tumor types;
- The availability of resources and facilities to efficiently and cost-effectively conduct NGS testing.

International recommendations

In a recent publication, ESMO recommended NGS for identifying tumor-agnostic alterations when matched therapies are accessible [2]. The updated 2024 guidelines expanded NGS indications beyond the 2020 recommendations [13], now encompassing advanced breast cancer, gastrointestinal stromal tumors (GIST), sarcomas, thyroid cancer, and cancers of unknown primary (CUP) (Tab. I). ESMO also stated that multigene sequencing outside these indications may be considered if patients are informed about the potential benefits and if the procedure does not impose significant additional costs on the public health system or lead to unsupported off-label drug use. The American Society of Clinical Oncology (ASCO) advises multigene sequencing for metastatic cancer patients with multiple actionable targets [14]. However, tissue-agnostic targeted therapies do not justify universal multigene testing for all solid tumors. The prevalence of specific molecular alterations (e.g., NTRK fusions) should guide decision-making, factoring in cost, available biological material, and clinical trial eligibility. Differences between ESMO and ASCO guidelines primarily stem from patient selection criteria for targeted therapies, reflecting distinct regulatory frameworks between the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA) [15]. In this context, the International Society of Liquid Biopsy (ISLB) is playing a crucial role in defining technical standards and quality benchmarks for liquid biopsy applications [4]. These efforts are essential for ensuring the reliability of liquid-based NGS, especially when tissue availability is limited or repeated sampling is not feasible.

Italian National guidelines and recommendations

In Italy, molecular predictive testing guidelines are collaboratively developed by representatives of Associazione Italiana di Oncologia Medica (AIOM), Società Italiana di Anatomia Patologica e Citologia Diagnostica (SIAPeC), and Società Italiana di Genetica Umana (SIGU). The Italian National Health System established essential healthcare services for predictive biomarkers in selected cancers through the 2017 Essential Levels of Care (Livelli Essenziali di Assistenza, LEA) framework [16]. In 2023, the Italian Ministry of Health and the Consiglio Superiore di Sanità (CSS) proposed regulatory criteria for the appropriate use of prognostic and predictive biomarkers in clinical practice [17]. Echoing ESMO and ASCO recommendations, the document emphasizes prioritizing NGS testing when multiple actionable alterations are linked to available targeted therapies, thereby reducing turnaround time and costs and optimizing sample use (Tab. I). The guidelines distinguish between targeted NGS panels for specific alterations and CGP for broader genomic insights necessary for diagnostic, prognostic, or predictive purposes. CGP is deemed appropriate in contexts such as homologous recombination deficiency (HRD) profiling in ovarian cancer, comprehensive profiling in CUP, pediatric solid tumors, and advanced cancers lacking further approved treatment options. For these cases, CGP should be recommended by a Molecular Tumor Board (MTB), which each Italian region must establish [18]. Patients considered for CGP must meet the following criteria:
- A life expectancy of at least 6 months.
- Good performance status (PS 0-1).
- Exhaustion of all standard treatment options.
- Absence of approved biomarkers or resistance to existing targeted therapies.
- Availability of sufficient biological material for genomic profiling.

MTB or multidisciplinary teams are responsible for patient selection. MTBs or laboratories accredited by NHS for NGS testing determine suitable NGS panels. MTBs provide treatment and clinical trial guidance.

RECOMMENDATION STATEMENTS

1. NGS testing should be prioritized in advanced cancers when multiple actionable alterations diagnostic and/or predictive for approved targeted therapies are available.

2. The use of CGP for patients with advanced cancers should be guided by an MTB.

NGS technologies

NGS platforms used for clinical purposes primarily rely on two sequencing technologies (ion semiconductor sequencing and reversible terminator/sequencing by synthesis) and chemistries (amplicon- or hybridization and capture-based) [19]. These technologies offer flexibility in sample throughput and varying turnaround times for data analysis. Despite different chemistries, these platforms follow a similar workflow, including library preparation, template generation and sequencing (Tabs. II, III).

Table II. Comparison of amplicon-based and hybrid capture-based library preparation approaches.

Library	Features	Advantages	Disadvantages
Amplicon-based	Selective amplification of specific genomic regions by PCR	High specificity for target genomic regions	Restricted to predefined genomic regions
	Specific primers for genomic targets of interest	Relatively low library preparation costs	Possible amplification bias
	Allows analysis to focus on predetermined genetic regions	Low initial DNA input	Difficulties in detecting complex structural variants
	Requires careful primer design	Fast library preparation	Risk of drop-out due to variations in primer sequences
		High sensitivity for specific variants	Poor coverage of non-amplified regions
		Suitable for analysis of known mutations or regions of clinical interest	
Hybrid capture-based	Hybridization probes to select specific genomic regions	Wider coverage than the amplicon method	Higher costs compared to the amplicon method
	Involves the preparation of an initial genomic library	Possibility of analyzing larger genomic regions	Greater procedural complexity
	Employs hybridization enrichment technologies	Improved uniformity of coverage	Requires larger amounts of initial nucleic acid
		Flexibility in the selection of regions of interest	Possible capture inefficiencies for some regions
		Reducing the genomic background	Increased library preparation time
		Suitable for analysis of complex gene panels	

Table III. Sequencing modalities primarily used in routine diagnostic practice. Main characteristics, applications, and output capabilities.

	Sequencing platform	Sequencing		Key applications	Read length (BP)	Data output per run	Run time	Number of reads
Illumina	iSeq 100 System	Polymerase-mediated incorporation of fluorescent nucleotides		Target gene panel	2 × 150 bp	1.2 Gb	~9.5–19 hr	
	MiniSeq System				2 × 150 bp	7.5 Gb	~5–24 hr	
	MiSeq System				2x300 bp	15 Gb	~5.5–56 hr	
	MiSeq i100 Seriesa				2 × 300 bp	30 Gb	~4–15.5 hr	
	NextSeq 550 System			Target gene panel - Exome and large panel sequencing	2 × 150 bp	120 Gb	~11–29 hr	
	NextSeq 1000 and 2000 Systems				2 × 300 bp	540 Gb	~8–44 hr	
	NovaSeq 6000 System			Target gene panel - Exome and large panel sequencing - Whole genome	2 × 250 bp	3 Tb	~13–44 hr	
	NovaSeq X Series				2 × 150 bp	8 Tb	~17–48 hr	
Thermofisher	Genexus System	H+ ion sensitive transistor	Ion Torrent™ GX5™ CHIP	Target gene panel	200 bp	3-12 Gb	13-24 hr	12-48 M
					400 bp	6-24 Gb	13-24 hr	
	Ion GeneStudioS5 System	H+ ion sensitive transistor	Ion Chip 510		200 bp	0.3-0.5Gb	4.5 hr	2-3 M
					400 bp	0.6-1Gb	10.5 hr	
			Ion Chip520		200 bp	0.6-1Gb	7.5 hr	4-6 M
					400 bp	1.2-2Gb	12 hr	
					600 bp	0.5-1.5Gb	12 hr	3-4 M
			Ion Chip 530		200 bp	3-4 Gb	10.5 hr	15-20 M
					400 bp	6-8 Gb	21.5 hr	
					600 bp	1.5-4.5 Gb	21 hr	9-12 M
			Ion Chip 540	Target gene panel - Exome and large panel sequencing	200 bp	10-15 Gb	19 hr	60-80 M

Table III. *Continues from the prevoius page.*

	Sequencing platform	Sequencing		Key applications	Read length (BP)	Data output per run	Run time	Number of reads
Thermofisher	Ion GeneStudioS5 Plus System	H+ ion sensitive transistor	Ion Chip 510	Target gene panel	200 bp	0.3-0.5Gb	3 hr	2-3 M
					400 bp	0.6-1Gb	5 hr	
			Ion Chip520		200 bp	0.6-1Gb	3.5 hr	4-6 M
					400 bp	1.2-2Gb	5.5 hr	
					600 bp	0.5-1.5Gb	5.5 hr	3-4 M
			Ion Chip 530		200 bp	3-4 Gb	5 hr	15-20 M
					400 bp	6-8 Gb	8 hr	
					600 bp	1.5-4.5 Gb	8 hr	9-12 M
			Ion Chip 540	Target gene panel - Exome and large panel sequencing	200 bp	10-15 Gb 20-30 Gb (2 run in 1 day)	10 hr 20 hr	60-80 M
			Ion Chip 550		200 bp	20-25 Gb	11.5 hr	100-130 M
	Ion GeneStudio S5 Prime System	H+ ion sensitive transistor	Ion Chip 510	Target gene panel	200 bp	0.3-0.5Gb	3 hr	2-3 M
					400 bp	0.6-1Gb	5 hr	
			Ion Chip520		200 bp	0.6-1Gb	3 hr	4-6M
					400 bp	1.2-2Gb	5.5 hr	
					600 bp	0.5-1.5 Gb	5.5 hr	3-4M
			Ion Chip 530		200 bp	3-4 Gb	4 hr	15-20M
					400 bp	6-8 Gb	6.5 hr	
					600 bp	1.5-4.5 Gb	7 hr	9-12M
			Ion Chip 540	Target gene panel - Exome and large panel sequencing	200bp	10-15 Gb	6.5 hr	60-80M
					200 bp (2 runs in 1 day)	20-30 Gb	10 hr	
			Ion Chip 550		200 bp	20-25 Gb	8.5 hr	100-130 M
					200 bp	40-50 Gb (2 runs in 1 day)	12 hr	
	Genexus Dx integrator System	H+ ion sensitive transistor	Ion Torrent™ GX5™ CHIP	Target gene panel	200 bp	3-12 Gb	13-24 hr	12-48 M
					400 bp	6-24 Gb	13-24 hr	

Library preparation

The DNA fragments produced during this phase depend on the sequencing platform and the type of sample. After fragmentation, the DNA ends are repaired, and adapters are ligated to the ends through a series of enzymatic steps. To generate the sequencing library, the amplicon-based approaches involve polymerase chain reaction (PCR) by emulsion with two primers flanking the DNA regions of interest ("amplicons"), while hybridization and capture-based methods employ probes to bind to the DNA sequence of interest [20]. The automation of library preparation using liquid handlers has significantly improved the reproducibility, efficiency, and scalability of NGS workflows [21]. These platforms enable high-throughput sample processing with minimal hands-on time, reducing the risk of contamination and variability introduced by manual pipetting. Automated systems can perform critical steps such as DNA fragmentation, end repair, adapter ligation, and clean-up, ensuring consistent sample preparation across multiple runs [22].

Template generation

In the emulsion PCR method, individual library molecules are mixed with magnetic beads coated with primers complementary to the adapter sequences. During PCR, each bead captures a single DNA fragment that serves as the substrate for the amplification, subsequently used for sequencing. Reversible terminator sequencing uses solid support where each DNA fragment attaches, forming a "bridge." This structure undergoes amplification through bridge PCR, generating dense clusters of identical DNA fragments that provide the substrate for high-throughput sequencing [23].

Sequencing

In ion semiconductor sequencing, each nucleotide addition releases a hydrogen ion, causing a localized pH change detected as an electrical signal by ion-sensitive sensors on a semiconductor chip. The sequence of voltage changes is recorded to determine the DNA sequence. In sequencing-by-synthesis, DNA polymerase incorporates terminator nucleotides conjugated to distinct fluorophores for each base (A, T, G, or C). During each cycle, the incorporated nucleotide emits a fluorescence signal captured by a high-resolution camera. These reversible terminator nucleotides allow strand synthesis to resume after the removal of the blocking group at the 3'-OH terminus [24].

Further technical specifications of different NGS platforms are summarized in Tables II and III and have been extensively described elsewhere [25]. Indeed, some distinctions between these approaches should be considered. These include nucleic acid input, read length, costs, and turnaround time. These differences can affect the selection of the analytical strategy, taking into account the volume, quality, and quantity of samples that can be processed and the detection accuracy for specific alterations and signatures [25]. Moreover, the workflow should be adapted to the analytical context and sample type. NGS technologies employed for circulating tumor DNA (ctDNA) analysis via liquid biopsy require specific modifications due to the unique properties of this analyte [26,27]. Although the overall preparation process remains consistent, library preparation for liquid biopsy requires ultra-low input DNA protocols to accommodate the limited availability of ctDNA, with amplification methods such as PCR-based or hybrid-capture techniques optimized for small DNA fragments (100–200 base pairs) of limited quantity. Efficient adapter ligation and barcoding are critical to minimize sample loss and high sequencing depth (≥10,000×) is essential to detect low-frequency variants (as low as 0.1%) within a background of non-tumor DNA [28].

RECOMMENDATION STATEMENTS

3. The choice of NGS platforms should consider factors such as platform availability, cost, expertise, turnaround time, DNA/RNA input, read length and workload.

4. Validated and/or certified workflows and technologies – preferably CE-IVD marked where applicable – should be adopted according to the clinical and analytical contexts, considering the requirement for the accurate evaluation of specific molecular alterations or signatures and sample type (e.g. tissue vs. liquid biopsy)

NGS panels

Panel size

The application of sequencing panels in oncology can be stratified into three primary tiers based on their complexity, scope, and utility: small panels, CGP, and whole exome or genome sequencing (WES/WGS) [29]. Each approach has distinct advantages and limitations, making them suitable for specific clinical or research contexts. As oncology increasingly shifts toward precision medicine, where individual mutations guide therapeutic decisions, careful attention must be paid to the specific reportable range of each panel [30]. This includes a clear understanding of the exact genomic regions and mutations covered by the panel, ensuring that clinically relevant alterations are accurately detected and reported. Small panels focus on a limited number of genes (typically 2–50), targeting actionable variants relevant to standard clinical practice [31]. These panels primarily detect single nucleotide variants (SNVs) and small insertions or deletions (indels), which encompass most standard-of-care drug targets. Their key strengths lie in their rapid turnaround times (TAT) (usually < 10 working days) and cost-effectiveness, enabling widespread adoption. CGP expands the scope, analyzing 300–700 genes and offering a broader molecular characterization of tumors. CGP panels detect multiple genomic alterations, including SNVs, indels, copy number variations (CNVs), and gene fusions, while also assessing key genomic signatures, including microsatellite instability (MSI), tumor mutational burden (TMB), and homologous recombination deficiency (HRD) [32-34]. These signatures can either be assessed using dedicated panels (e.g., MyChoice® CDx by Myriad Genetics) or derived from CGP data, though only a subset of panels have been clinically validated for these parameters. Orthogonal validation is strongly advised when implementing unvalidated signature analyses in clinical settings [35]. Indeed, complementary tests may be required for specific genomic alterations such as MSI and copy number variations (CNVs). MSI status can be confirmed using PCR-based assays or immunohistochemistry (IHC) for mismatch repair (MMR) protein expression, while CNVs can be validated through fluorescence in situ hybridization (FISH) [36]. CGP may pose challenges, including higher costs, longer TAT, and the need for sophisticated bioinformatics infrastructure. Its clinical utility is often most pronounced in cases where patients have exhausted standard treatment options that are referred to MTB, enabling the identification of rare alterations, novel biomarkers, or clinical trial opportunities [37,38]. For routine applications, smaller panels

remain more practical and cost-effective, particularly when targeting currently approved therapies. WES and WGS offer the most comprehensive genomic insights, enabling the identification of experimental drug targets and elucidating tumor biology [29,39]. Advances in WES/WGS technology, including compatibility with formalin-fixed paraffin-embedded (FFPE) samples, have enhanced their clinical potential [40,41]. However, these methods remain resource-intensive, requiring advanced bioinformatics, significant computational power, and incurring higher costs. Consequently, their use is largely confined to research applications or selected clinical cases where targeted panels fail to yield conclusive results. Despite their potential, the added clinical benefit of WES/WGS over CGP for routine oncology care remains a topic of ongoing investigation.

Gene fusion analysis

An additional critical consideration when ordering an NGS test involves the analysis of chromosomal rearrangements via RNA sequencing, which can identify oncogenic fusion genes that are potentially druggable [42]. While gene fusions can also be detected through DNA sequencing, this method primarily identifies the breakpoints of translocations often occurring within large intronic regions that may not be fully covered by gene panels [43]. In contrast, RNA sequencing circumvents these limitations by directly detecting fusion transcripts, reducing the risk of false-negative results. RNA-based gene panels provide comprehensive information on the expression of the fusion transcript, the fusion partner, and the potential functional impact [44]. Moreover, the sensitivity of NGS panels is influenced by the library preparation technologies employed. Thus, a thorough understanding of the genomic architecture of gene fusion of interest is essential not only for optimal panel design but also for accurate interpretation of results. For instance, a negative test result for a gene fusion may represent a false negative if the breakpoint region is inadequately covered by the gene panel used in the diagnostic assay.

Tissue vs liquid biopsy panels

The choice between tissue/cytologic samples (simplified as tissue) and liquid biopsy-based NGS panels is another critical starting point. Tissue testing, as the gold standard, provides a comprehensive view of tumor heterogeneity and enables detailed profiling of spatial genomic variations [45]. It is particularly recommended when thorough DNA and RNA profiling is essential for accurate diagnosis and treatment planning. However, tissue availability may be constrained by clinical or logistical factors, such as inaccessible tumor sites or insufficient sample quality [46]. In such scenarios, liquid biopsy is recommended as a non-invasive alternative to analyze ctDNA/ctRNA. Liquid biopsies are advantageous for real-time monitoring of tumor dynamics, but they have limitations, including challenges in assessing copy-number alterations and fusions due to technical variability and the lower abundance of ctDNA [47]. Despite these constraints, liquid biopsy should be prioritized when archival tissue is unavailable/inadequate, new biopsies are not feasible, and disease monitoring is required [48,49]. For patients without tissue-based genomic test results, actionable alterations identified in ctDNA can guide treatment decisions. Ultimately, the choice of analyte should be aligned with clinical objectives, tumor-specific characteristics, and practical considerations for sample acquisition.

In-house, outsourcing and in vitro diagnostic compliance

The decision to utilize commercial or customized and in-house or outsourced NGS panels shapes testing workflows and outcomes. Commercial in-house panels are pre-designed and extensively validated, offering streamlined solutions with reliable technical support, making them suitable for laboratories prioritizing ease of implementation and consistency [50]. Outsourced panels, which exploit the advanced capabilities of certified laboratories, are particularly useful for low-volume laboratories or those with limited technical capacity, though they may introduce delays in turnaround time and logistical challenges [12]. Regulatory compliance further guides panel selection. CE-marked in vitro diagnostic (IVD) panels, which meet European Union standards for diagnostic accuracy and reproducibility, are highly reliable for clinical use [51]. For the European Union In Vitro Diagnostics Regulation (EU IVDR), predictive biomarker assessments for clinical purposes should be conducted using a CE-marked IVD designed for that specific intended use. If a CE-marked IVD is unavailable, a validated alternative test can be utilized. Laboratories should critically assess the performance of IVD panels and perform robust validation of non-CE-marked assays and bioinformatics pipelines to ensure diagnostic accuracy [52,53]. Adopting panels with rigorous validation ensures consistency and reliability in NGS testing outcomes.

RECOMMENDATION STATEMENTS

5. Small panels should be preferred for rapid, cost-effective analysis of standard care actionable variants, while CGP can be used for broader molecular profiling in advanced cases or clinical trial enrollment. In both cases, the reportable range of the panels must be considered.

6. For the detection of oncogenic fusion genes, RNA-based sequencing panels should be prioritized over DNA-based approaches due to their superior sensitivity.

7. CE-marked IVD NGS panels should be used in clinical settings; commercial or customized assay with robust validation can be used if CE-IVD assays are not available for specific clinical requirements.

Bioinformatics

Parameters for NGS run evaluation

Advancing from raw data processing to in-depth data analysis and variant interpretation in clinical settings requires specialized algorithms and software applications [54]. Additionally, standardized templates for tracking NGS performance characteristics are currently available (https://www.amp.org/resources/validation-resources/) [25]. For clinical reliability and regulatory compliance, NGS bioinformatics tools should also meet CE-IVD/IVDR standards, ensuring validated algorithms for variant calling, annotation, and reporting. The NGS data analysis pipeline is divided into five core functions, typically categorized into three bioinformatics stages: primary analysis for base calling and signal processing; secondary analysis for read alignment and variant detection; tertiary analysis for variant annotation, filtering, and clinical interpretation.

Primary analysis

This phase focuses on converting raw signal data into readable sequencing reads. This includes signal detection, base quality scoring, and base calling, resulting in file formats such as FASTQ (Illumina) or unmapped binary alignment map (uBAM) files (Ion Torrent). In the former, fluorescence-based detection measures signal intensity during nucleotide incorporation. Signal data is processed through image alignment, cluster identification, and fluorescence correction to generate high-quality sequencing reads. In the case of Ion Torrent, signal detection begins with nucleotide incorporation, detected by a chip-embedded sensor. This signal is converted into voltage and stored as raw data (DAT files). Basecalling is subsequently performed by a dedicated module within the Ion Torrent Suite. Alternative basecallers employing diverse statistical and computational methods are also available for improved accuracy [55-57]. Base quality is commonly assessed using the Phred score, a logarithmic error probability scale. For example, a Phred score of Q10 indicates a 1 in 10 error probability (90% accuracy), while Q30 reflects a 1 in 1,000 error probability (99.9% accuracy) [58]. Basecalling performance and quality metrics are closely tied to sequencing platform-specific chemistries, necessitating technology-specific quality assessment strategies [59]. Critical quality metrics for raw sequencing reads are summarized in Table IV. Coverage depth directly influences NGS analytical sensitivity and accuracy with optimal levels varying depending on the specific assay and sample type. Selecting an appropriate depth requires careful balance between cost, turnaround time, and the desired analytical sensitivity. Notably, the presence of subclonal mutations and tumor and tissue heterogeneity necessitate significantly greater coverage depths compared to germline testing to ensure confident identification of clinically relevant somatic variants [52].

Secondary analysis

Secondary analysis involves aligning sequencing reads (from FASTQ or uBAM files) to a reference genome (commonly hg19 or GRCh37 for clinical applications) and identifying genetic variants. Though the human-readable SAM format is available, visualization tools such as Genome Browse (http://goldenhelix.com/products/GenomeBrowse/index.html) and Integrative Genomics Viewer (IGV) (https://igv.org/) facilitate alignment interpretation. The variant calling step identifies genomic alterations, including SNVs, insertions, deletions (indels) and structural variants (Tab. V), and the results are typically output in Variant Call Format (VCF) files [60]. Clinical-grade targeted sequencing panels often rely on proprietary variant calling and annotation pipelines, incorporating internal error modeling, region-specific blacklists, and technology-optimized parameters to ensure diagnostic accuracy. While open-source tools exist, they are generally optimized for research rather than clinical use. However, advancements in targeted sequencing are bridging this gap, improving the clinical applicability of open-source tools [61,62].

Tertiary analysis

This last step is exploited to determine the essential connection between variant data and their biological and clinical significance. This task requires continuous updates with the latest scientific discoveries and utilizes a broad array of specialized software and databases. Notable databases include ClinVar (https://www.ncbi.nlm.nih.gov/clinvar), which archives relationships between variants and human health according to the ACMG/AMP tiering system. For cancer-specific variants, the Catalogue of Somatic Mutations in Cancer (COSMIC, https://cancer.sanger.ac.uk/cosmic) focuses on somatic mutations in tumors and it is es-

Table IV. Overview of primary analysis in next-generation sequencing summarizing data processing, base calling, and quality scoring.

Parameter	Description	Notes
Read Quality Scores (Q-Scores) (%Q ≥ 20, %Q ≥ 30)	Phred scores (or equivalents) are fundamental. The score distribution for each base in each sequencing cycle must be analyzed, highlighting any quality drops toward the end of the reads. Advanced software offers visualizations like boxplots and heatmaps for rapid problem identification	The proportion of reads with Q30 (error probability < 0.1%) or higher is a critical measure of sequencing fidelity.
ISP loading/cluster density	The percentage of chip wells that contain Ion Sphere™ Particles (ISPs) or the total number of clusters generated during the NGS run	Both overloading/overcluster and underloading/undercluster should be avoided. The right loading concentration of chip/flowcell has to be validated per each panel inside each laboratory
Usable sequence / *Clusters* Passing Filter (PF)	The percentage of evaluable sequence/clusters passing filtering	An excess amount of loaded library may lead to Overcluster or Polyclonal beads, decreasing the percentage of Usable sequence / (PF)
Throughput (or Total Bases or total reads or Total Yield)	The number of filtered and trimmed base pairs reported in the output file	Low throughput might represent extreme DNA/RNA degradation or amplification heterogeneity across samples within the same run
Read length	The length of called reads measured in base pairs	The shape of the reads length graph should closely resemble the library size distribution trace, without the adapter sequences. Peaks that are too far from the library size may lead to ambiguous or suboptimal mapping
Mapped reads	Number of reads that were mapped to the full reference genome	A low rate may suggest read quality issues or contamination
Reads on target	Percentage of mapped reads that were aligned over a target region	It is crucial to evaluate the coverage of regions of interest and identify any exon dropouts or uncovered regions. The percentage of reads mapping to the intended genomic regions, indicative of assay specificity. This parameter is usually influenced by the usage of hotspot BED files
Mean Depth	Average base coverage depth over all bases targeted in the reference	Ensures adequate sequencing depth for detecting low-frequency variants
Uniformity	Percentage of target bases covered by the average base read depth	Assesses the even distribution of reads across target regions to minimize false negatives. Non-uniform coverage can compromise variant detection in some regions. The software must calculate metrics like the coverage coefficient of variation and visualize coverage across the genome/transcriptome
Primer Dimer	Unintended DNA fragments that can form during PCR	If a high relative primer dimer count is present, (re) check the library using a microfluidics instrument (e.g. tape station)
Amplicon Dropout	Detection of regions with poor amplification due to sequence or primer issue	Reduction of detectable alterations
RNA-Specific Parameters		
Gene Body Coverage	Uniformity of read distribution across transcripts to assess RNA integrity and library preparation	
Strand Specificity	Ensures proper orientation of reads, especially for stranded RNA libraries	Incorrect strand specificity may imply the removal of the read from downstream analysis
Fusion Detection Accuracy	Key for identifying gene rearrangements in oncology applications	Accuracy can be derived from the evaluation of depth at breakpoint junctions, and proportion of uniquely mapped reads
Prevalence of Exonic Regions	Ensures a realistic representation of transcriptional activity	

Table V. Standard nomenclature of NGS alterations and associated metrics.

Alteration Type	Standard Vocabulary	Metrics	Notes
Single Nucleotide Variant (SNV) or Multiple Nucleotide Variant (MNV)	HGVS Coding level: *c.* (exonic position of nucleotide) Protein level: *p.* (aminoacidic position within protein sequence) Genomic level: *g.* (genomic coordinate of nucleotide) RNA level: *r.* (transcript coordinate of nucleotide)	Variant Allele Frequency (VAF) The percentage of sequence reads observed matching a specific DNA variant divided by the overall coverage at that locus Depth (Vertical Coverage) The number of reads that align to known reference bases	The nucleotidic alteration in the sequence is always defined with respect to a unique RefSeq canonical transcript (*NM_*). The nucleotidic alteration may cause different outcomes at the protein level: * : Non-coding = : Synonimous dup: Duplicated aa del: Deleted aa delins: Deletion of 1+ aa and insertion of 1+ aa fs: change in ORF
Single Nucleotide Polymorphism (SNP)	dbSNP *rs:* unique identifier of a SNP	Depth (Vertical Coverage) The number of reads that align to known reference bases Frequency Percentage of incidence in a given population	SNVs are considered SNPs if their incidence in a given population is at least 1% Although annotation is performed mainly through dbSNP, SNP incidence relies on multiple databases
Copy Number Variation (CNV)	Ploidy ≥ 5/10 amplification (AMP) ≥ 3 gain = 2 diploid (normal) = 1 loss = 0 deletion (DEL)	Breakpoints Depth Good coverage of duplicated extremes Consistency Validation with CNV described in public databases Mapping Quality Good alignment scores	CNV are not directly observed through sequencing, but inferred based on sequencing depth of genomic regions, therefore the detection of CNV is strictly related to the software. Clinical interpretation of CNV events may vary significantly across diseases and genes
Fusions	HUGO The 5' HUGO gene name is listed first, followed by the 3' HUGO gene name, separated by a dash (-) Genomic Breakpoints The genomic coordinate of the two breakpoints is indicated and separated by a double column	Breakpoints Depth Good coverage of fusion breakpoints	In the clinical setting, fusions are typically observed on RNA to evaluate functionally active alterations

pecially useful to discriminate novel variants into potential germline or false positives. Finally, knowledge bases such as OncoKb (MSK's Precision Oncology Knowledge Base, https://www.oncokb.org/) and ESMO Scale for Clinical Actionability of molecular Targets (ESCAT) framework evaluate the clinical utility of genomic alterations in oncology, ranking them based on their potential for therapeutic actionability [63]. Special attention should be given to databases dedicated to those genes and/or cancer diseases characterized by a strong heritable component: the BRCA Exchange databases (https://brcaexchange.org/) is a specialized resource aggregating curated data on *BRCA1* and *BRCA2* variants, providing comprehensive classifications based on expert reviews and public health relevance. Additionally, the International Society for Gastrointestinal Hereditary Tumors (InSiGHT, https://www.insight-group.org/) database focuses on germline variants in mismatch repair (MMR) genes implicated in colorectal cancer syndromes, such as Lynch syndrome. In addition to evidence-based resources, a whole other set of software aims at predicting variants' pathogenicity by leveraging computational models to assess the likelihood that genetic variants impact protein function or contribute to disease [64-66].

Data management and storage

Effective management and storage of NGS data in clinical diagnostics require secure, scalable, and compliant systems to protect data integrity and patient privacy. For data security, sequencing data should be pseudo-anonymized and traceable via unique identifiers. Diagnostic reports should be stored as PDF files for clinical accessibility, while semi-processed data (e.g., BAM or

VCF files) can be archived in secure cloud-based or local storage systems to balance accessibility and storage costs. Storing large raw data (FASTQ) or efficient but unstructured data (PDF) in the long term is less preferable compared to the standard VCF format, as it ensures a better balance between informativeness, reusability and efficiency [67,68]. In accordance with the Clinical Laboratory Improvement Amendments (CLIA), records documenting all analytic system activities must be retained for at least two years [53]. In the context of germline genetic testing, current guidelines recommend retaining files that facilitate the regeneration and reanalysis of primary results with updated pipelines (e.g., BAM or FASTQ) for a minimum of 2 years. VCF files should be preserved for at least 20 years to allow for potential future reinterpretation of variant significance [69]. Lastly, the final clinical report and the type of patient informed consent (diagnostic-only, research, or broad data sharing) must be documented as metadata within the laboratory/institutional management system, ensuring ethical and legal compliance for data usage.

RECOMMENDATION STATEMENTS

8. NGS requires specialized software for processing raw data and generating clinically actionable results across primary, secondary, and tertiary analysis stages.

9. Clinical applications prioritize proprietary validated, preferably CE-marked, software for NGS variant calling due to its superior accuracy.

10. Tertiary analysis knowledge bases and computational models should be used to assess the clinical significance of genetic variants and their implications for patient care.

Quality controls and international standards certifications

Diagnostic laboratories must adhere to established validation protocols and practices to ensure quality control (QC) and quality assurance (QA). According to ISO 9001 and ISO 15189, the validation and verification of molecular tests or systems include the implementation of a robust QC regimen. This is particularly crucial for advanced diagnostic technologies such as NGS, where QC, international standards, reference materials, and participation in proficiency programs are essential [70-74].

Quality control

Systematic QC must be guaranteed in the evaluation of newly implemented molecular tests or systems, employee competency assessments, calibration of instruments, and monitoring of diagnostic test results in correlation with clinical findings, including diagnostic sensitivity and specificity. Indeed, any modification to the test system or process (e.g., new reagent lots, updated instruments) may compromise the integrity of results and variables such as procedural errors, instrument malfunctions, and environmental factors can also impact the accuracy of test outcomes [75]. To enhance QA, it is recommended to incorporate external controls (EC), no template controls (NTC), and reference materials (RM) [25]. External controls (EC) contain known positive or negative clinical specimens used to verify the integrity of reagents alongside unknown samples. Additionally, NTC should be included during library preparation to ensure reagent contamination is absent. Lastly, RM are employed for QC, system verification, error detection, test performance monitoring, and proficiency testing.

Proficiency testing

Each medical testing laboratory must enroll in approved proficiency programs for the relevant specialties to achieve accreditation [76]. External quality assurance (EQA) programs are critical for monitoring and improving NGS variant detection and clinical interpretation, ensuring high testing standards. The complexity of NGS has posed challenges for proficiency testing agencies in developing surveys that align with the analytical demands of multigene panels for somatic variants. Proficiency testing typically involves receiving blind samples from external agencies recognized under the Clinical Laboratory Improvement Amendments (CLIA) for conducting EQA. Participation in at least one proficiency testing scheme annually is recommended for laboratories specializing in NGS, including European-based programs (e.g. The European Molecular Genetics Quality Network - EMQN, Genomics Quality Assessment -GenQA) and national and/or regional-based programs (e.g. “valutazione esterna di qualità” (VEQ) [77].

ISO certifications: ISO 9001 and ISO 15189

ISO certifications ensure that laboratories adhere to international quality standards and undergo regular external evaluations. The primary certifications for medical laboratories are ISO 9001 and ISO 15189 [78]. The proper implementation of internal and external QCs is crucial for laboratory competence recognition. ISO 9001 Quality Management Systems is a widely recognized standard for quality management applicable to all organizations, including laboratories. It provides a framework for continuous improvement through process evaluation, leadership, goal-setting, and regular internal audits. Laboratories must be customer-oriented, responsive to needs, and capable of addressing non-conformities through corrective and preventive actions [79]. Moreover,

ISO 15189 Medical Laboratories – Requirements for Quality and Competence outlines comprehensive requirements for the quality management of medical and diagnostic laboratories. This standard is structured into management and technical components. Management requirements include laboratory organization, system quality management, complaint resolution, internal audits, and management reviews. Technical requirements focus on personnel qualifications, laboratory structure, environmental conditions, equipment, and examination procedures. A critical structural requirement for molecular diagnostics involves dedicated space division to prevent contamination and to maintain sample integrity. Data management and storage is another key point of ISO 15189 to guarantee patient data privacy and protection (Tab. VI).

RECOMMENDATION STATEMENTS

11. Periodical field-specific training and enhanced QC measures, including participation in EQA, are recommended to maintain professional competency and robust quality assurance in NGS testing.

12. UNI EN ISO 15189 and 9001 certifications are strongly recommended for NGS-focused molecular pathology laboratories to ensure quality standards and competence.

Socio-economic and organizational factors in NGS implementation

In addition to the essential aforementioned clinical, technical, and regulatory aspects, the successful deployment of NGS testing within molecular pathology is significantly influenced by organizational factors and regional variations, which must be considered for optimal implementation [80].

Turnaround time

The efficient delivery of sequencing results is critical for timely patient management. Currently, NGS turnaround times vary from days to weeks due to disparities in laboratory capacity, volume and workflow efficiency. In Italy, some centers achieve an average turnaround of ~10 working days, while others face delays due to infrastructure limitations and high demand. Continuous monitoring of disease staging, optimal tumor sample selection by pathologists, and proper specimen handling by technicians are essential for standardizing protocols and minimizing delays. Moreover, investments in advanced technologies can further streamline these processes [81].

Table VI. Essential aspects and requirements of ISO 9001 and ISO 15189 certifications for medical laboratories.

Aspect	ISO 15189	ISO 9001
Purpose	Specific standard for medical laboratories, focused on quality and technical competence requirements.	Generic standard for quality management systems, applicable to any sector.
Main Focus	Quality and competence in medical laboratory diagnostic services.	Quality management in business processes, with an emphasis on customer satisfaction.
Application Field	Medical and diagnostic laboratories.	Organizations of any type and sector.
Technical Requirements	Includes specific requirements for equipment, test methods, validation, personnel, and the work environment.	Does not include specific technical requirements but requires process and resource control.
Personnel Evaluation	Requires verification of technical competence of personnel.	Does not specify technical competence but requires training and general competence.
Method Validation	Requires specific validation of methods used to ensure accurate results.	Does not require technical validation but focuses on risk management and continuous improvement.
Equipment Management	Requires regular calibration and maintenance of diagnostic equipment.	Only requires management of equipment relevant to the quality of products or services.
Risk-Based Approach	Strongly risk-oriented in the context of erroneous diagnostic results.	Risk-based approach for all business processes, without specific sector focus.
Customer Involvement	Includes the patient as the final customer, with a focus on clinical requirements and safety.	Generic customer involvement, aimed at business customer satisfaction.
Documentation	Requires detailed procedures, quality manuals, and records for diagnostic compliance.	Requires general documentation to ensure traceability of quality processes.
Accreditation	Used as a basis for accreditation of medical laboratories (ISO 15189 is specific for accreditations).	Used as a basis for the certification of quality management systems.
Primary Benefits	Improves the quality and reliability of medical diagnoses.	Improves the efficiency of business processes and customer satisfaction.
Legal Obligation	Often required for regulatory compliance in healthcare (depending on the country).	Generally voluntary but required by some industries for competitiveness.

Workforce training and multidisciplinary team integration

A skilled workforce in genomics is vital for successful NGS integration. However, gaps in specialized NGS education persist within Italy's medical curricula and continuing education programs. Targeted training initiatives should prioritize practical experience with sequencing platforms and bioinformatics tools. Academic partnerships can support hands-on workshops and courses tailored to molecular pathology professionals [82]. Moreover, the integration of NGS into clinical practice necessitates robust multidisciplinary collaboration, bringing together the specialized knowledge of biologists, oncologists, pathologists, technicians and bioinformaticians. However, poor communication and unclear role definitions often hinder effective teamwork [83]. Formal networks that encourage interdisciplinary dialogue and cooperation are essential for maximizing the clinical impact of genomic data.

Costs and reimbursements

NGS incurs substantial, yet variable, costs influenced by geographic and healthcare system factors. Although initial testing costs are high, long-term savings emerge from improved patient outcomes [84,85]. Lately, the Italian Ministry of Health has enhanced and funded the use of NGS approaches for specific clinical conditions (e.g. metastatic non-small cell lung cancer, non-operable cholangiocarcinoma) (see GU Serie Generale n.253 del 28-10-2022; GU Serie Generale n.80 del 04-04-2023). However, reimbursement policies for genetic testing still lack uniformity across regions. Some areas provide structured reimbursement for oncological testing, while others impose financial burdens on patients due to inconsistent policies [86]. In Italy, norther regions are more advanced in implementing genomic tools, whereas southern regions face economic constraints limiting access [87]. These regional disparities hinder the uniform adoption of NGS technologies and compromise nationwide access to advanced diagnostics. In the absence of updated LEA, each region in Italy independently determines reimbursement policies for NGS – provided they are not under a financial recovery plan (piano di rientro). The national standardization of reimbursement frameworks and innovative funding and organizational models could address some of the current barriers and enhance the widespread availability of NGS testing. The hub-and-spoke network model involved a central hub for core facility analysis and spokes for sample collection and clinical result handling. This model would distribute the workload, ensuring standardized, efficient, and timely analyses, potentially streamlining logistical challenges. Conversely, the point-to-point model is characterized by independent operations at each site. This model may ensure accessibility and flexibility but faces issues such as costs related to resource disparity and redundant services [88-90]. Recently, The Italian Ministry of Health outlined criteria for identifying laboratories for Level I (small panels, ESCAT I targets) and II (CGP) molecular tests, including quality and structural, instrumental, and human resource standards [91]. Enabling an equitable distribution of these facilities and promoting inter-institutional collaboration will be fundamental to ensure NGS testing accessibility. Notably, reimbursement for NGS is included in the LEA, and its forthcoming implementation is expected to harmonize the use of NGS across the Country.

RECOMMENDATION STATEMENTS

13. The turnaround time for NGS-based predictive molecular testing should not exceed 10-15 working days.

14. Successful NGS implementation necessitates multidisciplinary laboratory collaboration, engaging trained technicians, pathologists, biologists, and bioinformaticians.

15. To enhance NGS testing implementation and accessibility, organizational models should promote the equitable distribution of technologies and resources while fostering inter-institutional collaboration.

Conclusions

This article provided a comprehensive framework for the analytical phase of NGS testing, addressing key aspects from clinical application and technologies to quality assurance and organizational factors. The recommendations reflect current best practices and are intended to guide laboratories in establishing robust and reliable NGS workflows. Recognizing the rapid evolution of NGS technology, the Working Group acknowledges the inherent limitations of any static set of recommendations and emphasizes the need for continuous review and refinement to incorporate emerging methods and platforms. By promoting standardized procedures, this document will contribute to harmonizing the quality of NGS-based diagnostics, ultimately improving patient care and maximizing the potential of predictive and precision pathology.

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Authors' contributions

Starting from an initial draft prepared by Konstantinos Venetis, all authors contributed substantially to the development of the manuscript and reviewed its final version. Elena Guerini Rocco coordinated the writing team and provided extensive input in both drafting and revising the text. Nicola Fusco served as the overall coordinator of the guidelines project, ensuring harmonization across all documents and conducting critical revision and editing of the manuscript. Umberto Malapelle and Fabio Pagni provided critical feedback and input throughout the writing process. Giancarlo Pruneri acted as the final reviewer.

Ethical consideration

Not applicable.

References

1 Khranovska N, Gorbach O, Skachkova O, Klimnyuk G. Application of Next-Generation Sequencing to Realize Principles of Precision Therapy in Management of Cancer Patients. Exp Oncol 2025;46(4):295-304. https://doi.org/10.15407/exp-oncology.2024.04.295.

2 Mosele MF, Westphalen CB, Stenzinger A, et al. Recommendations for the use of next-generation sequencing (NGS) for patients with advanced cancer in 2024: a report from the ESMO Precision Medicine Working Group. Ann Oncol 2024;35(7):588-606. https://doi.org/10.1016/j.annonc.2024.04.005.

3 van de Haar J, Roepman P, Andre F, et al. ESMO Recommendations on clinical reporting of genomic test results for solid cancers. Ann Oncol 2024;35(11):954-967. https://doi.org/10.1016/j.annonc.2024.06.018.

4 Fusco N, Jantus-Lewintre E, Serrano MJ, et al. Role of the International Society of Liquid Biopsy (ISLB) in establishing quality control frameworks for clinical integration. Critical Reviews in Oncology/Hematology 2025:104619. https://doi.org/https://doi.org/10.1016/j.critrevonc.2025.104619.

5 Buchta C, Marrington R, De la Salle B, et al. Behind the scenes of EQA - characteristics, capabilities, benefits and assets of external quality assessment (EQA). Clin Chem Lab Med 2025. https://doi.org/10.1515/cclm-2024-1289.

6 Linko S, Boursier G, Bernabeu-Andreu FA, et al. EN ISO 15189 revision: EFLM Committee Accreditation and ISO/CEN standards (C: A/ISO) analysis and general remarks on the changes. Clin Chem Lab Med 2025. https://doi.org/10.1515/cclm-2024-1451.

7 Fleming KA, Naidoo M, Wilson M, et al. High-Quality Diagnosis: An Essential Pathology Package. In: Jamison DT, Gelband H, Horton S, et al., eds. Disease Control Priorities: Improving Health and Reducing Poverty. 3rd ed. Washington (DC)2017.

8 Chandra S, Kusum A, Gaur DS, Chandra H. Analytical and Post Analytical Phase of an ISO 15189:2012 Certified Cytopathology Laboratory-A Five Year Institutional Experience. J Cytol 2022;39(1):37-43. https://doi.org/10.4103/JOC.JOC_90_20.

9 Satam H, Joshi K, Mangrolia U, et al. Next-Generation Sequencing Technology: Current Trends and Advancements. Biology (Basel) 2023;12(7) (In eng). https://doi.org/10.3390/biology12070997.

10 Cifuentes C, Lombana M, Vargas H, et al. Application of Comprehensive Genomic Profiling-Based Next-Generation Sequencing Assay to Improve Cancer Care in a Developing Country. Cancer Control 2023;30:10732748231175256. (In eng). https://doi.org/10.1177/10732748231175256.

11 Loong HH, Shimizu T, Prawira A, et al. Recommendations for the use of next-generation sequencing in patients with metastatic cancer in the Asia-Pacific region: a report from the APODDC working group. ESMO Open 2023;8(4):101586. (In eng). https://doi.org/10.1016/j.esmoop.2023.101586.

12 Hedblom AH, Pruneri G, Quagliata L, et al. Cancer patient management: Current use of next-generation sequencing in the EU TOP4. J Cancer Policy 2023;35:100376. (In eng). https://doi.org/10.1016/j.jcpo.2022.100376.

13 Mosele F, Remon J, Mateo J, et al. Recommendations for the use of next-generation sequencing (NGS) for patients with metastatic cancers: a report from the ESMO Precision Medicine Working Group. Ann Oncol 2020 (In eng). https://doi.org/10.1016/j.annonc.2020.07.014.

14 Chakravarty D, Johnson A, Sklar J, et al. Somatic Genomic Testing in Patients With Metastatic or Advanced Cancer: ASCO Provisional Clinical Opinion. J Clin Oncol 2022;40(11):1231-1258. (In eng). https://doi.org/10.1200/jco.21.02767.

15 Mun J, Lim B. The hallmarks of tissue-agnostic therapies and strategies for early anticancer drug discovery. Drug Discov Today 2024;29(12):104203. (In eng). https://doi.org/10.1016/j.drudis.2024.104203.

16 LEA 2017 GU 18/03/2017 https://www.trovanorme.salute.gov.it/norme/renderPdf.spring?seriegu=SG&datagu=18/03/2017&redaz=17A02015&artp=4&art=1&subart=1&subart1=10&vers=1&prog=001.

17 Proposta di regolamentazione per l'appropriatezza dell'utilizzo dei Test Multigenici NGS predittivi e prognostici nella pratica clinica. Consiglio Superiore di Sanità. 2023. https://www.pnrr.salute.gov.it/imgs/C_17_pubblicazioni_3358_allegato.pdf.

18 Istituzione dei Molecular Tumor Board e individuazione dei centri specialistici per l'esecuzione dei test per la profilazione genomica Next Generation Sequencing (NGS). Decreto del Ministero della Salute del 30 maggio 2023, G.U. n. 151 del 16 agosto 2023. https://www.gazzettaufficiale.it/eli/id/2023/08/16/23A04587/SG

19 Mardis ER. The Impact of Next-Generation Sequencing on Cancer Genomics: From Discovery to Clinic. Cold Spring Harb Perspect Med 2019;9(9) (In eng). https://doi.org/10.1101/cshperspect.a036269.

20 Sahoo OS, Aidasani H, Nayek A, et al. Role of next-generation sequencing in revolutionizing healthcare for cancer management.

MedComm – Future Medicine 2024;3(4):e70001. https://doi.org/https://doi.org/10.1002/mef2.70001.

[21] Socea JN, Stone VN, Qian X, Gibbs PL, Levinson KJ. Implementing laboratory automation for next-generation sequencing: benefits and challenges for library preparation. Front Public Health 2023;11:1195581. https://doi.org/10.3389/fpubh.2023.1195581.

[22] Kind D, Baskaran P, Ramirez F, et al. Automation enables high-throughput and reproducible single-cell transcriptomics library preparation. SLAS Technol 2022;27(2):135-142. https://doi.org/10.1016/j.slast.2021.10.018.

[23] Cappello F, Angerilli V, Munari G, et al. FFPE-Based NGS Approaches into Clinical Practice: The Limits of Glory from a Pathologist Viewpoint. J Pers Med 2022;12(5) (In eng). https://doi.org/10.3390/jpm12050750.

[24] Goodwin S, McPherson JD, McCombie WR. Coming of age: ten years of next-generation sequencing technologies. Nat Rev Genet 2016;17(6):333-51. (In eng). https://doi.org/10.1038/nrg.2016.49.

[25] Jennings LJ, Arcila ME, Corless C, et al. Guidelines for Validation of Next-Generation Sequencing-Based Oncology Panels: A Joint Consensus Recommendation of the Association for Molecular Pathology and College of American Pathologists. J Mol Diagn 2017;19(3):341-365. (In eng). https://doi.org/10.1016/j.jmoldx.2017.01.011.

[26] Wan JCM, Massie C, Garcia-Corbacho J, et al. Liquid biopsies come of age: towards implementation of circulating tumour DNA. Nat Rev Cancer 2017;17(4):223-238. (In eng). https://doi.org/10.1038/nrc.2017.7.

[27] Tébar-Martínez R, Martín-Arana J, Gimeno-Valiente F, Tarazona N, Rentero-Garrido P, Cervantes A. Strategies for improving detection of circulating tumor DNA using next generation sequencing. Cancer Treat Rev 2023;119:102595. (In eng). https://doi.org/10.1016/j.ctrv.2023.102595.

[28] Ranghiero A, Frascarelli C, Cursano G, et al. Circulating tumour DNA testing in metastatic breast cancer: Integration with tissue testing. Cytopathology 2023 (In eng). https://doi.org/10.1111/cyt.13295.

[29] Malhotra R, Javle V, Tanwar N, et al. An absolute approach to using whole exome DNA and RNA workflow for cancer biomarker testing. Front Oncol 2023;13:1002792. (In eng). https://doi.org/10.3389/fonc.2023.1002792.

[30] Tack V, Spans L, Schuuring E, et al. Describing the Reportable Range Is Important for Reliable Treatment Decisions: A Multiple Laboratory Study for Molecular Tumor Profiling Using Next-Generation Sequencing. The Journal of Molecular Diagnostics 2018;20(6):743-753. https://doi.org/https://doi.org/10.1016/j.jmoldx.2018.06.006.

[31] De Maglio G, Pasello G, Dono M, et al. The storm of NGS in NSCLC diagnostic-therapeutic pathway: How to sun the real clinical practice. Crit Rev Oncol Hematol 2022;169:103561. (In eng). https://doi.org/10.1016/j.critrevonc.2021.103561.

[32] Marques A, Cavaco P, Torre C, Sepodes B, Rocha J. Tumor mutational burden in colorectal cancer: Implications for treatment. Crit Rev Oncol Hematol 2024;197:104342. (In eng). https://doi.org/10.1016/j.critrevonc.2024.104342.

[33] Pepe F, Guerini-Rocco E, Fassan M, et al. In-house homologous recombination deficiency testing in ovarian cancer: a multi-institutional Italian pilot study. Journal of clinical pathology 2023 (In eng). https://doi.org/10.1136/jcp-2023-208852.

[34] Venetis K, Frascarelli C, Bielo LB, et al. Mismatch repair (MMR) and microsatellite instability (MSI) phenotypes across solid tumors: A comprehensive cBioPortal study on prevalence and prognostic impact. European Journal of Cancer 2025:115233. https://doi.org/https://doi.org/10.1016/j.ejca.2025.115233.

[35] de Jager VD, Timens W, Bayle A, et al. Future perspective for the application of predictive biomarker testing in advanced stage non-small cell lung cancer. Lancet Reg Health Eur 2024;38:100839. (In eng). https://doi.org/10.1016/j.lanepe.2024.100839.

[36] Bozzi F, Conca E, Silvestri M, et al. Detecting gene copy number alterations by Oncomine Comprehensive genomic profiling in a comparative study on FFPE tumor samples. Sci Rep 2025;15(1):4314. https://doi.org/10.1038/s41598-025-88494-3.

[37] Maio M, Ascierto PA, Manzyuk L, et al. Pembrolizumab in microsatellite instability high or mismatch repair deficient cancers: updated analysis from the phase II KEYNOTE-158 study. Ann Oncol 2022;33(9):929-938. (In eng). https://doi.org/10.1016/j.annonc.2022.05.519.

[38] Ray-Coquard I, Pautier P, Pignata S, et al. Olaparib plus Bevacizumab as First-Line Maintenance in Ovarian Cancer. N Engl J Med 2019;381(25):2416-2428. (In eng). https://doi.org/10.1056/NEJMoa1911361.

[39] Ramarao-Milne P, Kondrashova O, Patch AM, et al. Comparison of actionable events detected in cancer genomes by whole-genome sequencing, in silico whole-exome and mutation panels. ESMO Open 2022;7(4):100540. (In eng). https://doi.org/10.1016/j.esmoop.2022.100540.

[40] Kim R, Kim S, Oh BB, et al. Clinical application of whole-genome sequencing of solid tumors for precision oncology. Exp Mol Med 2024;56(8):1856-1868. (In eng). https://doi.org/10.1038/s12276-024-01288-x.

[41] Menzel M, Martis-Thiele M, Goldschmid H, et al. Benchmarking whole exome sequencing in the German network for personalized medicine. Eur J Cancer 2024;211:114306. (In eng). https://doi.org/10.1016/j.ejca.2024.114306.

[42] Schmid S, Jochum W, Padberg B, et al. How to read a next-generation sequencing report-what oncologists need to know. ESMO Open 2022;7(5):100570. (In eng). https://doi.org/10.1016/j.esmoop.2022.100570.

[43] Solomon JP, Benayed R, Hechtman JF, Ladanyi M. Identifying patients with NTRK fusion cancer. Ann Oncol 2019;30(Suppl_8):viii16-viii22. (In eng). https://doi.org/10.1093/annonc/mdz384.

[44] Venetis K, Sajjadi E, Ivanova M, et al. The molecular landscape of breast mucoepidermoid carcinoma. Cancer Med 2023 (In eng). https://doi.org/10.1002/cam4.5754.

[45] Bayle A, Peyraud F, Belcaid L, et al. Liquid versus tissue biopsy for detecting actionable alterations according to the ESMO Scale for Clinical Actionability of molecular Targets in patients with advanced cancer: a study from the French National Center for Precision Medicine (PRISM). Ann Oncol 2022;33(12):1328-1331. (In eng). https://doi.org/10.1016/j.annonc.2022.08.089.

[46] Kerr KM, Bubendorf L, Lopez-Rios F, et al. Optimizing tissue stewardship in non-small cell lung cancer to support molecular characterization and treatment selection: statement from a working group of thoracic pathologists. Histopathology 2024;84(3):429-439. (In eng). https://doi.org/10.1111/his.15078.

[47] Ma L, Guo H, Zhao Y, et al. Liquid biopsy in cancer current: status, challenges and future prospects. Signal Transduct Target Ther 2024;9(1):336. (In eng). https://doi.org/10.1038/s41392-024-02021-w.

[48] Raez LE, Brice K, Dumais K, et al. Liquid Biopsy Versus Tissue Biopsy to Determine Front Line Therapy in Metastatic Non-Small Cell Lung Cancer (NSCLC). Clin Lung Cancer 2023;24(2):120-129. (In eng). https://doi.org/10.1016/j.cllc.2022.11.007.

[49] Ranghiero A, Frascarelli C, Cursano G, et al. Circulating tumour DNA testing in metastatic breast cancer: Integration with tissue testing. Cytopathology 2023;34(6):519-529. (In eng). https://doi.org/10.1111/cyt.13295.

[50] Ferreira-Gonzalez A, Ko G, Fusco N, et al. Barriers and facilitators to next-generation sequencing use in United States oncology settings: a systematic review. Future Oncol 2024;20(35):2765-2777. (In eng). https://doi.org/10.1080/14796694.2024.2390821.

[51] Normanno N, Machado JC, Pescarmona E, et al. European Real-World Assessment of the Clinical Validity of a CE-IVD Panel for Ultra-Fast Next-Generation Sequencing in Solid Tumors. Int J Mol Sci 2023;24(18) (In eng). https://doi.org/10.3390/ijms241813788.

[52] Jennings LJ, Arcila ME, Corless C, et al. Guidelines for Validation of Next-Generation Sequencing–Based Oncology Panels: A Joint Consensus Recommendation of the Association for Molecular Pathology and College of American Pathologists. The Journal of Molecular Diagnostics 2017;19(3):341-365. https://doi.org/https://doi.org/10.1016/j.jmoldx.2017.01.011.

[53] Roy S, Coldren C, Karunamurthy A, et al. Standards and Guidelines for Validating Next-Generation Sequencing Bioinformatics Pipelines: A Joint Recommendation of the Association for Molecular Pathology and the College of American Pathologists. The Journal of Molecular Diagnostics 2018;20(1):4-27. https://doi.org/https://doi.org/10.1016/j.jmoldx.2017.11.003.

[54] Pereira R, Oliveira J, Sousa M. Bioinformatics and Computational Tools for Next-Generation Sequencing Analysis in Clinical Genetics. J Clin Med 2020;9(1) (In eng). https://doi.org/10.3390/jcm9010132.

[55] Cacho A, Smirnova E, Huzurbazar S, Cui X. A Comparison of Base-calling Algorithms for Illumina Sequencing Technology. Brief Bioinform 2016;17(5):786-95. (In eng). https://doi.org/10.1093/bib/bbv088.

[56] Ledergerber C, Dessimoz C. Base-calling for next-generation sequencing platforms. Brief Bioinform 2011;12(5):489-97. (In eng). https://doi.org/10.1093/bib/bbq077.

[57] Zhang S, Wang B, Wan L, Li LM. Estimating Phred scores of Illumina base calls by logistic regression and sparse modeling. BMC Bioinformatics 2017;18(1):335. (In eng). https://doi.org/10.1186/s12859-017-1743-4.

[58] Ewing B, Hillier L, Wendl MC, Green P. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res 1998;8(3):175-85. (In eng). https://doi.org/10.1101/gr.8.3.175.

[59] Ross MG, Russ C, Costello M, et al. Characterizing and measuring bias in sequence data. Genome Biol 2013;14(5):R51. (In eng). https://doi.org/10.1186/gb-2013-14-5-r51.

[60] Danecek P, Auton A, Abecasis G, et al. The variant call format and VCFtools. Bioinformatics 2011;27(15):2156-8. (In eng). https://doi.org/10.1093/bioinformatics/btr330.

[61] Feng B, Lai J, Fan X, et al. Systematic comparison of variant calling pipelines of target genome sequencing cross multiple next-generation sequencers. Front Genet 2023;14:1293974. (In eng). https://doi.org/10.3389/fgene.2023.1293974.

[62] Aldea M, Vasseur D, Italiano A, Nikolaev SI. WGS/WES-RNAseq compared to targeted NGS in oncology: is there something to unlock? Ann Oncol 2023;34(12):1090-1093. (In eng). https://doi.org/10.1016/j.annonc.2023.09.3118.

[63] Mateo J, Chakravarty D, Dienstmann R, et al. A framework to rank genomic alterations as targets for cancer precision medicine: the ESMO Scale for Clinical Actionability of molecular Targets (ESCAT). Ann Oncol 2018;29(9):1895-1902. (In eng). https://doi.org/10.1093/annonc/mdy263.

[64] Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. Nat Methods. United States2010:248-9.

[65] Schubach M, Maass T, Nazaretyan L, Röner S, Kircher M. CADD v1.7: using protein language models, regulatory CNNs and other nucleotide-level scores to improve genome-wide variant predictions. Nucleic Acids Res 2024;52(D1):D1143-d1154. (In eng). https://doi.org/10.1093/nar/gkad989.

[66] Ng PC, Henikoff S. Predicting deleterious amino acid substitutions. Genome Res 2001;11(5):863-74. (In eng). https://doi.org/10.1101/gr.176601.

[67] Boscolo Bielo L, Guerini Rocco E, Crimini E, et al. Molecular tumor board in patients with metastatic breast cancer. Breast cancer research and treatment 2025;210(1):45-55. https://doi.org/10.1007/s10549-024-07535-z.

[68] Corradi A, Bonizzi G, Sajjadi E, et al. The Regulatory Landscape of Biobanks In Europe: From Accreditation to Intellectual Property. Curr Genomics 2025;26(1):15-23. https://doi.org/10.2174/0113892029313697240729091922.

[69] Rehm HL, Bale SJ, Bayrak-Toydemir P, et al. ACMG clinical laboratory standards for next-generation sequencing. Genetics in medicine : official journal of the American College of Medical Genetics 2013;15(9):733-47. https://doi.org/10.1038/gim.2013.92.

[70] Normanno N, Apostolidis K, Wolf A, et al. Access and quality of biomarker testing for precision oncology in Europe. Eur J Cancer 2022;176:70-77. (In eng). https://doi.org/10.1016/j.ejca.2022.09.005.

[71] Esposito A, Crimini E, Criscitiello C, et al. The Safety and Suitability of DNA Sequencing of Tissue Biopsies Performed on Patients Referred to a Phase I Unit. Cancers (Basel) 2024;16(24). https://doi.org/10.3390/cancers16244252.

[72] Guerini-Rocco E, Venetis K, Cursano G, et al. Standardized molecular pathology workflow for ctDNA-based ESR1 testing in HR+/HER2- metastatic breast cancer. Crit Rev Oncol Hematol 2024;201:104427. https://doi.org/10.1016/j.critrevonc.2024.104427.

[73] Bonizzi G, Capra M, Cassi C, et al. Biobank for Translational Medicine: Standard Operating Procedures for Optimal Sample Management. J Vis Exp 2022(189) (In eng). https://doi.org/10.3791/63950.

[74] Bonizzi G, Zattoni L, Capra M, et al. Standard operating procedures for biobank in oncology. Front Mol Biosci 2022;9:967310. (In eng). https://doi.org/10.3389/fmolb.2022.967310.

[75] Agarwal R. Quality-Improvement Measures as Effective Ways of Preventing Laboratory Errors. Laboratory Medicine 2014;45(2):e80-e88. https://doi.org/10.1309/LMD0YIFPTOWZONAD.

[76] Kessler HH, Raggam RB. Quality assurance and quality control in the routine molecular diagnostic laboratory for infectious diseases. Clin Chem Lab Med 2012;50(7):1153-9. (In eng). https://doi.org/10.1515/cclm-2011-0707.

[77] Gutowska-Ding MW, Deans ZC, Roos C, et al. One byte at a time: evidencing the quality of clinical service next-generation sequencing for germline and somatic variants. Eur J Hum Genet 2020;28(2):202-212. (In eng). https://doi.org/10.1038/s41431-019-0515-1.

[78] Buchta C, Coucke W, Mayr WR, et al. Evidence for the positive impact of ISO 9001 and ISO 15189 quality systems on laboratory performance - evaluation of immunohaematology external quality assessment results during 19 years in Austria. Clin Chem Lab Med 2018;56(12):2039-2046. (In eng). https://doi.org/10.1515/cclm-2018-0482.

[79] Helbig M, Kahla-Witzsch HA, Helbig S, Knecht R, Gstöttner W. [Quality assessment according to DIN EN ISO 9001:2000: certification in a university ENT department]. Hno 2006;54(12):922-8. (In ger). https://doi.org/10.1007/s00106-006-1467-7.

[80] Mardis ER. Next-generation DNA sequencing methods. Annu Rev Genomics Hum Genet 2008;9:387-402. (In eng). https://doi.org/10.1146/annurev.genom.9.081307.164359.

[81] Morash M, Mitchell H, Beltran H, Elemento O, Pathak J. The Role of Next-Generation Sequencing in Precision Medicine: A Review of Outcomes in Oncology. J Pers Med 2018;8(3) (In eng). https://doi.org/10.3390/jpm8030030.

[82] Whitley KV, Tueller JA, Weber KS. Genomics Education in the Era of Personal Genomics: Academic, Professional, and Public Considerations. Int J Mol Sci 2020;21(3) (In eng). https://doi.org/10.3390/ijms21030768.

[83] Ma A, O'Shea R, Wedd L, Wong C, Jamieson RV, Rankin N. What is the power of a genomic multidisciplinary team approach? A systematic review of implementation and sustainability. Eur J Hum Genet 2024;32(4):381-391. (In eng). https://doi.org/10.1038/s41431-024-01555-5.

[84] Mirza M, Goerke L, Anderson A, Wilsdon T. Assessing the Cost-Effectiveness of Next-Generation Sequencing as a Biomarker Testing Approach in Oncology and Policy Implications: A Literature Review. Value Health 2024;27(9):1300-1309. (In eng). https://doi.org/10.1016/j.jval.2024.04.023.

[85] Matias-Guiu X, Temprana-Salvador J, Garcia Lopez P, et al. Implementing digital pathology: qualitative and financial insights from eight leading European laboratories. Virchows Arch 2025. https://doi.org/10.1007/s00428-025-04064-y.

[86] De Lorenzo F, Marletta M. 14° Rapporto sulla condizione assistenziale dei malati oncologici. Roma: QS Edizioni, 2022.

[87] De Lorenzo F, Marletta M. 16° Rapporto sulla condizione assistenziale dei malati oncologici. Roma: QS Edizioni, 2024.

[88] Venetis K, Cursano G, Pescia C, et al. Liquid biopsy: Cell-free DNA based analysis in breast cancer. The Journal of Liquid Biopsy 2023:100002. https://doi.org/https://doi.org/10.1016/j.jlb.2023.100002.

[89] Fasola G, Barducci MC, Pelizzari G, et al. Implementation of Precision Oncology in Clinical Practice: Results of a National Survey for Health Care Professionals. The Oncologist 2023;28(6):e324-e330. https://doi.org/10.1093/oncolo/oyad020.

[90] Barducci MC, Tozzi VD, Pelizzari G, et al. Precision oncology implementation in a regional-based health care system: A professional consensus to define the pathway. Journal of Cancer Policy 2024;42:100515. https://doi.org/https://doi.org/10.1016/j.jcpo.2024.100515.

[91] APMP G. APMP & Regioni. Percorso regionale per l'implementazione della medicina di precisione nei territori italiani. Roma: Deloitte Consulting, 2023.