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# Molecular Pathology in the New Age of Personalized Medicine

*Valeria Cecilia Denninghoff*

## Abstract

Personalized medicine is a new approach that allows the identification of patients that can benefit from targeted therapies because of the molecular characteristics of the tumors they present. The molecular profile of the tumor can be studied at the genomic (DNA), transcriptomic (RNA) or protein (protein) level. The next generation sequencing is a useful tool for the study of molecular profile from DNA/RNA. This tool requires molecular pathologists highly trained in pre-analytic processes, tumor area microdissection for tumor cell enrichment, methodology analysis and results. The in-depth study of molecular alterations in patients allows optimizing molecular diagnosis and selecting candidates for receive novel treatments against specific molecular targets. These patients are expected to benefit from multidisciplinary approach and learning. The aim of this chapter is to show the implications of molecular pathology in personalized medicine with an actual approach from the methodological limitations of formalin-fixed paraffin embedded (FFPE) tissues and their pre-analytical conditions.

**Keywords:** molecular pathology, personalized medicine, next generation sequencing, NGS, clinical benefit, multidisciplinary approach

## 1. Introduction

Personalized medicine is a new approach that allows the identification of patients that can benefit from targeted therapies, since the molecular characteristics of their tumors could be identified. Over the last decade, new drugs have been incorporated into the treatment, including the development of immunotherapy and treatment against specific molecular targets [1]. Thus, patients can receive specific treatments according to the biology of their tumor, turning oncology a tool for personalized medicine. In order to do so, the development of new DNA/RNA sequencing technologies was required, as well as the development of specific antibodies identifying mutated or altered proteins, and the design of new in situ hybridization techniques. The latter has enabled the selection via genetic biomarkers of patients, who can benefit from therapies targeted against specific molecular alterations [2]. Based on the detection of these point molecular alterations, with a clear oncogenic role, treatments have been developed to block the activation of mutated, amplified proteins or product of translocations by specific drugs. The identification of patients with therapeutic molecular targets in their tumors is currently a standard of care. Notwithstanding that, the initial morphological diagnosis and the eventual tumor classification by immunohistochemistry (IHC), as well as the acquisition, handling and processing of tumor tissue play a pivotal role.

In advanced-stage patients, a relatively small amount of tissue obtained at a single procedure must be used most efficiently for all studies [3]. In this sense, consensus exists about making histopathological diagnosis using as little material as possible, which should be kept for molecular studies [4, 5]. The combination of less invasive techniques that provide very small samples to carry out an increasing number of determinations is controversial, since it does not allow to increase the amount of tumor cells. Consequently, more sensitive and specific molecular determinations are required [6].

Although several methods are being developed, such as free tumor DNA detection in peripheral blood, most of these determinations are currently experimental and few are validated for clinical use [7, 8]. Therefore, until more sophisticated techniques for these and other molecular markers are validated, the amount/size of the samples should be considered.

The aim of this chapter is to show the implications of molecular pathology in personalized medicine with an actual approach from the methodological limitations of formalin-fixed paraffin embedded (FFPE) tissues and their pre-analytical conditions.

## **2. Pre-analytical processes**

In molecular pathology, several variables should be considered for optimal results, and pre-analytical conditions are evaluated.

### **2.1 Cold ischemia**

One of the crucial phases in tissue management is the period of time immediately after the sample is extracted from the patient until it is placed in a fixation solution (cold ischemia). In an experimental animal model, significant differences in pH values were found between organs at the same cold-ischemia time, and in the same organ at different times. However, no differences were seen in the RNA quality assessed by its integrity number or absorbance ratios [9]. These results reveal a high pH in tissues undergoing ischemia. Firstly, although RNA integrity number (RIN) is a powerful tool to analyze the ribosomal profile and to further infer RNA quality from fresh and frozen tissues (and to compare samples RIN values given the same organism/tissue/extraction method), it is not enough to predict the integrity of mRNA transcripts or to describe the real biological conditions. Secondly, acidic duodenal pH has been reported to alter gene expression in the pancreas of a cystic fibrosis mouse. Upon correction of duodenal pH, either genetically (breeding CFTR-null with gastrin-null mice) or pharmacologically (proton pump inhibitor omeprazole), expression levels of genes measured by quantitative RT-PCR were significantly normalized [10]. Whether alkalosis is secondary to ischemic cell damage, or it may contribute to ischemic cell damage, is yet unknown. Thus, tissue alkalosis in cold-ischemia time may be an underlying mechanism of gene expression changes. Therefore, tissue-pH regulation after organ removal may minimize biological stress in human tissue samples. To date, no consensus exists about the optimal preservation solution. Further optimization of the composition of preservation solutions is required to prolong organ preservation time, and to maximize the yield of successful transplantations by improving the quality and function of organs [11]. Most laboratories have neither control nor record of how long it takes between tissue removal and immersion in the fixer, and its arrival in the laboratory. In addition, most automatic tissue processor machines include a fixation step that further increases the fixation time, which is not often considered.

## **2.2 Tissue fixation**

Once the tissue has been obtained it should be fixed and 10% Neutral buffered formalin (NBF) fixation is recommended. Pre-fixation in alcohol-based fixative, decalcifying acidic solutions, acidic fixatives (such as Bouin) or those containing metallic salts may alter DNA antigenicity or integrity. Setting a period of more than 6 hours and less than 48 hours is recommended [12]. Short or excessive fixation time may have deleterious effects on DNA and protein antigenic epitopes [13, 14]. The most frequently described effect of formalin in DNA is its fragmentation into small pieces. The use of polymerase chain reaction (PCR) techniques in formalin fixed paraffin embedded (FFPE) tissues is associated with a higher incidence of sequence artifacts and risk of misinterpretation in PCR results, compared with the use of fresh samples [15, 16]. After the inclusion of the tissue in paraffin, the sample remains stable and is preserved against oxidative damage or other degenerative effects. However, in addition to fixation, the type of storage is another documented source that can damage DNA and cause artifacts in the PCR. For a better preservation of DNA, FFPE blocks should be stored below 27°C in humidity-free conditions. Although humidity can affect DNA stability, the acceptable humidity control range is not described. In our experience, up to ten-year-old FFPE blocks have been used. Provided that storage is accurately done and the pre-analytical parameters indicated in this chapter are met, blocks can be preserved up to this time [17]. Since FFPE tissue is currently used for genetic analysis, results should always be carefully interpreted. Mutations detected from FFPE samples by sequencing must be confirmed by independent PCR reaction. Determining the nature and duration of fixation is a great challenge to pathology laboratory, which receives samples from other centers. Therefore, it was suggested that the cold ischemia time, the type and time of tissue fixation should be registered in the pathology report [18].

## **3. Tumor area microdissection for tumor cell enrichment**

For a molecular analysis, the following data are required: type of biopsy (primary tumor or metastasis), type of block, and percentage of tumor cells needed for each method.

### **3.1 PCR amplicon size**

As above mentioned, fixation breaks the genetic material into small fragments, and then PCR of FFPE tissue needs a design of specific-sequences primers that flank targets with molecular weight less than 300 bp. Should the designed primers flank a fragmented-amplicon, they fail to perform the enzyme amplification because they need the continuity of the DNA/RNA mold to generate a strand, thus leading to lower sensitivity or false-negative results. Thus, the input for a PCR reaction performed from FFPE tissue requires mandatory quantification with DNA/RNA calculator spectrophotometer. Thus, each methodology uses a different sample input to obtain the analytical sensitivity (LOD). Every PCR requires a balance between its reaction components, and then the sample input has a direct relationship with the concentration of the primers.

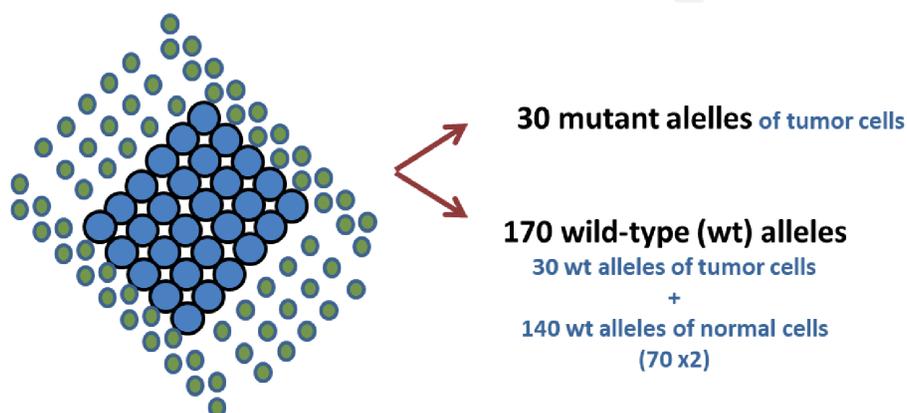
Therefore, somatic mutations, which are generated in tumors and are not present in normal cells, require a minimum percentage for each method.

### 3.2 Tumor cell enrichment

Based on the premise that somatic mutations occur, for the most part, in one of the alleles present in human genome, knowing that in humans there are two equal alleles on somatic chromosomes, one of maternal and one of paternal origin, we must understand that if we seek a tumor marker, we must enrich our input in this allele (**Figure 1**).

Sequencing of tumors is now routine and guides personalized cancer therapy. Mutant allele fractions (MAFs, or the ‘mutation dose’) of a driver gene may reveal the genomic structure of tumors and influence response to targeted therapies [19]. Mutation fraction can be defined as the ratio between mutant and wild-type (wt) alleles in a tumor sample. Allelic fraction is generally applied to a single mutation in a tumor, and is therefore distinct from allelic frequency, which examines the frequency of an allele in a population. To date, however, these terminuses are unfortunately exchanged. Dideoxynucleotide sequencing is a routine method for identifying genetic changes. Since both alleles are amplified in this method, enough input of mutant allele (as compared to the input of normal allele) must be detected. However, this detection requires at least 10–20% of allelic presence. Mutations below this threshold due to normal cells high contamination or tumor heterogeneity could not be detected by this method [20]. Low percentages of neoplastic cells are sometimes associated with unreliable results. Therefore, the percentage of tumor cells must be estimated either through microdissection technique or selection of block interest region [5, 21]. The normal tissue and the lymphocyte infiltration areas must be removed from the tissue for analysis since both are nucleated elements that provide normal DNA. Areas of necrosis should be also removed, since the cell causing necrosis cannot be identified and may be normal or neoplastic. As we know, cell/tumor free DNA drained by biological mechanisms such as secretion, apoptosis and necrosis can be amplified by new generation methods that require smaller chain fragments, this allows us to infer that necrotic cell DNA can be amplified too, considering that an amount of intact nucleic acid chains still present in necrotic masses, unknowing the normal/tumor cell origin. In case microdissection is performed, higher sensitivity is obtained and more chance to detect a tumor specific mutation.

Depending on the method of extraction, hematic areas might be removed. However, they fail to provide normal DNA, because they are anucleated cells, but hemoglobin is one of the main polymerase inhibitors in PCR [22]. Regarding the use of clots, a DNA purification method is required to extract hemoglobin. In this sense, specific columns for FFPE tissues are of value. In several cases, Fine Needle Aspiration (FNA) is the first (and often the sole) diagnostic technique, given its low invasiveness, with



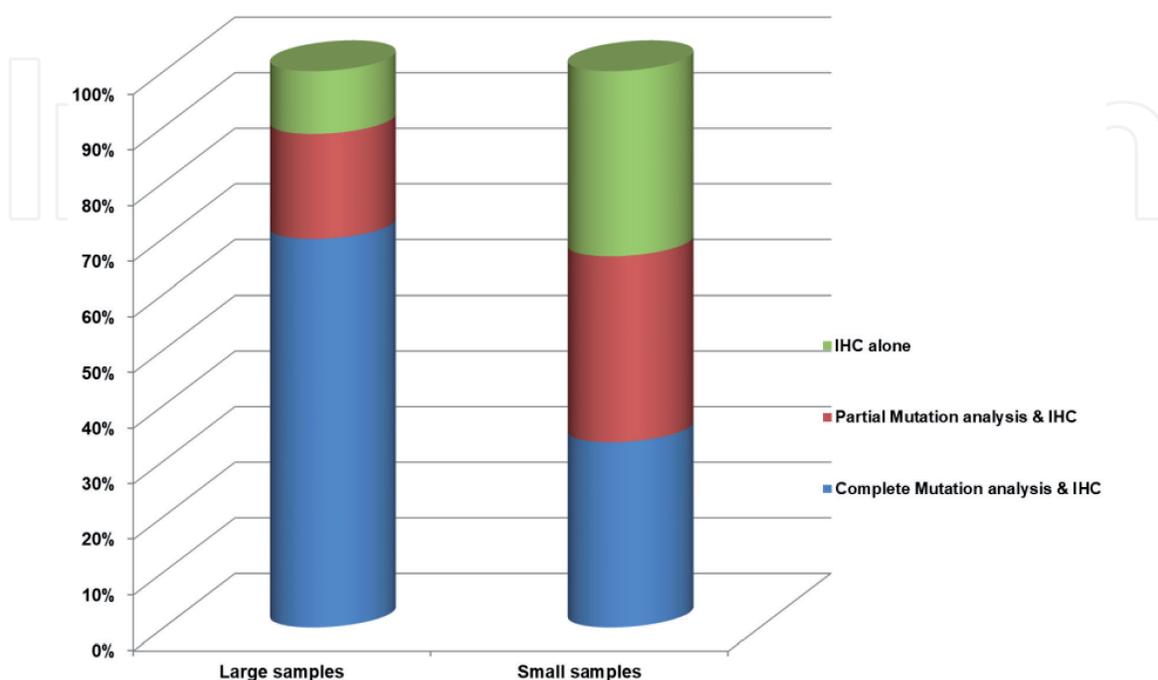
**Figure 1.**  
*Mutant allele fractions (MAFs, or the ‘mutation dose’).*

the clot being all the material available for molecular studies. Here formalin fixation is recommended, and although some reports propose 70% ethanol as an alternative, as above mentioned, DNA antigenicity or integrity may be altered by alcohol-based fixatives [5, 6, 23]. To increase the sensitivity of Sanger sequencing, and to discriminate from technical background, at least 70% of tumor cells are required [5]. The chromatogram obtained failed to discriminate specific signal from background. Such chromatogram type may be determined by pre-analytical conditions (pre-fixing, fixative type or fixation time).

As expected, there was a statistically significant difference between large and small samples DNA concentration. However, no significant differences were observed in concentration, fragments number or tumor initial percentage among different small sample types [18]. We can infer that all these types of tissue samples are similarly useful and depend on interdisciplinary medical team (surgeons, radiologists, clinicians, pathologists and oncologists) [6]. Large samples are blocks from surgical specimens, while small samples could be a core biopsy (yielding tissue samples approximately 1 mm in diameter), biopsies from bronchoscopy, nodal biopsies obtained by mediastinoscopy, and fine needle aspiration resulting in cytological specimens and clots. However, no significant differences were observed in concentration, fragments number or tumor initial percentage among different small sample types. **Figure 2** shows that the amount of tissue obtained from small biopsies is often inadequate for a complete evaluation [18].

Over the last decade, genomic research of various solid tumors has suddenly progressed through the discovery of several molecular biomarkers that eventually impact on the prognosis and treatment of most common cancers. Recent technical innovations, such as “next or second generation” sequencing or “massively parallel” sequencing, have the potential to detect many abnormalities in a single assay, and are probably the solution to tissue shortage [24, 25].

This definitely results into multiple activities for surgeons and pathologists, who must obtain and process samples, write a pathology report, choose the material for molecular biology. In furtherance, those molecular biosciences technicians performing studies must draw up guidelines to standardize these practices, and algorithms to cover cyto- and histopathological diagnoses, IHC and molecular studies [4, 5, 23, 24, 26].



**Figure 2.**  
Performance of IHC and molecular study of large and small biopsies.

## 4. Methodological analysis and results

Detection of tumor-derived mutations in FFPE is challenging because the tumor DNA is often scarce, fragmented, at a very low concentration and diluted by the presence of a background of non-mutant DNA (both tumor and non-tumor origin). Once the area of tumor cells is selected to be processed, the method of purification of the macromolecules must be chosen. Although manual non-expensive forms (phenol-chloroform-PK) exist, they fail to provide the necessary amount and quality of DNA. There are affinity columns for DNA, RNA or DNA/RNA together, which can be used on a low scale; and finally automated nucleic acid extraction equipment. Some years ago manual extraction was used for FFPE tissue because the columns were developed only for fresh samples. In the last decade the advent of personalized medicine boosted the development of new methodologies for this purpose. Heydt *et al.* used FFPE tissue samples for the comparison of five automated DNA extraction systems, the BioRobot M48, the QIAcube and the QIASymphony SP all from Qiagen (Hilden, Germany), the Maxwell 16 from Promega (Mannheim, Germany) and the InnuPure C16 from Analytik Jena (Jena, Germany). The results revealed that the Maxwell 16 from Promega seems to be the superior system for DNA extraction from FFPE material. This study also evaluated DNA quantification systems using the three most common techniques, UV spectrophotometry, fluorescent dye-based quantification, and quantitative PCR. The comparison of quantification methods showed inter-method variations, but all methods could be used to estimate the right amount for PCR amplification and for massively parallel sequencing. DNA extracts were quantified as follows: NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific), Quant-iT dsDNA HS Assay on the Qubit 2.0 fluorometer (Life Technologies), QuantiFluor dsDNA Sample Kit on the QuantiFluor-ST fluorometer (Promega) and Quant-iT Pico-Green dsDNA reagent (Life Technologies) on the LightCycler 480 Instrument (Roche). No difference was observed in mutation analysis based on the results of the quantification methods. These findings emphasize that it is particularly important to choose the most reliable and constant DNA extraction system, especially when using small biopsies and low elution volumes [27]. Once DNA/RNA has been obtained and quantified, analysis requires highly sensitive and specific assays. Different techniques with their own advantages and disadvantages can be used to identify and monitor mutations.

### 4.1 Real-time qPCR assays

A real-time PCR or quantitative PCR (qPCR) amplifies, both quantitatively and semi-quantitatively, a targeted DNA molecule during the PCR process. There exist at least two methods for the detection of PCR products: non-specific fluorescent dyes that bind double-stranded DNA molecules by intercalating between the DNA bases. This method is used in qPCR because the fluorescence can be measured at the end of each amplification cycle to determine, either relatively or absolutely, how much DNA has been amplified. The other method is sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter, which permits detection only after hybridization of the probe with its complementary sequence (TaqMan).

There is also a revolutionary method that uses PlexZyme™ technology. The revolution in this technology is given by a structure called partzyme (A and B). Each partzyme has 3 different regions: (I) the region that joins the target sequence of DNA, (II) the catalytic constituent region, and (III) the region that joins the probe. Once the primers generate the amplicons, both partzymes join their complementary sequences through the region (I), acquiring a characteristic structure thanks to the region (II) that allows the region (III) to be exposed. The fluorescently

labeled reporter probe also binds to the partzymes in the region (III) exposed, and once the active catalytic core is formed, the probe is cleaved, producing a signal that is indicative of successful amplification of the target gene. This technology can produce a robust quintuplex with five target assays into a single reaction tube that contained 10 partzymes (5 A and 5 B), 10 primers (5 forwards and 5 revers), and 5 probes, with a 5 different fluorophores. All consumables required for sample preparation and RT-PCR amplification and detection are provided in a single cartridge loaded into the Idylla™ system. Handling time is less than two minutes per sample, with the liquid-tight, disposable cartridges greatly reducing the risk of contamination (Biocartis NV, Belgium).

#### **4.2 ddPCR assay**

In the non-sequencing space, digital PCR (ddPCR), is a highly sensitive and specific technique for the detection of mutations. DNA molecules are split into droplets that form a water oil emulsion. Droplets are like individual test tubes or wells on a plate where a PCR reaction occurs from a DNA template. Each drop is analyzed or read to determine the fraction of positive droplets in the total sample and can accurately and sensitively quantify a mutation. The creation of thousands of drops means that a single sample can generate thousands of data, which are statistically analyzed. For digital PCR the assays are limited to specific single mutations or sets of highly related mutations at the same locus. The analysis of broader genomic regions using ddPCR is not feasible. However, discriminatory multiplex ddPCR assays can be developed, which enable very rapid and cost-effective monitoring for a limited number of mutations in serial plasma samples [28].

#### **4.3 Sanger capillary sequencing**

Sanger sequencing is a DNA sequencing method based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication [29–30]. This method was first developed by Frederick Sanger and colleagues in 1977, and became the most widely used sequencing method for over 40 years. However, the Sanger method remains widely used for smaller-scale projects and for validation of NGS results.

#### **4.4 Next-generation sequencing (NGS)**

In this decade, the treatment of cancer patients has evolved with the addition of new massive sequencing technologies. This contributed to the study of tumor biology with an accurate and highly covered diagnostic method that allows the selection of those patients likely to benefit most from target-specific targeted therapies. NGS, massively parallel or deep sequencing, refers to a DNA sequencing technology that has revolutionized genomic research. NGS can be used to sequence the whole human genome within a single day. In contrast, the previous Sanger sequencing technology used to decipher the human genome took over a decade to deliver the final draft [31]. Over the last years, massively parallel sequencing has rapidly evolved and has now transitioned into molecular pathology routine laboratories. This is an interesting platform for the simultaneous analysis of multiple genes with low input material. Therefore, laboratories working with FFPE material and high sample throughput largely require high-quality DNA obtained from automated DNA extraction systems. The spectrum of DNA variation in a human genome comprises small base changes (substitutions), insertions and deletions of DNA, large genomic deletions of exons or whole genes and rearrangements, such as

inversions and translocations. Traditional Sanger sequencing focuses on the discovery of substitutions and small insertions and deletions.

There are a number of different NGS platforms using different sequencing technologies, but all these platforms sequence millions of small fragments of DNA in parallel. The aim of bioinformatics analyses is to piece together these fragments by mapping the individual reads to the human reference genome (pipelines). Each of the three billion bases in the human genome is sequenced several times, in order to provide accurate data and an insight into unexpected DNA variation. NGS can be used to sequence either whole genomes or specific genomic areas of interest, including all 22,000 coding genes, the whole-genome sequencing (WGS), the whole exome sequencing (WES). This is a genomic technique for sequencing all of the protein-coding regions of genes in a genome, known as the exome; or small numbers of individual genes (NGS panels).

Parallel sequencing requires target enrichment, which is a pre-sequencing step that only allows part of a whole-genome be sequenced, or regions of interest, without sequencing the entire genome of a sample. The two most commonly used techniques for NGS target enrichment are capture hybridization and amplicon-based (multiplex PCR). In capture hybridization, genomic DNA is cut to produce small fragments that join sequencer-specific adaptors and indexes to prepare the library, and then the sample is hybridized with biotinylated RNA library primers. Target regions are extracted with magnetic streptavidin beads, amplified and sequenced. Capture hybridization is a screening method for large genetic panels and a large DNA input (more than 1 ug DNA), with a laborious and complex workflow, but a better performance. In amplicon sequencing, custom oligo capture probes are designed to flank DNA specific regions without fragmenting. Extension/ligation takes place between hybridized probes. Finally, the uniquely labeled amplicon library is ready for cluster generation and sequencing. The extension/ligation occurs between hybrid probes which determines a uniquely tagged amplicon library ready for cluster generation and sequencing. Amplification sequencing is used for small gene panels or somatic mutation hotspots (target from kb to Mb), with lower DNA input (100 ng). It has a simple and fast protocol (combining sample preparation and enrichment in one assay), but it is more liable to false positive and negative calls. Considering the WGS method in the same fresh and FFPE samples, hybrid capture sequencing showed higher sensitivity compared to amplicon sequencing, while maintaining 100% specificity using Sanger sequencing as a validation method. Amplicon method has higher target rates. Hybridization capture-based approaches demonstrated that many of them could be false positives or negatives [32]. These results reveal advantages and disadvantages of both methods. Therefore, a greater number of trials must be undertaken to demonstrate both clinical usefulness and socioeconomic benefits. On occasions, an extremely sensitive method is not worth using given its clinical implications.

The basic premise of cancer genomics is that cancer is caused by somatically acquired mutations, and is therefore a disease of the genome. Capillary-based cancer sequencing has been ongoing for over a decade. However, these investigations were restricted to relatively few samples and small numbers of candidate genes. Tumor heterogeneity and the addition of new molecular targets have become a challenge that needs a multidisciplinary approach and learning, with the study of the molecular profile of the tumor at the genomic (DNA), transcriptomic (RNA) or protein (protein) level. NGS technique is a useful and novel tool for the study of molecular profile from DNA/RNA. To do the library using amplicon methods it is only necessary to obtain 10 ng of DNA just from the tumor, and 10 ng of RNA, which is feasible, even from small samples, fixed in formalin and included in paraffin [31].

Thus, three of the major technical drawbacks of the massive analysis required for the approach of multiple specific biomarkers for the treatment are resolved. These

drawbacks include the small size of biopsy sample and material scarcity, paraffin fixation of tissues and its effect on DNA/RNA and the impossibility to collect and store fresh material in standard clinical practice. Therefore, this type of studies is necessary to optimize the quality of patient care, avoiding errors and false positives or negatives. Thus, the use of NGS panels with small and overlapping amplicons would solve all these drawbacks, always associated with a bioinformatics algorithm (pipeline) that allows the overlap of the fragments obtained with a reference sequence.

## 5. In-depth study of molecular alterations

The prevalence of molecular alterations with targeted treatment may vary according to different variables, such as the region of the world, race and gender [33–34]. About 86% of tumors have molecular alterations that can potentially be treatable with approved or developing drugs, of which approximately 30% have clinically available drugs. The distribution of these alterations in patients with metastatic disease varies compared to those observed in resected tumors at earlier stages [35].

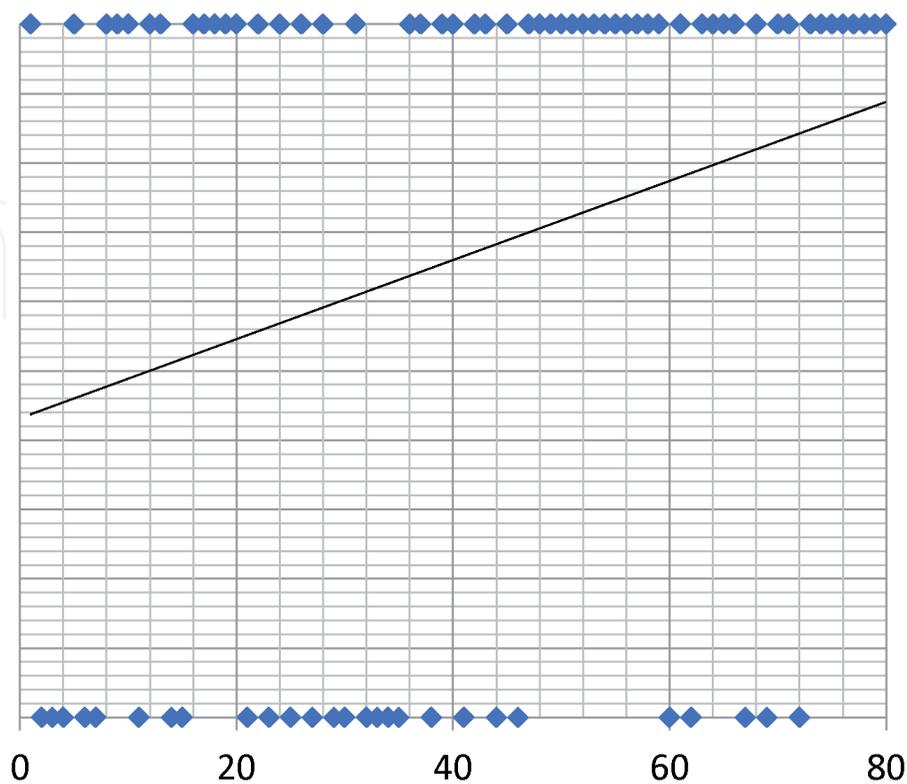
Different analysis options may be combined according to the molecular target to be identified, the type of molecular alteration and the type of sample required. Regarding the KRAS gene, a GTPase which functions as an upstream regulator of the MAPK and PI3K pathways, it is frequently mutated in various cancer types including pancreatic, colorectal and lung cancers [36].

KRAS was one of the first markers to be used as a therapeutic target in colorectal cancer (CRC) in clinical practice since the approval of cetuximab in the second line in 2008. Both the European Medical Agency (EMA) and the Food and Drug Administration (FDA) in 2008 approved the use of anti-EGFR monoclonal antibodies in patients with tumors with non-mutated KRAS (KRAS-wt). The selection of patients for anti-EGFR treatment based on the mutational status of codons 12 and 13 of the KRAS gene is highly specific to non-responder patients. At that time, the tissue was not macro-dissected, biopsies containing more than 70% of tumor cells were processed by sequencing for the reasons mentioned above, and approximately 30% of cases could not be evaluated since they failed to meet these criteria. Codon 12 and 13 of exon 2 of the KRAS gene were studied and the type of mutation found was irrelevant. For exon 2, 40% of the CRC patients were mutated and 60% were wt (codon 12 and 13). Results showed that 95% of patients with mutated CRC for KRAS did not benefit from anti-EGFR treatment. However, it was not sensitive enough because only half of patients with KRAS-wt tumors responded to treatment [37]. Then, the 59 and 61 codons of exon 3 and the 117 and 146 codons of exon 4 were eventually added. Automated qPCR methods were developed, which covered these hot-spots and dually reported wt or mutated. Nowadays, these binomial methods (wt/mutated) would not serve to identify the G12C amino acid change (c.34G > T p.Gly12Cys). Target therapies like KRAS G12C covalent inhibitors, such as AMG-510, are currently in early phase clinical trials and show promising results for the treatment of KRAS G12 mutant lung cancer patients. However, KRAS G12C colorectal cancer patients have not shown the same response. KRAS mutation testing was carried out using 13 technologies and assays. Limits of detection (LD) of the 13 methods were showed in the following table. Of 13 assays evaluated in this work, 9 showed relatively similar levels of accuracy and reliability in detecting KRAS mutations at low levels with varying sensitivities (50 copies mutant allele frequency by each technology). The best performances were obtained by three assays: OncoPrint Focus Assay, Idylla KRAS Mutation Test and UltraSEEK, with high sensitivity and specificity across the entire cell line panel. The worst performances in detection were Illumina Nextera Rapid Capture Custom Lung Panel and Sanger capillary sequencing [38].

	Real-time quantitative PCR			MALDI-TOF	NGS						ddPCR	Sanger	
	1	2	3	4	5	6	7	8	9	10	11	12	13
LD	5	5	5	10	0,1	5	5	5	10	5	5	0.001	20
<ol style="list-style-type: none"> <li>1. <b>therascreen KRAS RGQ PCR Kit</b></li> <li>2. <b>cobas KRAS Mutation Test</b></li> <li>3. <b>Idylla KRAS Mutation Test</b></li> <li>4. <b>iPLEX Pro</b></li> <li>5. <b>UltraSEEK</b></li> <li>6. <b>ThunderBolts</b></li> <li>7. <b>Oncomine Focus Assay</b></li> <li>8. <b>Sentosa SQ NSCLC Panel</b></li> <li>9. <b>Illumina Nextera Rapid Capture Custom Lung Panel</b></li> <li>10. <b>Ion AmpliSeq Cancer Hotspot Panel v2</b></li> <li>11. <b>TruSight Tumor 15 panel</b></li> <li>12. <b>PrimePCR ddPCR Mutation Assays KRAS</b></li> <li>13. <b>ABI3730 sequencing</b></li> </ol>													

The NGS study may infer biological mechanisms that may explain primary resistance (absence of response to tyrosine kinase inhibitors and disease progression as a better response). This information is required for decision-making of the allelic frequency data for DNA sequence variants, amplified reads for fusions, or the number of copies of amplified genes, since in order to determine that a sequence variant has a clear oncogenic role in the tumor, its representative presence is required. One of the most common false positives with NGS, partly due to its high sensitivity, is the amplification and sequencing of variants from clonal hematopoiesis. Obtaining DNA from FFPE is a methodology used for more than decades, with satisfactory results, since the DNA obtained was degraded by fixation-paraffinization process, as well as its opposite effect which is the deparaffinization of tissue. Obtaining RNA from this type of sample is most controversial given its increased lability, and was recently accepted due to the incorporation of new purification strategies. Therefore, obtaining RNA from FFPE was the greatest difficulty of this DNA/RNA NGS method, and required this minimum learning curve to achieve optimal 80% performance (**Figure 3**). The effectiveness of RNA isolation was calculated, taking into account criterion  $>5000$  reads as evaluable sample, for each run/chip. Increased performance was achieved as the long runs occurred. The initial yield was less than 50%, reaching 80% maximum, because the fixation of the tissue as well as the deparaffinization process are counterproductive effects for obtaining RNA. Pre-analytical pathological processes for NGS take a crucial role.

This has been especially relevant in RNA sequencing from paraffin block. A learning curve is required before using this methodology in the clinical field. The acquisition of macromolecules management is critical. On the other hand, multidisciplinary work is crucial for the correct interpretation of the information provided by these new technologies. Crude data alone, without associated bioinformatics information, should not be used for the treatment of patients. The main pitfall of NGS in the clinical setting is the infrastructure, such as computer capacity and



**Figure 3.**  
RNA performance (1=  $>5000$  reads, 0  $<5000$  reads).

storage, and personnel trained in comprehensive analyses and interpretation of the subsequent data. In addition, and in order to obtain clinically relevant information in a clear and robust interface, the volume of data needs to be proficiently managed. However, to make NGS cost effective one would have to run large batches of samples which may require supra-regional centralization. The objective of implementing new technologies is to develop personalized treatment strategies that result in prolongation of survival of patients with a better quality of life.

## 6. Conclusion

The analysis of the biology of tumors, using NGS, allows to expand the number of molecular alterations to be studied, and allows to detect more patients who can benefit from targeted treatments, modifying the survival in patients with detected and treated molecular alterations. A continuous and inexorable shift in surgical pathology can be observed, with histological diagnosis being just one of its components. The molecular profile is nowadays an essential tool for anatomic pathology practice, which invariably requires highly trained specialists. The in-depth study of molecular alterations in patients allows optimizing molecular diagnosis and selecting patients to receive novel treatments, targeted against specific molecular targets for the clinical benefit of patients, through a multidisciplinary approach and learning.

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## Conflict of interest

The author declares no conflict of interest.

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