Molecular alterations in the screening and care of tumours. Interreg Sudoe. European Regional Development Fund Work package 1.2.

Standardised Operation Procedure (SOP) for the study of genetic and epigenetic alterations in tumoral and circulAting free DNA/RNA

Report prepared by Molecular Pathology Group, Complejo Hospitalario de Navarra, Navarrabiomed

1. Introduction

In the latest years there is an **increasing interest in the field of Personalised Medicine for the study of new biomarkers** that help clinicians to make a decision about the oncologic therapy of the patients with cancer. Directed therapies against molecular targets combined with conventional treatments, i.e. chemotherapy and radiotherapy are more effective than conventional treatments alone, and help to prolonge disease survival rates.

These molecular alterations are **genetic and epigenetic changes that contribute to cancer progression and can determine the efficacy of oncological treatment with approved drugs**, e.g. the level of expression of hormone receptors (estrogen and progesterone receptors) for the hormonal treatment in breast cancer.

Epigenetic alterations are common in cancer, including **aberrant DNA methylation and microRNA expression, and altered post-translational modifications of histones**. Aberrant DNA hypo- and hypermethylation are very frequent and have been considered biomarkers of diagnosis and survival. Nevertheless, its value as a determinant of response to therapy is more limited, with few examples of predicitive markers such as *MGMT* hypermethylation related to response to temozolomide in glioblastoma multiforme.
Nevertheless, **many genetic alterations (point mutations, copy number variations and fusions) have been described to have clinical value as determinants of the effect of first-line therapies in several types of cancer**, such as colorectal cancer, lung cancer and melanoma. Lung adenocarcinoma (ADC) is the paradigm of cancer with numerous driver alterations that influence on directed therapies. The analysis of these mutations is mandatory for the best treatment appproach. The onset of new resistance mutations to directed therapies such as EGFR T790M mutation in lung TKIs-treated ADC makes a change in the treatment of the patients**. Most of these analysis must be performed in circulating free DNA obtained from peripheral blood** given the general status of the patient and the lack of availability of samples to be analysed. Free-circulating nucleic acids, such as tumor-specific extracellular DNA fragments, mRNAs and miRNA are present in serum or plasma, and have the potential to provide biomarkers for diagnosis and treatment. They are present usually as short fragments, (<1000 bp: DNA), <1000 nucleotides (RNA) and 20-25 nucleotides for miRNA.

The **application of high throughput technologies** is essential for the analysis of multiple epigenetic and genetic markers from the same sample for the choice of new lines of therapy.

The methodology for the study of genetic and epigenetic alterations in circulating-free DNA and tumoral tissue by high throughput technologies must be clear and reproducible**. This document includes the Standardisation Operation Procedure as a step-by-step procedure for the analysis of multiple targets for the study of epigenetic alterations by methylation arrays and genetic alterations by next generation sequencing (NGS).**

For details about the definition and application of these technologies, please refer to the presentation « Onconet_Work package 1.2._Annexed document » downloaded in the Onconet webpage.

2. Description of the Standardised Operation Procedure (SOP)

2.1. SOP for epigenetic analysis in tumoral DNA/ circulating free DNA

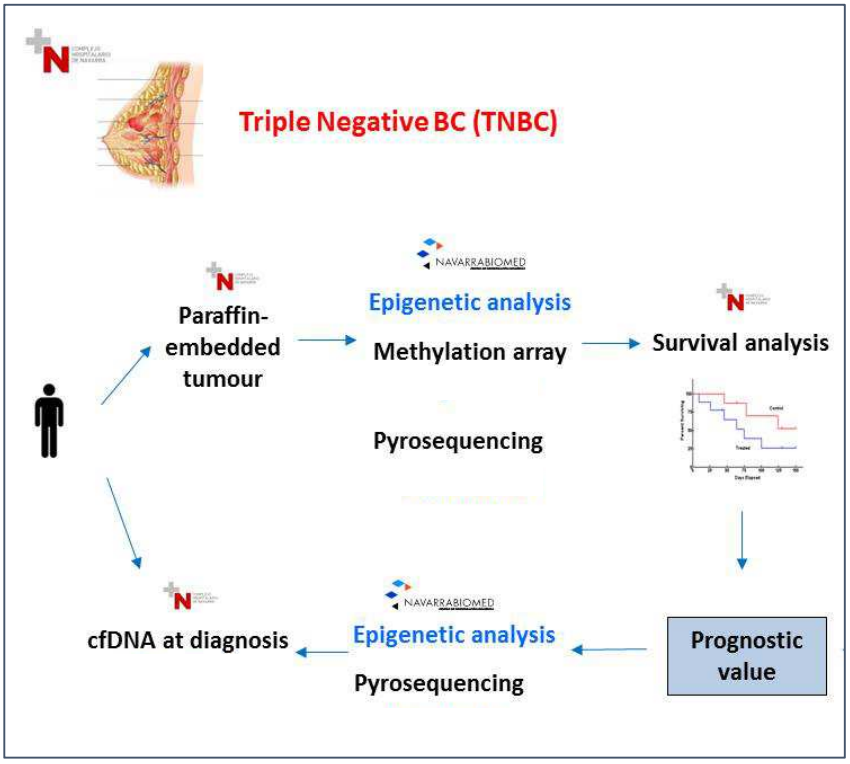


Figure 1. SOP for epigenetic studies

2.1.1. Tumoral-Normal DNA/cfDNA extraction

- **Tissue DNA extraction for methylation array**

  Frozen tissue is the elegible material for methylation array, and non-column based extraction methods are preferable.

  The procedure includes the following steps:

  - Cut a tissue fragment (0.2 cm x 0.2 cm) and place it into an eppendorf (1.5 ml).
  - Add 2.5 ml Tris 1 M-EDTA 0.25 M-NaCl 2.5 M (10:10:0.15).
  - Cut the tissue mechanically into a homogenate and place it into a 15 ml tube.
  - Add 180 µl Proteinase K 10 mg/ml, and 275 µl SDS al 10% and leave it overnight under agitation and digestion at 37º C.
  - Add 50 µl proteinase K 10 mg/ml and incubate the tubes for one hour at 55 ºC.
  - Add 180 µl RNAase A and incubate the tubes for one hour at 37 ºC.
  - Add 3 ml phenol in a extraction hood, and mix softly by inversion.
  - Centrifuge 5 minutes, 3300 rpm at room temperature.

- Pipette the upper aqueous phase very carefully and place it into a new eppendorf 1.5 ml tube.
- Add 1.5 ml phenol and 1.5 ml chloroform in an extraction hood, mix softly by inversion and centrifuge 5 minutes 3300 rpm at room temperature.
- Add 3 ml chloroform and repeat the previous step.
- Place the supernatant into a new 1.5 ml eppendorf tube, add 1/10 V NaCl 2.5 M and mix by inversion.
- Add 1 volume (V) EtOH 100%, and leave the DNA to precipitate overnight at –20 ºC.
- Centrifuge 14000 rpm 5 minutes at 4 ºC, and remove the supernatant.
- Resuspend the DNA pellet in 50-150 µl Tris 10 mM/EDTA 0,1 mM, depending on the initial size of the tissue.
- DNA quantification is performed by fluorimetric assay by using a specific Kit for quantifying amplifiable human genomic DNA.

- **Tissue DNA extraction for pyrosequencing**

Formalin-fixed paraffin-embedded (FFPE) tissue is suitable for the confirmation of methylation by the pyrosequencing technique. The procedure for the DNA purification from FFPE is performed by a column-based method that consists of the following steps:

- **Deparaffinization**
  - Cut 5-20 µm sections from tissue blocks using a microtome and place the sections in a 1.5 ml microcentrifuge tube.
  - Add 1 ml 100% xylene to the samples, vortex briefly and spin down to ensure all the sample is in contact with the xylene.
  - Heat the sample for 3 minutes at 50ºC to completely remove paraffin.
  - Centrifuge the sample for 2 min at maximum speed and remove carefully the xylene without disturbing the pellet.
  - Wash the pellet twice with 1 ml 100% ethanol, vortexing briefly to mix.
  - Briefly centrifuge at maximum speed and remove ethanol carefully without disturbing the pellet.
  - Air dry the pellet for 15-45 min at room temperature (RT).

- **Protease digestion**
  - Add digestion buffer (100 µl if sample size is ≤40 µm and 200 µl if the sample size is 40-80 µm) and 4 µl proteinase K per sample.
  - Swirl the tube gently to mix and briefly centrifuge to bring the tissue down into the solution.
  - For RNA isolation incubate the sample in a heat block for 15 minutes at 50ºC, then 15 minutes at 80ºC.
  - For DNA isolation incubate the sample for 16 hours at 50ºC.

- **Nucleic Acid Isolation**
  - For DNA and RNA, combine the amounts of isolation additive and ethanol 100% according to the volume of digestion buffer previously used and mix by pipetting up and down.

	Volume of digestion buffer	
	100 µl	200 µl
Isolation additive	120 µl	240 µl
100% ethanol	275 µl	550 µl

  - For each sample, place a filter cartridge in a collection tube supplied.

- Pipet up to 700 µl of the sample- ethanol mixture onto the filter cartridge, close the lid and centrifuge at 10000 g for 30 seconds.
- Discard the flow through and put the filter cartridge into the collection tube.
- Add 700 µl of washing solution A to the filter and centrifuge at 10000 g for 30 seconds and discard the flow through and put the Filter Cartridge into the collection tube.
- Repeat the same step bellow with washing solution B.
- Discard the flow through and put the filter cartridge into the collection tube.
- Spin for an additional 30 seconds to remove residual fluid from the filter.

- **Nuclease digestion and final nucleic acid purification**
  - For RNA isolation add a mix of 6 µl 10x DNase buffer, 4 µl DNase and 50 µl of nuclease-free water to the center of the filter and incubate for 30 min at RT
  - For DNA isolation add a mix of 10 µl RNase A and 50 µl nuclease-free water to the center of the filter and incubate for 30 min at RT
  - Add 700 µl of washing solution A to the filter and centrifuge at 10000 g for 30 seconds and discard the flow through and put the Filter Cartridge into the collection tube.
  - Repeat the same step bellow with washing solution B.
  - Transfer the filter cartridge to a new collection tube and apply 60 µl of Elution solution or Nuclease-free water at RT (for RNA elution) and preheated to 95ºC (for DNA elution) to the center of the filter.
  - Incubate the sample at RT for 1 minute and centrifuge at maximum speed for 1 minute to pass the elution with the RNA or DNA through the filter.

2.1.2. Epigenetic analysis in tumour. Methylation array

Methylation array for genome-wide DNA methylation screening is performed using different technologies. Generally, one or two probes are used to interrogate CpG locus depending on the probe design for a particular CpG site. Here the protocol used for the interrogation of the CpG locus by two probes, one "unmethylated" and one "methylated", is going to be described. The 3' end of each probe is designed to match either the protected cytosine (methylated design) or the thymine base (methylated design) resulting from bisulfite conversion of genomic DNA with kits according to manufacturer recommendations.

This technique must be performed in tumoral and non-neoplastic tissues in order to detect alterations that are only present in cancer.

The procedure is performed in nine consecutive steps:

- **Bisulfite conversion**
  DNA should be quantified by fluorimetric detection and 1000 ng are transformed by bisulfite conversion in the following steps:
  - Denaturation of the genomic DNA and addition of the conversion reagent.
  - Incubation in a thermocycler 95° C for 30 seconds and 50° C for 1 hour for 16 cycles.
  - Wash off the conversion reagent.
  - Desulphonate the column with desulphonation buffer and incubate at room temperature for 15 minutes.
  - Wash off the desulphonation buffer and add elution buffer.
  - Centrifuge to elute and transfer the bisulfite-converted DNA samples to the 96-well bisulfite plate.
  - Heat-seal the plate and store it at -20 ºC.

- **Isothermal amplification**
  - Transfer 4 µl of the converted DNA sample from the plate to the corresponding wells in the 96-well plate of analysis (PA).
  - Dispense 4 µl 0.1N NaOH into each well of the PA that contains amplification mix and sample, and seal the plate with the 96-well cap mat.
  - Vortex the plate at 1600 rpm for 1 minute and centrifuge to 280g for 1 minute.
  - Incubate for 10 minutes at room temperature and invert the sealed plate at least 10 times to mix contents.
  - Incubate in hybridization oven for 20–24 hours at 37° C.

- **DNA Fragmentation by an endpoint enzymatic process**
  - Centrifuge the plate to 50g for 1 minute.
  - Add 50 µl of fragmentation solution to each well containing sample and seal the PA with the 96-well cap mat.
  - Vortex the plate at 1600 rpm for 1 minute and centrifuge it to 50 g for 1 minute.
  - Place the sealed plate on the 37C heat block for 1 hour.

- **DNA Precipitation**
  - Add 100 µl precipitation solution to each plate well.
  - Seal the plate with the cap mat and vortex the plate at 1600 rpm for 1 minute.
  - Incubate at 37ºC for 5 minutes, centrifuge to 50g at 22C for 1 minute.
  - Add 300 µl 100% 2-propanol to each well, invert at least 10 times to mix contents thoroughly and incubate at 4ºC for 30 minutes.
  - Centrifuge to 3000 g at 4° C for 20 minutes, decant supernatant by quickly inverting the plate, and leave the uncovered, inverted plate on the tube rack for 1 hour at room temperature to air dry the pellet.

- **DNA Resuspension**
  - Add 46 µl resuspension reagent to each well of the plate containing a DNA pellet.
  - Apply foil seal to plate for 5 seconds, place the sealed plate in the hybridization oven and incubate for 1 hour at 48°C.
  - Vortex the plate at 1800 rpm for 1 minute and pulse centrifuge to 280xg.

- **DNA Hybridization**

  The amplified and fragmented DNA samples anneal to specific oligomers that are covalently linked to the different bead types.
  - Denature the samples at 95° C for 20 minutes in the heat block, then place it at room temperature for 30 minutes and centrifuge the plate to 280 xg for one minute.
  - Place each chip in a chamber insert and using a multi-channel precision pipette, dispense 15 µl of each DNA sample onto the appropriate section.
  - Place the hybridization chamber into the hybridization oven at 48° C with agitation.
  - Incubate at 48° C 16-24 hours and wash chips.

- **Single-Base Extension**

  This reaction incorporates labeled nucleotides into the extended primers hybridized to DNA on the chip.
  - Dispense 150 µl extension solution, incubate for 30 seconds and repeat 5 times.
  - Dispense 450 µl extension solutions and incubate for 10 minutes each.
  - Pipette 450 µl 95% formamide/1 mM EDTA and incubate for 1 minute.

- **Chip staining**
  - Dispense different volumes of staining solutions and incubate for different periods of time.
  - After washing the chips dry the chips in the vacuum desiccator for 55 minutes.
  - Clean the underside of each chip with a wipe.

- **Image and analyses of the chips**
  - Image chip on the laser-based, high-resolution benchtop optical imaging scanner.
  - Methylation Module is used to analyze array data to assign site-specific DNA methylation β-values to each CpG site.
  - The level of DNA methylation at each CpG locus (β-value) is calculated as M/(M+U), ranging from 0 (100% unmethylated, U) to 1 (100% methylated, M). Differentially expressed probes are obtained by limma t-test (False Discovery Rate<0.05). Those probes with delta β-value $|\Delta\beta|>0.2$ and located in CpG islands in the 5´UTR, 1500-200bp upstream the transcription start site (TSS) or in the first exon are considered.

2.1.3. Epigenetic analysis in tumour and cfDNA. Pyrosequencing

Pyrosequencing is the elegible method to confirm the differential methylation status (hyper- and hypomethylation) of candidate genes obtained by the methylation array. This is a method of DNA sequencing based on the "sequencing by synthesis", in which the incorporation of nucleotide into single stranded DNA by DNA polymerase generates the release of pyrophosphate ion. The presence of ATP sulfurylase and luciferase generates light, and this signal is proportional to the number of complementary nucleotides that are present on the template strand.

This technique should be applied to the same samples (DNAs from frozen tissues) used for the methylation array in order to confirm the results. Once confirmed, an additional series of samples must be further analyzed to confirm the biological role of the alterations. Fragmented DNA extracted from FFPE samples are analyzable by this technique because pyrosequencing reads short DNA fragments (20-40 bp). Pyrosequencing was carried out following the next steps:

- **DNA bisulfite conversion**
  DNA should be quantified by fluorimetric detection and 1000 ng are transformed by bisulfite conversion in the following steps:
  - Denaturation of the genomic DNA and addition of the conversion reagent (bisulfite).
  - Incubation in a thermocycler 95° C for 30 seconds and 50° C for 1 hour for 16 cycles.
  - Wash off the conversion reagent.
  - Desulphonate the column with desulphonation buffer and incubate at room temperature for 15 minutes.
  - Wash off the desulphonation buffer and add elution buffer.
  - Centrifuge to elute and transfer the bisulfite-converted DNA samples to sterile 1.5 ml eppendorfs.

- **PCR amplification**
  PCR must be performed to amplify target DNA. PCR amplification is performed in the samples using byotinilated specific primers for each gene, hot-start DNA polymerase and bisulphite modified DNA. Amplification conditions are generally as follows:

Stage	Step	Temp.	Time
Initial activation	Enzyme activation	95ºC	15 minutes
Number of cycles: 42	Denaturation	95ºC	20 seconds
	Annealing	53ºC	30 seconds
	Extension	72ºC	20 seconds
Final extension	Final extension	72ºC	5 minutes

The amplicons are resolved by electrophoresis using 2% (w/v) agarose gel in 1x Tris-borate-EDTA buffer, stained and visualized in a standard transilluminator.

- **Retain the PCR products by High-Performance Streptavidin-Sepharose microbeads**
  - Before start keep all reactives and solutions at RT (15-25ºC).
  - Swirl gently the Streptavidin-Sepharose microbeads until a homogeneus solution is obtained.
  - Prepare a master mix for DNA retention as is summarized in table . Prepare 10% more volume than necessary for all the reactions.

Component per reaction	Volume
High Performance Streptavidin Sepharose microbeads	2 µl
Binding buffer	40 µl
Nuclease-free water	28 µl

  - Add 70 µl of the master mix to a 24-well PCR plate.
  - Add 10 µl of a well-optimized biotinylated PCR product to each well.
  - Seal the plate with an adhesive film and agitate constantly for at least 5-10 min at RT (15-25ºC) using a mixer (1400 rpm).

- **Separation of DNA strands and release of samples into the workstation**
  - During immobilization, prepare vaccum workstation for the sample preparation filling reservoirs with 50 ml ethanol (reservoir 1), 40 ml denaturation solution (reservoir 2), 50 ml wash buffer (reservoir 3), and 50 ml/70 ml nuclease-free water (reservoirs 4/5).
  - Prewarm one of the plate holder (without a plate) on a heating block at 80ºC.
  - Fill the five troughs supplied with the corresponding solutions.
  - Dilute the sequencing primer to 0.3 µM in annealing buffer.
  - Heat the PCR plate at 80ºC for 2 min using the plate holder.
  - Remove the plate from the holder and allow the samples cool down to RT.
  - After immobilization of templates, place PCR plate and the pyrosequencing plate in the workstation.
  - Apply vacuum to the tool by opening the vacuum switch.
  - Agitate gently the plate for at least 1 minute and lower carefully the filter probes into the PCR plate to capture the Sepharose microbeads containing the immovilized PCR template, capturing the beads quickly to avoid their precipitation.
  - Transfer the vacuum brush to the reservoirs (see first step of this protocol) in these steps:
    - 70% ethanol. Flush the filter probes for 5 seconds.
    - Denaturation solution : Flush the filter probes for 5 seconds.
    - Wash buffer : Flush the filter probes for 10 seconds. Raise the tool to beyond 90ºC vertical for 5 sec to drain all the extra liquid from the filter probes.

- Collect the microbeads into the pyrosequencing plate by switching off the vacuum and gently shaking the vacuum brush to release the beads in the plate containing sequencing primer by side to side.
- With the vacuum switch off, transfer the brush to the 50 ml high-purity water trough, and agitate for 10 seconds.
- Wash the filter probes by lowering them into 70 ml high-purity water trough and applying vacuum.
- Raise the tool to beyond 90ºC vertical for 5 sec to drain all the extra liquid from the filter probes.
- Switch off the vacuum and place the tool in the parking position. All the residual liquids and solutions should be discarded.
- Heat the pyrosequencing plate in the 80ºC pre-heated thermal support for 2 minutes.
- Remove the plate from the plate holder and allow the samples to cool down at RT.

- **Processing a run on the pyrosequencer**
  - All the reactives including the pyrosequencing cartridge should be at RT.
  - Resuspend the enzyme and substrate mixtures need for the pyrosequencing reaction in 620 µl each of nuclease-free water and mix by swirling the vial gently.
  - Load the pyrosequencing cartridge with the volumes of nucleotides, enzyme, and substrate mixes determined by the pre-run information in the pyrosequencing program, avoiding air bubbles.

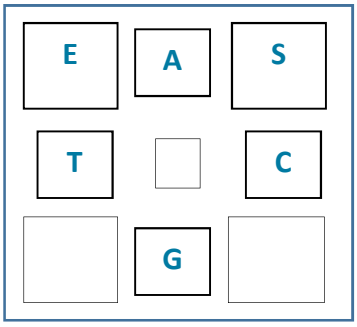


Figure 2. Illustration of the pyrosequencing cartridge. E: enzyme mixture ; S: substrate mixture; A, T, C, G: nucleotides.

  - Open the cartridge gate and insert the filled reagent cartridge with the label facing out, and close the gate.
  - Place the plate on the heating block inside the instrument and close the plate holding frame and the instrument lid.
  - Select the run file stored in the USB and start the run. The file contains the sequence to analyze and the dispensation order of the nucleotides, that is loaded automatically. The run will last 20 to 40 minutes, depending on the fragment size.
  - Once the run finsihes, open the instrument lid, open the cartridge gate and remove the reagent cartridge by lifting it up and pulling it out.
  - Open the plate holding frame and remove the plate from the heating block.
  - Close the lid, discard the plate and clean the reagent cartridge according to the instructions of the datasheet.

2.2. SOP for NGS analysis in tDNA/cfDNA-cfRNA

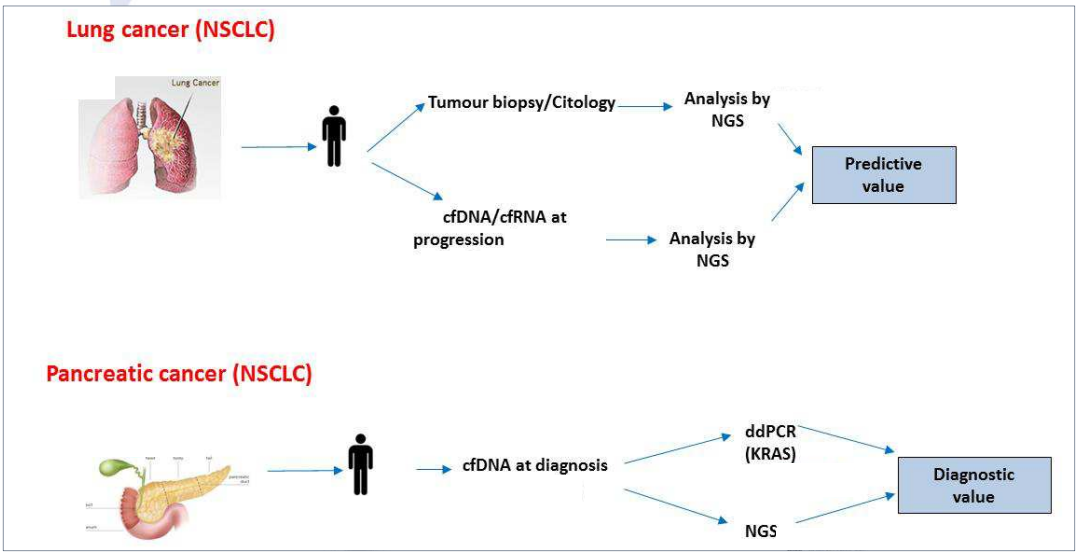


Figure 3. SOP for the analysis of genetic alterations in tumoral/circulating free DNA/RNA for the analysis of predictive and diagnostic value of new biomarkers

Figure 3. Workflow for NGS analysis in tDNA

2.2.1. Nucleic acid extraction for NGS analysis

- **tDNA and tRNA**

The extraction of DNA and RNA is performed simultaneously from the same FFPE sections, in order to use the same biological material for the analysis. It is essential to select appropiate tumoral area for NGS analysis. A percent of tumoral component (higher than 70%) is advisable.

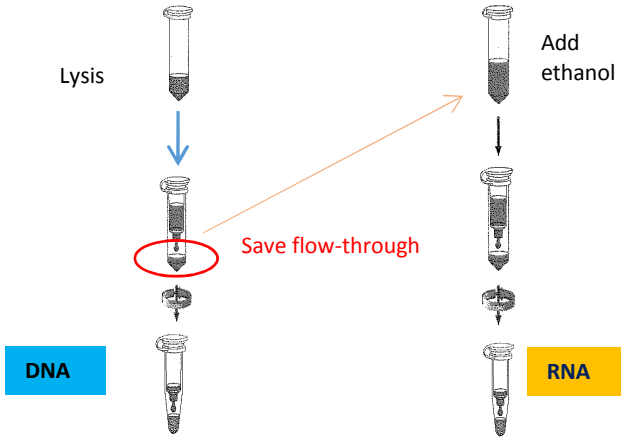


Figure 4. Graph showing the simultaneous DNA/RNA extraction.

RNA in histological FFPE samples typically is fragmented and chemically modified to a degree that renders it incompatible with many molecular analysis techniques. The use of a kit that optimizes the protease digestion conditions to release, a maximal amount of RNA fragments of all sizes in a short period of time is recommended.

DNA tends not to fragment as RNA, but the is quite dense and is much more reactive to formaldehyde. A long period of tissue digestion (overnight) is required to release substancial amounts of DNA from tissue samples.

The most critical issues for the concentration and quality of the DNA/RNA are the condition of tissue prior to fixation and embedding, the age of the sample and the process of fixation and embedding as well as storage of the sample.

The procedure of DNA/RNA extraction is performed in nine consecutive steps:

- **Deparaffinization**
  - Cut 5-20 µm sections from tissue blocks using a microtome and place the sections in a 1.5 ml microcentrifuge tube.
  - Add 1 ml 100% xylene to the samples, vortex briefly and spin down to ensure all the sample is in contact with the xylene.
  - Heat the sample for 3 min at 50ºC to completely remove paraffin.
  - Centrifuge the sample for 2 min at maximum speed and remove carefully the xylene without disturbing the pellet.
  - Wash the pellet twice with 1 ml 100% ethanol, vortexing briefly to mix.

- Briefly centrifuge at maximum speed and remove ethanol carefully without disturbing the pellet.
- Air dry the pellet for 15-45 min at RT.

- **Protease digestion**
  - Add digestion buffer (100 µl if sample size is ≤40 µm and 200 µl if the sample size is 40-80 µm) and 4 µl proteinase K per sample.
  - Swirl the tube gently to mix and briefly centrifuge to bring the tissue down into the solution.
  - For RNA isolation incubate the sample in a heat block for 15 minutes at 50ºC, then 15 minutes at 80ºC.
  - For DNA isolation incubate the sample for 16 h at 50ºC.

- **Nucleic Acid Isolation**
  - For DNA and RNA, combine the amounts of isolation additive and ethanol 100% according to the volume of Digestion buffer previously used and mix by pipetting up and down.

	Volume of digestion buffer	
	100 µl	200 µl
Isolation Additive	120 µl	240 µl
100% ethanol	275 µl	550 µl

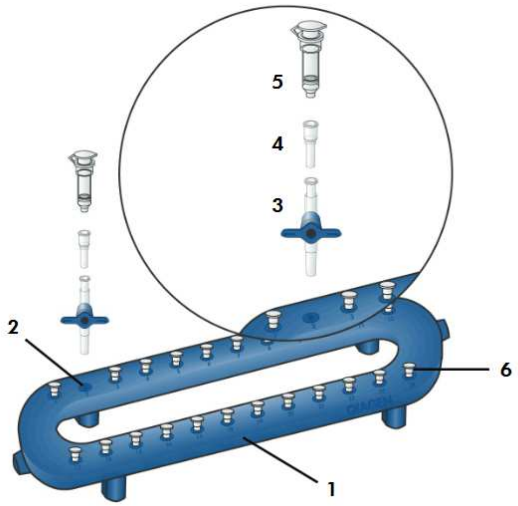
  - For each sample, place a filter cartridge in a collection tube supplied.
  - Pipet up to 700 µl of the sample- ethanol mixture onto the filter cartridge, close the lid and centrifuge at 10000 g for 30 seconds.
  - Discard the flow through and put the filter cartridge into the collection tube.
  - Add 700 µl of washing solution A to the filter and centrifuge at 10,000 g for 30 seconds and discard the flow through and put the Filter Cartridge into the collection tube.
  - Repeat the same step bellow with washing solution B.
  - Discard the flow through and put the Filter Cartridge into the collection tube.
  - Spin for an additional 30 seconds to remove residual fluid from the filter.

- **Nuclease digestion and final Nucleic Acid Purification :**
  - For RNA isolation add a mix of 6 µl 10x DNase buffer, 4 µl DNase and 50 µl of nuclease-free water to the center of the filter and incubate for 30 minutes at room temperature.
  - For DNA isolation add a mix of 10 µl RNase A 50 µl nuclease-free water to the center of the filter and incubate for 30 minutes at RT.
  - Add 700 µl of washing solution A to the filter and centrifuge at 10,000 g for 30 seconds and discard the flow-through and put the filter cartridge into the collection tube.
  - Repeat the same step bellow with washing solution B.
  - Transfer the Filter Cartridge to a new collection tube and apply 60 µl of Elution solution or Nuclease-free water at RT (for RNA elution) and preheated to 95ºC (for DNA elution) to the center of the filter.
  - Incubate the sample at RT for 1 minute and centrifuge at maximum speed for 1 minute to pass the elution with the RNA or DNA through the filter.
  - DNA and RNA quantification must be performed by a fluorimetric method (e.g. Qubit).

- **Circulating free nucleic acid extraction (cfDNA and cfRNA):** Plasma obtained in optimal conditions is the best eligible start material for cfDNA/cfRNA extraction.

- **Blood plasma extraction:**
  - 10 ml of total blood is obtained in a special collection tube (preferable)/ BD Vacutainer EDTA K2 tube for the obtention of 4 ml plasma.
  - Two consecutive rounds of centrifugation (2000 g, 10 minutes) are performed to obtain optimal plasma for the analysis. In the case of special collection tubes, time from blood drawing to centrifugation is not critical ; on the contrary, EDTA-based blood extractions must be processed in short periods of time (less than two hours).

- **cfDNA/cfRNA extraction:**
  - Lysis of the sample and binding of the nucleic acid fraction:
    - Before start, insert components into the vacuum station as follows:



1. Vacuum manifold
2. Slot
3. Valve
4. Connector
5. Purification mini-column
6. Tube extender

Figure 5. Vacuum manifold used for extraction (modified from manufacturer).

- Bring the volume of samples to 3 ml with PBS and add 300 µl Proteinase K into a 50 ml tube.
- Add 3 ml of plasma to the 50 ml tube, and then add 2.4 ml lysis buffer containing 1 µg carrier RNA to allow optimal binding of the circulating nucleic acids to the silica membrane.
- Mix by vortexing for 30 seconds and incubate immediately at 60ºC for 30 minutes.
- Add 5.4 ml buffer to the lysate and mix by vortexing for 15-30 seconds.
- Incubate for 5 minutes on ice and apply the lysate into the tube extender of the column
- Switch on the vacuum pump and leave it on until all the sample have passed through the column.
- Switch off the vacuum pump, remove and discard the tube extender.

- Washing:
  - Apply 600 µl wash buffer 1 to the column and switch on the vacuum pump until all the volume has passed through the filter.
  - Apply 750 µl 96-100% ethanol to the column and switch on the vacuum pump. When finished, switch off the vacuum pump.
  - Close the lid of the mini-column, remove from the vacuum support and discard the item 4.
  - Place the mini-column into a 2 ml collection tube and centrifuge at 20,000 g (14,000 rpm) for 3 minutes.
  - Place the mini-column into a new 2 ml collection tube and incubate at 56ºC for 10 min to dry the membrane.
  - Place the mini-column into a new 1.5 ml elution tube and apply 50 µl elution buffer to the center of the column. Close the lid and incubate at RT for 3 min.
  - Centrifuge at 20,000 g (14,000 rpm) for 1 min to elute the nucleic acids.
  - Quantification of circulating free nucleic acids is performed by flourimetric detection. It is expected that 5-50 ng cfDNA are obtained from a 10 ml blood extraction.

2.2.2. NGS. Library preparation (Tumour DNA)

Two libraries, one based on DNA and the other one based on RNA must be generated in order to analyze all the molecular alterations.

The ideal number of samples to analyze are multiples of 8 in order to optimise all the disposable material and gene panels.

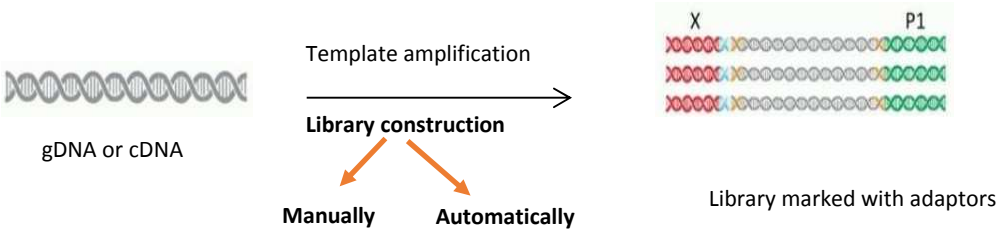


Figure 6. Workflow for library preparation

The operation procedure consists of:

- **Retrotranscription from RNA to cDNA:**

  The quantity of RNA for reverse transcription PCR requires 10 ng of DNase-treated total RNA. Increasing this quantity will usually result in higher quality libraries.

  - For each sample, add the following components into a 96-well PCR plate:

Component	Volume
5x RT reaction mix	2 µl
10 x enzyme mix	1 µl
Total RNA (10 ng)*	≤ 7 µl
Nuclease-free water	Up to 10 µl

*Substitute an equal volume of nuclease-free water or low TE to prepare a no-template control (NTC)

- Seal the plate with an adhesive film, vortex, centrifuge at 100g for 30 sec and load the plate in the thermal cycler to synthesize cDNA under the following conditions:

Temperature	Time
42ºC	30 min
85ºC	5 min
10ºC	∞

- **Set up of the cDNA target amplification reactions:**

For each target amplification reaction, use 300–30000 copies of DNA (10 ng gDNA) from FFPE tissue. Increasing the amount of DNA results in higher-quality libraries, especially when DNA quality or quantity is unknown.

- For each sample, add the following components into a 96-well PCR plate placed on a pre-chilled block:

Component	Volume
5x reaction mix*	4 µl
5x cDNA panel assay*	2 µl
cDNA synthesis reaction**	10 µl
Nuclease-free water	4 µl

*Pipet slowly, mix thoroughly and centrifuge briefly to collect.
**Centrifuge the samples at 100 g for 30 seconds before pipetting.

- Mix the total volume up and down at least 5 times before sealing the plate.
- Place a compression pad, load the plate in the thermal cycler and run the following program to amplify the target regions:

Stage	Step	Temperature	Time
1 cycle	Enzyme activation	98ºC	2 min
Set number of cycles*	Denature	98ºC	15 sec
	Anneal and extend	60ºC	4 min
1 cycle	---------	10ºC	Hold

*The recommended cycle number is based on 10 ng RNA input. Adjust the cycle number according to the following table

Input nucleic acid	Cycle number (10 ng)	Cycle number adjustement	
		1 ng RNA input	100 ng RNA input
FFPE RNA	30	+3	-3

- **Set up of the DNA target amplification reactions**

  - For each sample, add the following components to each cDNA synthesis reaction:

Component	*Volume*
5x reaction mix	4 µl
5x DNA panel assay	4 µl
DNA (10 ng)*	≤ 12 µl
Nuclease-free water	Up to 20 µl

*Substitute an equal volume of nuclease-free water or low TE to prepare a no-template control (NTC).

  - Mix the total volume up and down at least 5 times before sealing the plate.
  - Place a compression pad, load the plate in the thermal cycler and run the following program to amplify the target regions:

Stage	*Step*	*Temperature*	*Time*
1 cycle	Activate enzyme	99ºC	2 min
Set number of cycles*	Denature	99ºC	15 sec
	Anneal and extend	60ºC	4 min
		4ºC	Hold

The recommended cycle number is based on 10 ng DNA input. Adjust the cycle number according to the following table:

Input nucleic acid	*Cycle number (10 ng)*	*Cycle number adjustement*	
		1 ng DNA input	100 ng DNA input
FFPE DNA	20	+3	-3

- **Partial digestion of the amplicons**
  - Transfer the amplicons from the DNA plate to the RNA/cDNA plate.
  - Add 2 µl of enzymatic reagent to each amplified sample.
  - Mix the total volume up and down at least five times before sealing the plate with an adhesive film.
  - Place a pad, load the plate in the thermal cycler and then run the following program:

Temperature	*Time*
50ºC	10 min
55ºC	10 min
60ºC	20 min
10ºC	For up to 1 hour

- **Ligation of the barcode adapters**

When sequencing multiple libraries on a single chip, different barcode adapters must be ligated to each library. It is important to note that DNA and RNA libraries from the same sample also require different barcodes and also handling barcode adapters should be careful to avoid cross-contamination. The mixture for ligation of the adapters include:

Component	Volume
Switch solution	4 µl
Barcode adapters	2 µl
DNA ligase	2 µl
Total volume (including 22 µl of digested amplicons)	30 µl

- Mix the total volume up and down at least 5 times before sealing the plate.
- Place a pad, load the plate in the thermal cycler and then run the following program:

Temperature	Time
22ºC	30 min
68ºC	5 min
72ºC	5 min
10ºC	For up to 24 hours

- **Purification of the barcoded libraries and elution**
  - Centrifuge the plate to collect the contents in the bottom, carefully remove the plate seal and add 45 µl of magnetic beads to each library. It is important that the stock of beads are well dispersed after vortex thoroughly and also to pipet the solution slowly.
  - Pipet up and down 5 times to mix the bead suspension with the DNA and incubate for 5 min at RT.
  - Place the plate in a magnetic rack and incubate for 2 min or until the solution clears.
  - Carefully discard the supernatant without disturbing the pellet.
  - Remove the plate from the magnet and add 150 µl of freshly prepared 70% ethanol. Gently pipet up and down five times for washing.
  - Return the plate to the magnet and incubate for 2 min or until the solution clears.
  - Carefully discard the supernatant and repeat a second wash.
  - Ensure ethanol is completely removed because ethanol drops inhibit library amplification.
  - Keep the plate in the magnet and air-dry the beads at RT for 2-5 min, avoidiing them to overdry.
  - Remove the plate from the magnet and add 50 µl of low Tris:EDTA to dilute the pellet
  - Mix by pipetting the elution up and down at least 5 times and incubate for 2 min at RT.
  - Place the plate to the magnet at least for 2 min or until the solution clears.
  - Transfer the elution (supernatant) to a 1.5 ml eppendorf for storage.
  - Libraries can be stored at 4-8ºC for up to 1 month, and at -20ºC for longer periods.

- **Equalization of the barcoded libraries:** This procedure is performed to mix all the libraries at the same concentration:

Component	Volume
Primers	40 µl
High Fidelity mix	10 µl
DNA (10 ng)*	10 µl
Nuclease- free water	40 µl

  - Add 52 ul of the mixture to the beads put into each well, resuspend and introcue it into the magnet.
  - Retire 50 ul, put them into other wells and proceed with the amplification to equalize*.

- After the amplification in the thermocycler add 10 ul of capture solution, incubate 5 minutes at RT
- Add 6 µl beads and resuspend and incubate the mixture 5 minutes at RT.
- Put the mixture in the magnet 3 minutes and take the supernatant into a new tube.
- Wash twice with 150 ul wash solution, add 100 ul elution solution and resuspend the spheres.
- Put the plate into the thermocycler and incubate it at 35ºC for 5 minutes.
- Put the plate into the magnet for 3 minutes and remove the supernatant where DNA will be eluted.
- Proceed with the immobilization in solid phase.

  *During the amplification prepare the beads :

  - Mix 3 µl spheres/reaction and 6 ul wash solution Agitate for 5 seconds, centrifugue to obtain all the spheres, and put the tube into the magnet for a minute.
  - Remove the the supernatant, and add the same volume of wash solution added in the previous step.

- **Combination and quantification of the equalized libraries**

The concentration of the libraries must be performed by a fluorometer with a dsDNA Assay Kit with high sensitivity.

- Measure the concentration of 10 µL of each amplified library on the Qubit™ Fluorometer.
- Amplified libraries typically have concentrations of 300-1500 ng/ml, althoguh libraries below 300 ng/ml can still provide good quality sequences.

When analysing genomic DNA and RNA alterations the libraries that are prepared from the same sample must be combined in such a way different read depths for the paired DNA and RNA libraries can be produced. The ratio 80:20 (DNA:RNA) of the paired libraries (100 pM) to be sequenced on the same chip.is the eligible one.

The steps from partial digestion of the amplicons to equalization of the libraries should be performed by experienced personnel in order to to avoid variability in the concentration of the prepared DNA and RNA libraries. If that is not the case, **it is advisable to automatise these steps.**

2.2.3. NGS. Library preparation (cfDNA/cfRNA)

The protocol for NGS analysis in free nucleic acids is very similar to the one described for tumoral DNA (see above). In the next steps the main differences of the NGS procedure are enumerated:

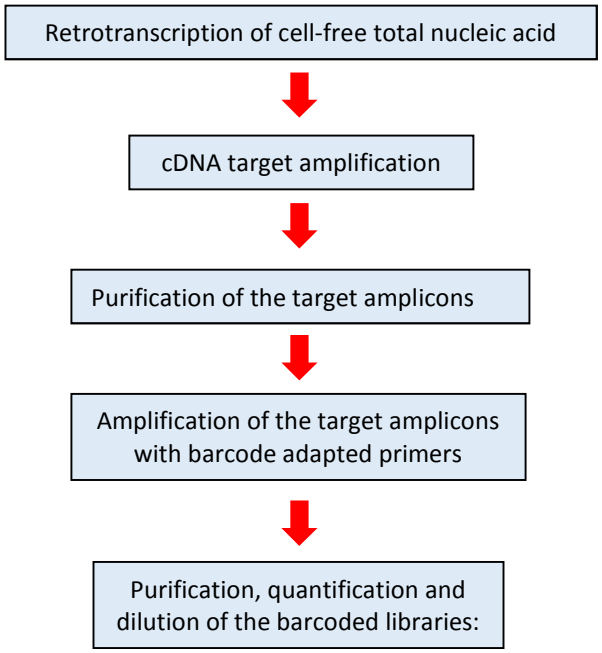


Figure 7. Workflow for NGS analysis in cfDNA/cfRNA

- **cfDNA input for the NGS technique**: 20 ng of cfDNA enables the detection of rare variants present at 0.1%, being advisable to load a maximum of 50 ng of cfDNA.

- **Retrotranscription from cell-free total nucleic acid to cDNA:** For each sample, add the following components into a 96-well PCR plate:

Component	*Volume*
5x RT reaction mix, including enzyme	2.6 µl
Cell free total nucleic acid (20 ng)*	≤ 10.4 µl
Nuclease-free water	Up to 13 µl

*Substitute an equal volume of nuclease-free water or low TE to prepare a no-template control (NTC)

- **cDNA target amplification reactions**

Component	*Volume*
5x reaction mix*	15 µl
5x cDNA panel assay*	2 µl
cDNA synthesis reaction**	13 µl

*Pipet slowly, mix thoroughly and centrifuge briefly to collect.
**Centrifuge the samples at 100 g for 30 seconds before pipetting.

Stage	*Step*	*Temperature*	*Time*
1 cycle	Activate enzyme	98ºC	1 min
2 cycles	Denature	98ºC	15 sec
	Anneal	64ºC	2 min
	Anneal	62ºC	2 min
	Anneal	60ºC	4 min
	Anneal	58ºC	2 min
	Extension	72ºC	2 min
1 cycle	Final extension	72ºC	2 min
	Store	4ºC	Up to 1 hour

- **Partial digestion of the amplicons:** This step is not performed.

- **Purification of the amplicons:** A new step of purification is performed before barcoding.
  - Centrifuge the plate to collect the contents in the bottom and carefully remove the plate seal.
  - Measure carefully the volume of reaction mixture and add nuclease-free water to equalize all the volumes to 30 ul.
  - Add 45 µl of magnetic beads to each library. It is important that the stock of beads are well dispersed after vortex thoroughly and also to pipet the solution slowly, as in the case of NGS for tDNA.
  - Vortex 15 seconds and then incubate for 5 minutes al RT.

  The rest of the procedure is identical to except fo :
  - The use of 80% ethanol instead of 70% and incubate at RT 30 seconds. Repeat twice, and stand the mixture in the magnet.
  - The addition of 24 ul of low TE buffer to the pellet.

- **Amplification of the target amplicons with barcode primers:**

Component	*Volume*
Purified cDNA obtained from previous section	23 µl
Tag sequencing	1 µl
cfDNA library primer P1	1 µl
cfDNA library PCR master mix	25 µl
Total volume (including 22 µl of digested amplicons)	30 µl

Stage	Step	Temperature	Time
1 cycle	Enzyme activation	98ºC	1 min
18 cycles	Denaturation	98ºC	15 sec
	Annealing	64ºC	15 sec
	Extension	72ºC	15 sec
1 cycle	Final extension	72ºC	5 min
		4ºC	Up to 24 h

- **Purification, quantification and dilution of the barcoded libraries:**

  - Add 57.5 µl of magnetic beads to each library instead of 45 µl.
  - It is essential to quantify the library in triplicates by quantitative PCR (Q-PCR) with a commercial quantitation kit. For this procedure five standards by 10-fold serial dilutions of the E. coli DH10B Control library must be prepared.
  - Two dilutions of each sample library must be prepared (1 :100 and 1 :1000 from the first one by combining library with nuclease-free water, and vortex to mix.
  - Add per sample 10 µl of 2x Master Mix to 1 µl 20x control assay.
  - Mix 11 µl pf Master Mix to 9 µl of 1 :1000 dilution in a 96-well plate:

Stage	Temperature	Time
1 cycle	50ºC	2 min
	95ºC	20 sec
18 cycles	95ºC	1 sec
	60ºC	20 sec
1 cycle	72ºC	5 min
Hold	4ºC	Up to 24 h

  - After Q-PCR calculate the concentration of the undiluted library by multiplying the determined concetration *1000.

2.2.4. NGS. Templating and chip load (Tumoral DNA and cfDNA/cfRNA)

At this point it is essential to set the conditions for templating and sequencing.

- The kit used and the parameters of analysis, samples, chips, and reagents throughout the workflow.
- Sample information (type of sample, tumor location, percentage of tumoral cellularity, sex of the patient).
- Barcodes used for the sample.
- Number of flows/run (number of injections of nucleotides and mix reaction).
- Reference files for the sequence analysis. These are:
  - DNA Reference Library: hg19(Human (hg19)).
  - DNA Target Regions: DNA.designed.bed (Tumour DNA)/ DNA.designed.bed (liquid biopsy), provided by the manufacturer.
  - DNA Hotspot Regions: RNA. designed.bed / RNA.designed.bed (liquid biopsy), provided by the manufacturer.

Depending on the platform used, clusters generation is performed externally to the sequencer. The procedure is done by clonal amplification based on emulsion PCR .
Templating consists of several steps:

- Preparation of the emulsion PCR by mixing oil with enzyme, nucleotides, primers, polymerase and $MgCl_2$.
- Do the amplification in the amplification cell of the euipment.
- Washing and recuperation of the sample (white balls) after the amplification by emulsion.
- Enrichment of the emulsion beads by amplification (**Figure 9**).

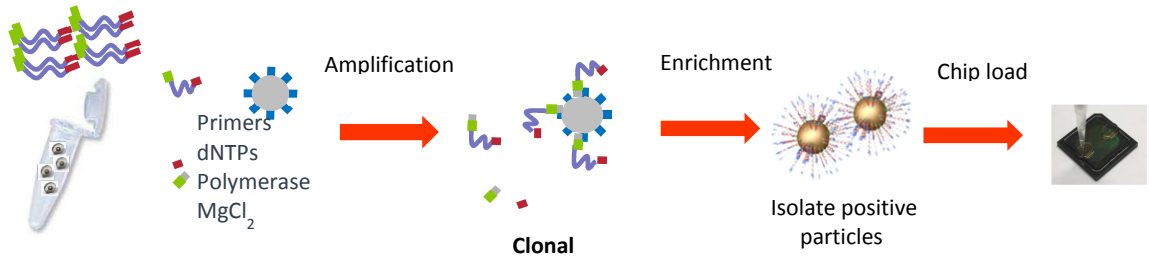


Figure 8. Workflow for DNA templating and chip load

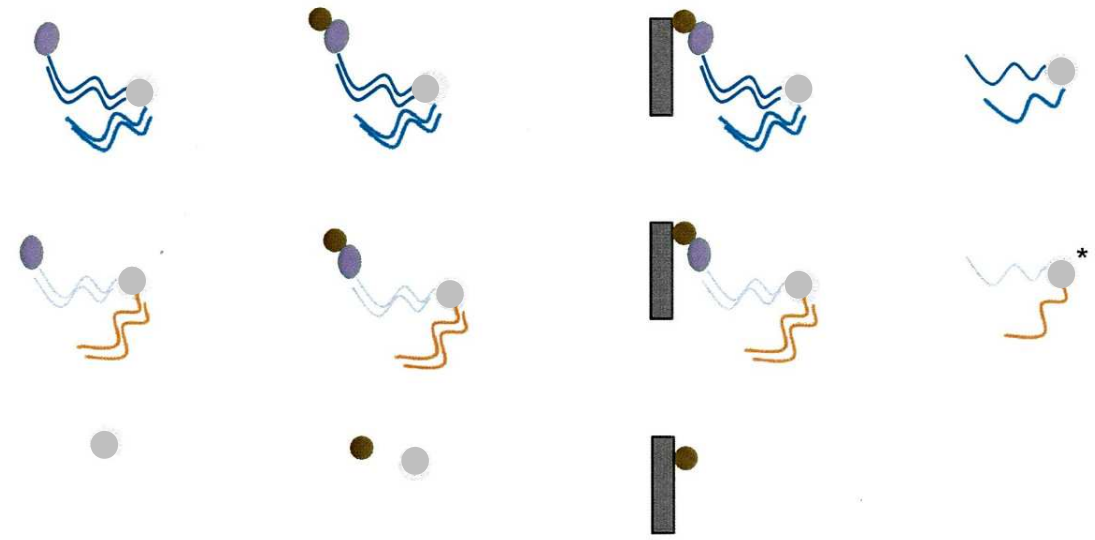


Figure 9. Workflow for enrichment

Manual chip loading must be very careful with micropippette, avoiding bubbles.
Sequencing is performed when the chip is loaded.

2.2.5. Bioinformatic analysis for the study of genetic alterations (Tumoral DNA and cfDNA/cfRNA)

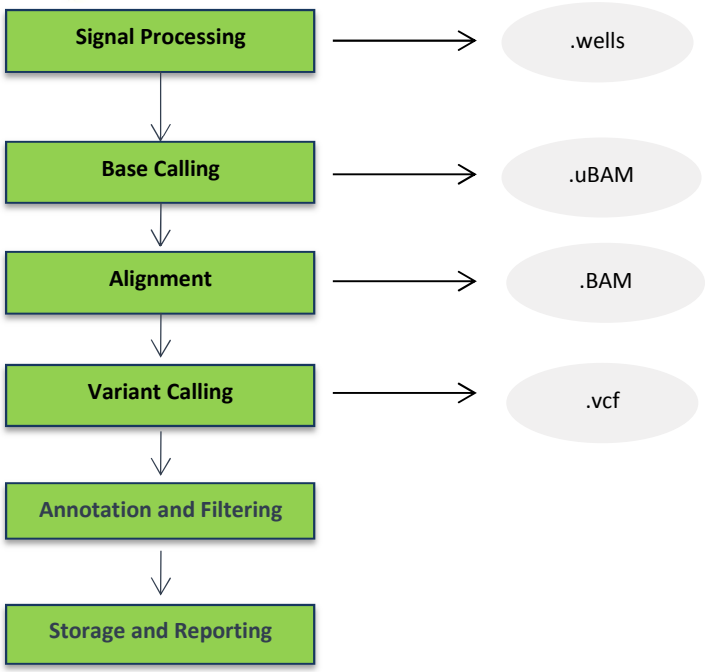


Figure 10. Workflow for bioinformatic analysis

- Check the run quality:
  - Total bases and total reads
  - Mean and median read Length>60 bp
  - Mapped reads
  - On target (approx. 100%)
  - Mean depth ,that describes the number of times that a given nucleotide in the genome has been read.
  - Average number of reads that align to, or "cover," known reference bases
  - Uniformity (approx. 100%)

- Filter the candidates (single nucleotide variants-SNVs/smaill deletions and insertions-Indels, copy number variations-CNVs, based on key parameters, mainly:
  - Allele frequency.
  - Quality.
  - Coverage (it should be >500).
  - Strand bias.
  - Signal shift.
  - Base quality.

- Find all positions with evidence for a variant, focus on these positions and evaluate if there is enough evidence for this variant.