Instructions for use

Targeted Liquid Biopsy OncoKit

Ref. IMG-415

RUO

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This product is designed for use in research. Health in Code provides no guarantee, whether explicit or implicit, that extends beyond the proper functioning of the components of this kit. Health in Code's sole obligation, in relation to the aforementioned guarantees, shall be to either replace the product or reimburse the cost of it, per the client's preference, provided that materials or workmanship prove to be defective.

Health in Code is not liable for any cost or expense, direct or indirect, or damage or harm incurred by the customer or user as a result of use of the product by the buyer or user.

All Health in Code products undergo strict quality control. Targeted Liquid Biopsy OncoKit has passed all internal validation tests, thus guaranteeing the reliability and reproducibility of each assay.

If you have any questions about the use of this product or its protocols, feel free to contact our Technical Department:

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Modifications to the Instructions for Use (IFU)		
Version 02	OCT 2022	Update of sections 3, 5 and 10. Update of quality QC of pre-capture and post-capture libreries (sections 7.6.1 and 7.6.2)
Version 01	JUN 2022	Document creation

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01 General information

Liquid biopsy consists in obtaining circulating tumor cells (CTCs) or free circulating DNA from tumor cells from non-solid tissue matrices, such as blood, cerebrospinal fluid, etc.

Circulating tumor DNA generally accounts for 0.01 to 10% of free circulating DNA; therefore, its detection requires a high analytical capacity and a high sensitivity, which calls for technologies that can provide the necessary accuracy and sensitivity.

Despite the development of numerous technologies to detect biomarkers in peripheral blood using different platforms, the lack of standardization and the variability among methodologies to assess liquid biopsy are limiting factors for the integration of these technologies in clinical settings.

Nevertheless, the clinical efficiency of free circulating DNA testing has already been demonstrated for different tumors, including lung, colorectal, prostate, melanoma, breast, and pancreatic cancer, among others.

References

- *Cescon DW, Bratman S, Chan SM et al. Circulating tumor DNA and liquid biopsy in oncology. Nat Cancer. 2020; 1(3): 276–290. doi:10.1038/s43018-020-0043-5*
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- *Zhang YC, Zhou Q, Wu YL. The emerging roles of NGS-based liquid biopsy in non-small cell lung cancer. J Hematol Oncol. 2017;10(1):167. Published 2017 Oct 23. doi:10.1186/s13045-017-0536-6*
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02 Intended use

Targeted Liquid Biopsy OncoKit has been designed for full automated testing of the blood fraction containing free circulating tumor DNA (ctDNA) in patients affected by lung cancer.

Massive sequencing (NGS) allows detecting the main mutations associated with factors predictive of response to treatment and is intended for individualized monitoring, as well as to early detection of resistance and early diagnosis of relapse. The design of Targeted Liquid Biopsy OncoKit allows testing for the main biomarkers associated with sensitivity to targeted therapy and for those that can appear as resistance mechanisms or as a result of clonal evolution itself, through a single test performed on at least 10 ng ctDNA. This information is of great help both in selecting the most suitable treatment for each diagnosis and in detecting relapses. Likewise, since ctDNA is present already at early stages of the disease and the panel includes the main driver biomarkers, the panel is useful for the early detection of lung cancer.

For this purpose, Targeted Liquid Biopsy OncoKit integrates:

- Sequencing of whole exonic regions of **13 genes**:

  ALK, BRAF, EGFR, ERBB2/Her2, KIT, KRAS, MAP2K1, MET, NRAS, PIK3CA, RB1, ROS1, TP53

- Sequencing of ***hotspot*** regions of ***NTRK1*** (F589L, G595R, G667C/S and G608D) and ***NTRK3*** (G623R and G696A).

- Capture of **8 fusion genes de fusión** with all possible rearrangements covered.

  To this end, the Targeted Liquid Biopsy OncoKit panel includes those intronic regions where breakpoints have commonly been identified in the literature. The covered genes and regions are the following: ***ALK*** (introns 18 and 19), ***EGFR*** (introns 24, 25, and 26), ***ETV6-NTRK3*** (introns 4 and 5), ***NRG1*** (introns 1, 4, and 5), ***NTRK1*** (introns 9, 10, 11, 12, and 13), ***NTRK2*** (intron 15), ***RET*** (introns 9, 10, and 11) and ***ROS1*** (introns 31, 32, 33, 34, and 35).

- Detection of copy number variations (**CNVs**) in the whole genome, allowing for the detection of alterations in genes, chromosome arms, or whole chromosomes. Moreover, this analysis has been improved using a low-density SNP array through the capture SNPs distributed throughout the whole genome. This allows both validating the obtained results and detecting alterations where loss of heterozygosity has occurred but copy number has been neutralized by a duplication (**Copy-neutral LOH**).

Thus, Targeted Liquid Biopsy OncoKit allows analyzing lung cancer clinical samples via a protocol that integrates the high sensitivity capture of the regions of interest with hybridization probes, with molecular ctDNA barcoding technique with a unique adaptor and later high-throughput sequencing (NGS). This type of protocol allows removing optical (sequencing) and PCR duplicates during bioinformatics analysis without removing duplicates from different DNA molecules. Results obtained with this type of analysis significantly improve sensitivity, therefore improving variant detection in low-quality DNA samples.

03 Technical characteristics

Targeted Liquid Biopsy OncoKit has been validated in the Illumina's NextSeq 500/550Dx System platform via the analysis of DNA reference samples from the Coriell Institute and Horizon Dx, and from patient samples that have been previously genotyped with other technologies. In said validation, the specific detection of the variants present in the selected genes (see above) has been verified, and so were the repeatability and reproducibility of the technique.

The protocol includes molecular barcoding through the addition of **unique molecular identifiers (UMIs)**. It consists of short sequences, or "barcodes," that are added to each fragment during the preparation of the libraries. These UMIs are used in the bioinformatics analysis phase and their main functions are:

- The **reduction of sequencing errors** introduced by the polymerase during the amplification phase.
- The **usability of all existing reads**, eliminating the concept of PCR duplicates and, therefore, improving the sensitivity of the bioinformatics analysis.

Technical specifications:

- Sample type: ctDNA from peripheral blood and pleural fluid
- Necessary amount of DNA: 10-50 ng.
- Limits of detection:
  - The detection limit of the analysis of structural and point variants, small insertions, and deletions is 1%.
  - The limit of detection for CNV analysis is 2.9 total copies for gains, i.e. 4 copies at 50% tumor representation.
- Mean coverage: 7300X.
- Mean coverage after UMI analysis: 2500X.
- Coverage: 96.4% of bases covered at 100X depth.
- Uniformity: 96.8% of bases covered at >20% of mean coverage.
- Specificity: >99%.
- Sensitivity: >99%.
- Repeatability: >99%.
- Reproducibility: >97%.

This product is compliant with the quality specifications of the ISO 9001 standards both regarding the manufacturing and validation process and the materials used for its production.

04 Safety warnings and precautions

- It is recommended to strictly follow the instructions in this manual, especially regarding the handling and storage conditions of the reagents.
- Do not mouth-pipette.
- Do not smoke, eat, drink, or apply cosmetics in areas where kits and samples are handled.
- Any cuts, abrasions, and other skin injuries must be properly protected.
- Do not pour the remains of reagents down the drain. It is recommended to use waste containers established by the legal norm and manage their treatment through an authorized waste management facility.
- In the event of an accidental spill of any of the reagents, avoid contact with the skin, eyes, and mucous membranes and rinse with a large amount of water.
- Safety data-sheets (MSDS) of all dangerous substances contained in this kit are available on request.
- This product requires the manipulation of samples and materials of human origin. It is recommended to consider all materials of human origin as potentially infectious and manipulate them according to level 2 of the OSHA norm on biosafety and bloodborne pathogens or other practices related to biosafety of materials that contain or are suspected to contain infectious agents.
- This kit has been validated with specific equipment and under specific conditions that may vary widely among laboratories. Therefore, it is recommended that each laboratory verify compliance with the technical specifications of the manufacturer when the kit is to be used for the first time.
- The manufacturer assumes no responsibility for any damage or failure of the assay caused by substituting reagents included in the kit for ones not provided by Health in Code.
- The manufacturer does not guarantee the assay's reproducibility when the user uses reagents that have not been validated by Health in Code but are considered by the user equivalent to those provided in the kit.
- The manufacturer is not liable for the obtained results when the bioinformatics analysis is carried out on an analysis platform different from Data Genomics.

05 Content and storage conditions of the kit

This kit contains sufficient reagents for the preparation of 24 libraries. The reagents included in this kit are as follows:

- **Elution Buffer**: Buffer to elute DNA.
- **Reagent Plate**: Plate containing all the necessary reagents for DNA end repair reactions and Illumina adaptor binding, as well as for the amplifications within the library preparation protocol.
- **Beads and Buffers Plate**: Plate containing the required magnetic particles and wash buffers to perform the necessary capture and purification within the library preparation protocol.
- **Index Strip**: Oligonucleotides with a unique 8-nucleotide sequence compatible with Illumina adaptors. They are necessary to label libraries for each sample, leading to a unique combination that will allow for their analysis after sequencing. The kit includes 24 different indexes distributed in single-use strips.
- **Targeted Liquid Biopsy Probe Strips**: Synthetic biotinylated oligonucleotides complementary to the kit's target regions which allow for the hybridization with said regions and which are later captured via streptavidin magnetic particles due to the biological property of bonding between biotin and streptavidin molecules.
- **Input Strips**: 8-well strips for DNA of the samples.
- **Magnis Library Output Strips, QC Strips, and Foil Seals**: 8-well strips to collect the generated libraries, strips to collect the pre-capture libraries, which can be used for an optional quality check, and seals for the well strips included in the kit.
- **Magnis 96-Well PCR Plate**: Plate for amplification reactions.
- **Magnis Deep-Well HSM Plate**: Plate for the capture and purifications necessary for the library preparation protocol.
- **Magnis Thermal Cycler Seal**: Seal for the 96-well plate.
- **Magnis Tip Waste Bin**: Container for tip waste created during the protocol.

The components of the kit are listed below:

Box 1 of 4			
Reagents	Color indicator	Quantity	Conservation
Sample input strips	Red	3 strips	15–25°C
Beads / buffer plates	White	3 plates	4°C
Elution Buffer	Green disc	2 x 1 mL	4°C

Table 1. Reagents in box 1 of Targeted Liquid Biopsy OncoKit

Box 2 of 4			
Reagents	Color indicator	Quantity	Conservation
Reagents Plate	Black/White	3 plates	-20°C
Index strip*	Black	3 strips	-20°C

Table 2. Reagents in box 2 of Targeted Liquid Biopsy OncoKit

NOTE: Each kit includes three of four possible index combinations: A1, A2, A3, and A4.

Box 3 of 4			
Reagents	Color indicator	Quantity	Conservation
Targeted Liquid Biopsy Probes strips	White	3 strips	-80°C

Table 3. Reagents in box 3 of Targeted Liquid Biopsy OncoKit

Box 4 of 4			
Reagents	Color indicator	Quantity	Conservation
Magnis Library Output Strips	Green	1 strip	15–25°C
QC Strips	Blue	1 strip	15–25°C
Foil Seals	-	5	15–25°C
Magnis 96-Well PCR Plate	Transparent	1 plate	15–25°C
Magnis Deep-Well HSM Plate	White	1 plate	15–25°C
Magnis Thermal Cycler Seal	-	1	15–25°C

Table 4. Reagents in "Magnis Empty Consumables" box; box 4 of 4

NOTE: Each kit contains three "Magnis Empty Consumables" boxes, one for each run of 8 samples on the Magnis instrument.

06 Equipment, reagents and material not included in the kit

Equipment:

- 10 µL, 20 µL, 200 µL, and 1000 µL micropipettes
- Vortex mixer (compatible with 1.5 mL tubes; with adjustable speed from 300 to 3,000 rpm)
- Centrifuge (compatible with 1.5 mL tubes and 0.2 mL strips; with adjustable speed of at least 1,000 rpm)
- Centrifuge compatible with 96-well plates
- Fluorometer (recommended: Qubit; ThermoFisher)
- Fragment analyzer (optional: TapeStation System by Agilent Technologies; LabChip GX Touch/GXII Touch by PerkinElmer)
- Automated library preparation system Magnis NGS Prep System by Agilent Technologies (cat. no. G9710AA)
- Illumina sequencer (recommended: NextSeq)
- Maxwell RSC DNA Extraction System

Reagents:

- Extraction kit: Maxwell RSC ccfDNA LV Plasma Kit (cat. no. AS1840; Promega)
- Nuclease-free water
- Fluorometer reagents. Recommended: Qubit dsDNA BR Assay Kit (cat. no. Q32853; Invitrogen), Qubit dsDNA HS Assay kit (cat. no. Q32854; Invitrogen)
- NaOH 0.2N (cat.no. 1091401000; Fluka)
- TRIS-HCl 200 mM pH 7
- PhiX Control v3 (cat. no. FC-110-3001; Illumina)
- Fragment analyzer reagents. Optional:
    - TapeStation D1000 Reagents (cat. no. 5067-5583; Agilent), High Sensitivity D1000 Reagents (cat. no. 5067-5585; Agilent), Cell-free DNA Reagent (cat. no. 5067-5631) and Genomic DNA Reagents (cat. no. 5067-5366).

NOTE: This kit does not include the reagents necessary for NGS sequencing.

Materials:

- Filter pipette tips (10 µL, 20 µL, 200 µL, and 1000 µL)
- Sonicator consumables. Recommended: microTUBE AFA Fiber Pre-Slit tubes (cat. no. 520045; Covaris)
- Sterile filter tips compatible with Magnis NGS Prep System (Ref: 19477-022; Agilent)
- Sterile 1.5 ml tubes

- Latex gloves
- Fluorometer consumables Recommended: Qubit™ assay tubes (Ref: Q32856; Invitrogen)
- Fragment analyzer consumables. Optional:
    - TapeStation D1000 ScreenTape (cat. no. 5067-5582; Agilent), High Sensitivity D1000 ScreenTape (cat. no. 5067-5584; Agilent), Cell-free DNA ScreenTape (cat. no. 5067-5630) and Genomic DNA ScreenTape (cat. no. 5067-5365).

NOTE

Targeted Liquid Biopsy OncoKit is prepared to be used in combination with the *Imegen-gDNA Reference Samples* (REF: IMG-368) kit, which includes good-quality germline DNA, both male and female, used for normalization necessary for the CNV analysis

07 Assay protocol

The reagents included in Targeted Liquid Biopsy OncoKit, which are to be used by the Magnis NGS Prep System, come pre-dosed for 24 libraries, used in 3 assays 8 libraries each, thus optimizing the performance of the equipment.

For correct coverage normalization, which is necessary for the CNV analysis (see section 8 of this document), it is necessary to have the reference germline fastq libraries of the same sex as the test sample. Due to the intrinsic variability of the technique observed in different laboratories, it is necessary that the germline reference samples be processed in the same laboratory, with the same equipment, and following the same protocol as the test samples. To generate fastq files of germline reference samples of both sexes available, Health in Code provides the user with the IMG-368 kit, which contains one female and one male sample to be processed with Targeted Liquid Biopsy OncoKit.

The steps necessary to carry out the preparation of 8 libraries using Targeted Liquid Biopsy OncoKit are outlined below.

07.1 | Preparation of the Magnis system for the execution of a protocol

01 Ensure that there are no materials from previous runs on the instrument deck, as they could interfere with the setup and launch processes.

02 Close the instrument door.

03 Turn on by pressing the power button on the front of the instrument (LED indicator lights will turn on). Wait while the system launches. This may take a few minutes.

04 UV decontamination is recommended prior to each run. In order to do so:

- ◊ On the *Home* screen, press *Decontamination*.

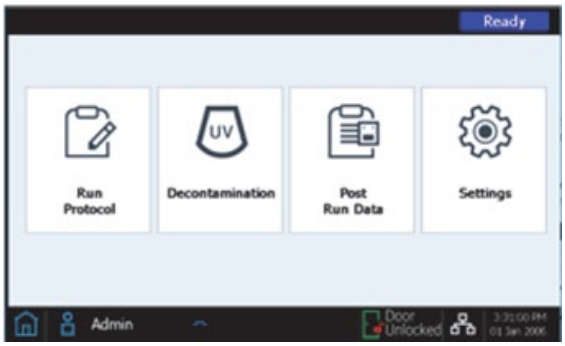


Figure 1. Magnis NGS Prep system Home screen

- ◊ On the Decontamination screen, press *Quick cycle*, then press *Start* (LED indicators will turn off during UV decontamination so that UV light can be emitted).

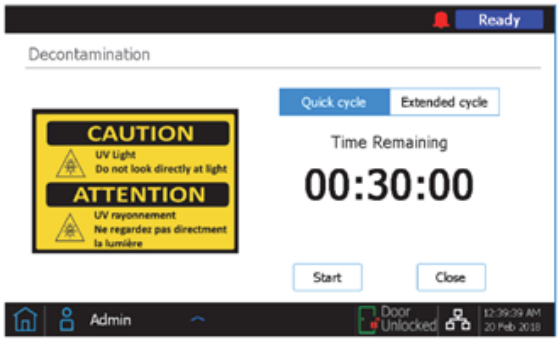


Figure 2. Magnis NGS Prep Decontamination screen

> ↘ Warning:
>
> Do not look directly at the UV light while decontamination is in progress.

NOTE: During the 30-minute decontamination process, proceed with the protocol.

05 Once the decontamination cycle is complete (LED indicators glow blue), press *Close* to return to the *Home* screen.

07.2 | Preparation of the DNA template strand

The steps necessary for the preparation of eight samples by Targeted Liquid Biopsy OncoKit are outlined below.

All reagents and consumables for DNA preparation, dilution, and fragmentation should be stored and used in areas separate from where polymerase chain reaction procedures are performed.

01 Thaw the extracted DNA samples at room temperature.

02 Shaking and quantifying DNA samples:

- For the quantification of ctDNA, the use of the commercial "Cell-free DNA" fragment analysis kit by Agilent Technologies is recommended. In this way, by measuring only DNA with a size range between 100 and 300 bp, only the ctDNA that will become the template, and not the co-extracted gDNA, will be quantified during the following steps of the protocol.

> ↘ NOTE: DNA samples where free circulating DNA does not constitute at least 50% of the extraction and where its total amount is smaller than 10 ng per 50 µl can lead to decreased test specificity and sensitivity.

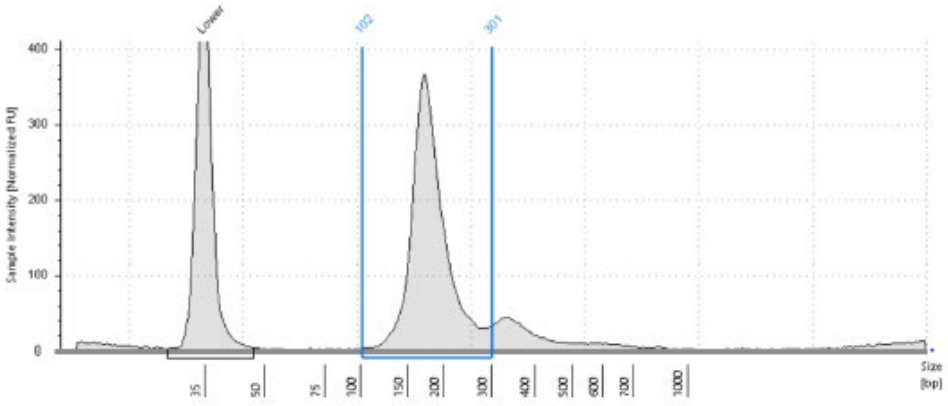


Figure 3. Result of fragment analysis of an extraction with 82% of circulating free DNA

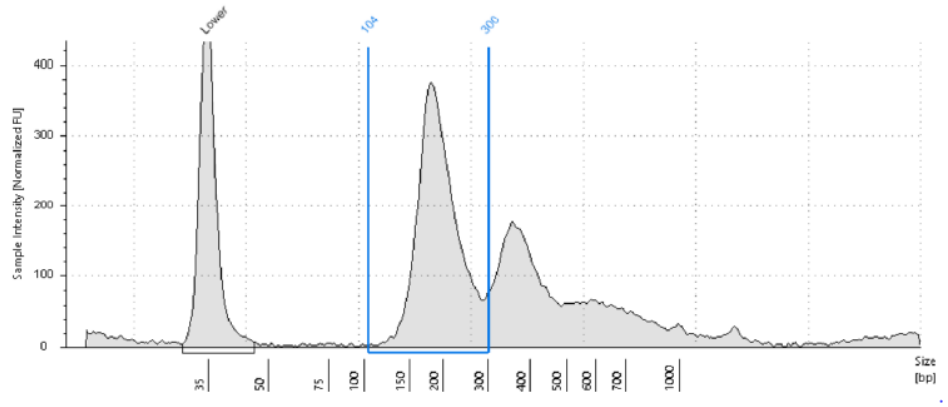


Figure 4. Result of fragment analysis of an extraction with 50% of circulating free DNA

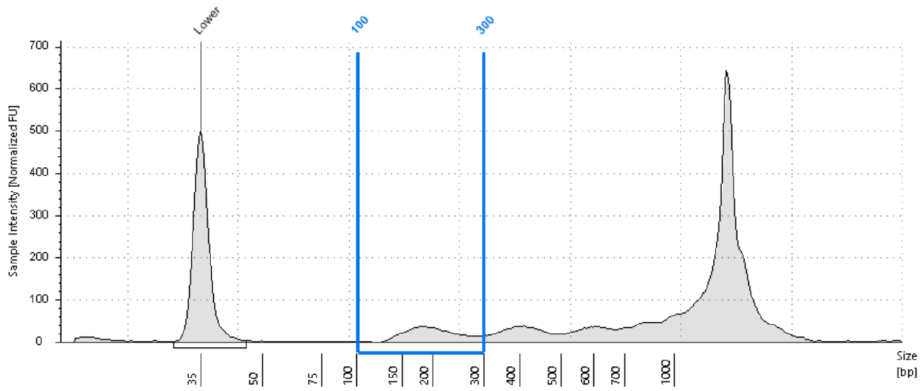


Figure 5. Result of fragment analysis of an extraction with 8% of circulating free DNA

03 In a 50 µL volume, prepare the highest possible amount of template DNA within the total 10–50 ng range.

- If working on a ctDNA sample, use water as eluant and follow the protocol.
- If working on a gDNA sample, use *Elution Buffer* as eluant and follow the protocol for DNA fragmentation from section 7.2.1 onwards.

Optional: If Health in Code's integrated tracking system is used (*Sample tracking components*; Ref. IMG-340), this step must be carried out replacing 2 µL of nuclease-free water by the same amount of one tracing reagent per sample.

04 Vortex all samples, spin, transfer onto the red Sample Input Strip, and keep cold until use.

NOTE: The samples must be placed in the Magnis NGS Prep System device as shown in Figure 6, with Sample 1 loaded in the well located farthest from the barcode.

NOTE: Please do not add any text or labels that could obscure the barcode of the *Sample Input Strip*.

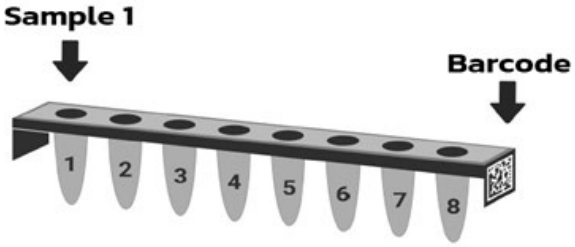


Figure 6. Required placement of samples in the Sample Input Strip

07.3 | Preparation of the reagents and plasticware used by the Magnis system

Reagents to be used in this step:

Reagent	Color	Conservation
Reagents Plate	Blue plate	-20°C
Beads / Buffer Plate	White plate	4°C
Index Strip	Black strip	-20°C
Targeted Liquid Biopsy Probe Strip	White strip	-80°C
Box "Magnis Empty Consumables"	N/A	15-25°C

01 Preparation of the *Reagents plate*:

- Thaw the plate at room temperature, keeping the plate in its white cardboard sleeve.
- Once the contents of all wells are thawed, vortex the plate while keeping it in its white cardboard sleeve. Begin by pressing the long side of the plate on the vortex head for 10 seconds. Afterwards, rotate the plate 90° and press the short side of the plate on the vortex head for 10 more seconds. Repeat the rotation/mixing sequence until completed on all four sides of the plate.
- Spin the plate (still in its white cardboard sleeve) in a centrifuge set at 250 x g for 1 minute.
- Make sure that no bubbles have formed at the bottom of the plate wells. If bubbles are present, repeat the spin step.
- Keep plate in its packaging and at cold temperature to be used on the same day.

02 Preparation of the *Beads/Buffers plate*:

- Keep the sleeved plate at room temperature for about 30 minutes.
- Vortex the plate (still in its cardboard sleeve). Start by pressing the long side of the plate on the vortex head for 10 seconds. Afterwards, rotate the plate 90° and

- press the short side of the plate on the vortex head for 10 more seconds. Repeat the rotation/mixing sequence until completed on all four sides of the plate.
- Spin the plate (still in its cardboard sleeve) in a centrifuge set at 150 x g for 10 seconds. Do not exceed recommended spin times to prevent beads from pelleting.
- Keep plate in its cardboard sleeve at room temperature to be used on the same day.

03 Preparation of the *Index strip*:

- Determine and record the set of indexes that will be used for the run. The provided strips are inscribed with A1, A2, A3, or A4 on the strip tube end opposite to the barcode. The following table shows the order of the indexes of each strip and their sequence.

A1 Strip		A2 Strip		A3 Strip		A4 Strip	
Index	Sequence	Index	Sequence	Index	Sequence	Index	Sequence
A01	GTCTGTCA	A02	GCGAGTAA	A03	AGCAGGAA	A04	CCGTGAGA
B01	TGAAGAGA	B02	GTCGTAGA	B03	AGCCATGC	B04	GACTAGTA
C01	TTCACGCA	C02	GTGTTCTA	C03	TGGCTTCA	C04	GATAGACA
D01	AACGTGAT	D02	TATCAGCA	D03	CATCAAGT	D04	GCTCGGTA
E01	ACCACTGT	E02	TGGAACAA	E03	CTAAGGTC	E04	GGTGCGAA
F01	ACCTCCAA	F02	TGGTGGTA	F03	AGTGGTCA	F04	AACAACCA
G01	ATTGAGGA	G02	ACTATGCA	G03	AGATCGCA	G04	CGGATTGC
H01	ACACAGAA	H02	CCTAATCC	H03	ATCCTGTA	H04	AGTCACTA

Table 5. Sequences of the indexes included in the kit

- Thaw the selected Index strip at low temperature, mix for 5 seconds on a vortex mixer and spin.
- Check the wells of the strip to make sure that liquid accumulates at the bottom of the wells and that no bubbles are present.

IMPORTANT: If the index strip used in an assay has not been registered, it can be reviewed on the *Post-Run Data* screen of Magnis Prep System. On this screen, open the *Labware Info* tab and locate the *Index Strip* row. The strip number is a value between 1 and 12 in the Index Strip column, located on the right-hand side of the screen. The specific *Index* associated with each number from 1 to 12 is shown in the following table.

Index Strip number from the Post-Run Data screen	1	2	3	4	5	6	7	8	9	10	11	12
Index Strip inscription	A1	A2	A3	A4	A1	A2	A3	A4	A1	A2	A3	A4

Table 6. Correlation between the Index on the Post-Run Data screen and the inscription on the strip

04 Immediately before it is used, thaw the *Targeted Liquid Biopsy probe strip* at cold temperature. Vortex for 5 seconds and spin. It is important to check that bubbles have not formed at the bottom of the well.

NOTE: The probe is pre-dosed in the first well of the strip, which does not include any legible labels indicating the specific identity of the probe design. It is recommended that special care be exercised to guarantee the traceability of this reagent both during storage and during the protocol.

05 Finally, prepare a box of *Magnis Empty Consumables* to be used during deck setup.

07.4 | Running the library preparation protocol

07.4.1 | Start of the protocol

01 On the Home screen shown on the touchscreen, press *Run Protocol*. The system will lock the instrument door and carry out an Instrument Health Check (IHC), which may take several minutes.

02 Once this check is completed, the message *Enter Run* Info will automatically appear on the screen. In the *Protocol menu*, select SSEL XTHS-RevB-ILM.

03 **Recommended**: mark the *Aliquot sample for QC* checkbox if you want the instrument to take an aliquot of each pre-capture library. This will enable quality control later on.

> NOTE: Quality control of the pre-capture libraries will only be available once the run has been fully completed.

Figure 7. Magnis NGS Prep System Enter Run Info screen

04 Move on to the next screen.

05 Select appropriate sample type, FFPE DNA. This label refers to low-quality DNA, whether extracted from paraffin-embedded tissue or free circulating DNA.

06 Select among the input DNA amount options (10, 50, 100, and 200 ng) in the *Input Amount* menu. In case an amount other than 50 ng and within the 10-50 ng range is used, please select the 10-ng option.

> NOTE: Template DNA quality and quantity settings will determine the number of cycles of the subsequent amplifications performed by the instrument. For this reason, it is essential to enter accurate information so that all samples have the same amount of input DNA.

07.4.2 | Deck setup

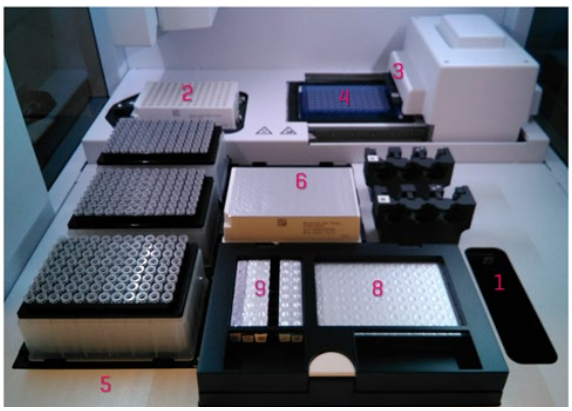
The deck can be very easily set up by following the steps on the Magnis touchscreen.

For each deck loading step, the deck position to be loaded will be shaded in blue on the touchscreen. Once each step is completed, move on to the next screen.

In order to guarantee the correct placement of the reagents and consumables in the Magnis instrument, please verify that the barcode of each item is facing the user, i.e., towards the front part of the instrument, Except for the *Magnis Thermal Cycler Seal*, whose barcode must be facing upward, and the three necessary boxes of tips not included in the kit, which do not have a barcode.

After removing the lid of the boxes of new and completely full tips, it is important to verify that the boxes are properly secured to the platform.

The following figure shows a completely loaded deck with each material numbered from 1 to 10, following the steps displayed by the Magnis instrument.



Step 1/10: Loading the disposable container into the waste bin
Step 2/10: Loading Magnis Deep-Well HSM
Step 3/10: Inserting the Magnis Thermal Cycler Seal into the slot of the thermal cycler module
Step 4/10: Loading the Magnis 96-Well PCR Plate onto the thermal cycler block
Step 5/10: Loading 3 full tip boxes
Step 6/10: Loading the Beads/Buffers plate
Step 7/10: Checking the chiller module for optimal temperature
Step 8/10: Loading the Reagent Plate into the chiller module
Step 9/10: Loading the strip tubes onto the chiller module (QC Strip is optional)
Step 10/10: Close the Magnis instrument door

Figure 8. Magnis NGS Prep instrument deck loaded for the run and quick loading guide

Below, the setup steps shown on the Magnis touchscreen are explained in detail:

01 Place the disposable container *Magnis Tip Waste Bin* (included in the "*Magnis Empty Consumables*" box) in the waste bin located in the lower-left corner. The barcode must be facing the user, as shown on the touchscreen. Close the waste bin.

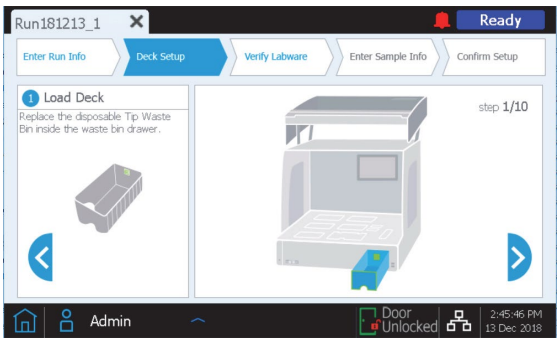


Figure 9. Step 1 of 10 on the Deck Setup screen of the Magnis NGS Prep System

02 Place the *Magnis Deep-Well HSM Plate* (included in the "*Magnis Empty Consumables*" box) as shown on the instrument's touchscreen. To do this, first insert the left edge of the plate into the spring-loaded slot and then lower the right edge of the plate down until it sits flat on the platform. Once flat, shift the plate slightly to the right and ensure that it is secured inside the holder.

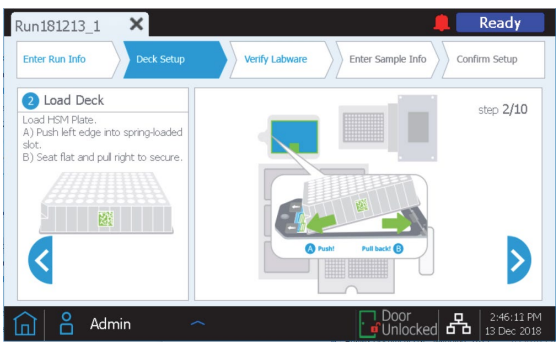


Figura 10 . Paso 2 de 10 de la pantalla Deck Setup del equipo Magnis NGS Prep

03 Place the *Magnis Thermal Cycler Seal* (included in the "*Magnis Empty Consumables*" box) as shown on the instrument's touchscreen. To do this, peel the protective film from the white foam pad located below the metal plate. After the full sheet of film has been removed, insert the *Thermal Cycler Seal* into the slot of the thermal cycler, with the barcode facing up, and slide until it clicks into place.

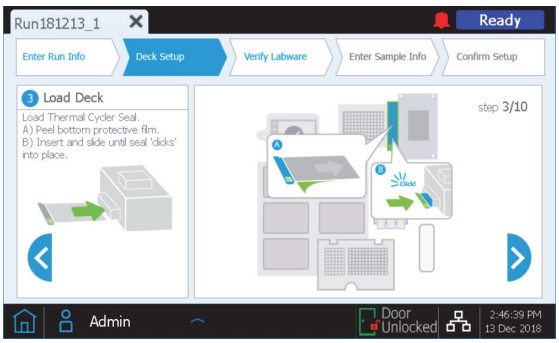


Figure 11. Step 3 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

04 Place the *Magnis 96-Well PCR Plate* (included in the "*Magnis Empty Consumables*" box) as shown on the instrument's touchscreen. To do this, insert the plate wells into the thermal cycler block wells, with the plate barcode facing the user. Make sure that the plate is fully seated by pressing evenly, first on the center of the plate and then on the corners.

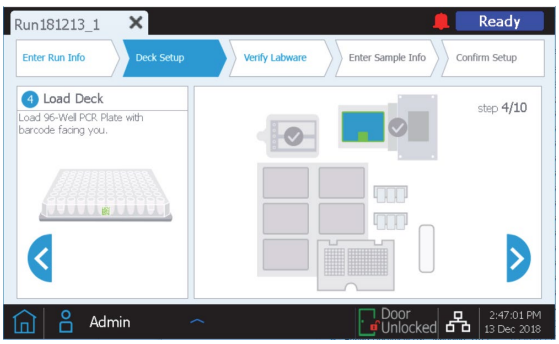


Figure 12. Step 4 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

05 Load a fresh, full tip box at each deck position indicated on the instrument's touchscreen. After removing the lid, verify that each box of tips is properly secured in its platform position.

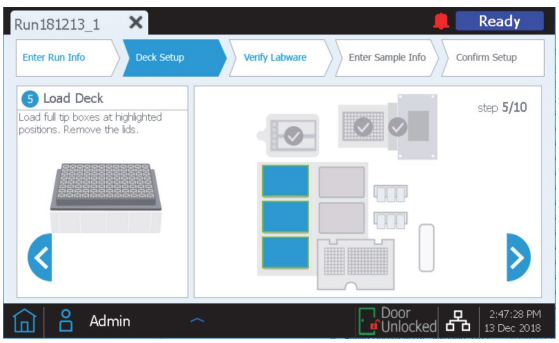


Figure 13. Step 5 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

06 Place the *Beads/Buffers Plate* (prepared in section 7.3 of this document). Remove the white cardboard sleeve and then load the plate as shown on the instrument's touchscreen, with the barcode facing the user. To do this, first insert the left edge

of the plate in the spring-loaded slot and then lower the right edge of the plate down until it sits flat on the platform. Once flat, shift the plate slightly to the right and ensure that it is secured inside the holder.

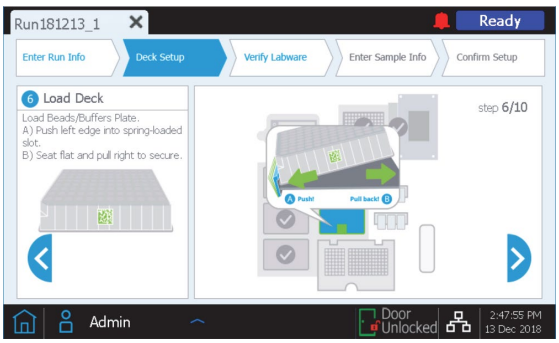


Figure 14. Step 6 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

07 The instrument's chiller module must reach a temperature of 12 °C before loading the Magnis instrument. If said temperature has not been reached at this point, the touchscreen will appear as shown in Figure 15. However, if the chiller has already reached the necessary temperature, this screen will not appear.

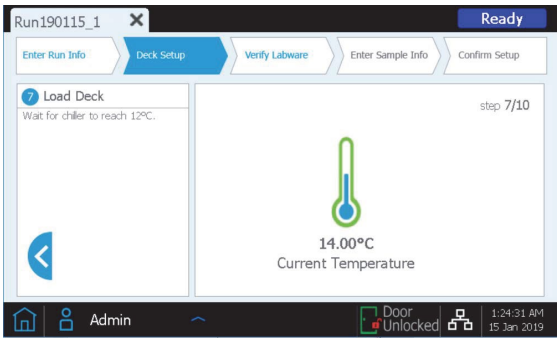


Figure 15. Step 7 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

08 Open the door of the chiller module by pressing on the half-circle button indicated with a green arrow on the touchscreen. *Place the Reagent Plate* (prepared in section 7.3 of this document) in the chiller module. Remove the white cardboard sleeve and then load the plate as shown on the instrument's touchscreen, with the barcode facing the user. Firmly press downwards, applying pressure evenly across the plate.

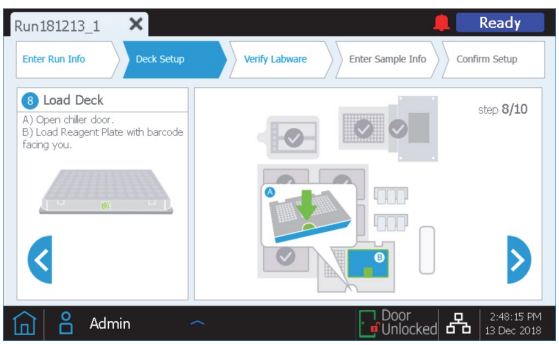


Figure 16. Step 8 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

09 Load the strip tubes for the run in the indicated positions of the chiller, as shown on the instrument's touchscreen. Ensure that each strip is secured by pressuring firmly and evenly on the edges of the strip tubes. Avoid touching or damaging the foil covers. All the tube strips must have a barcode facing the user.

- Load the *Sample Input Strip* (red strip) containing the DNA samples prepared in section 7.2 of this document into the chiller holder position labeled with **S**.
- Load the *Index Strip* (black strip), prepared in section 7.3 of this document, into the chiller holder position labeled with **IDX**.
- Load the Targeted Liquid Biopsy Probe Strip (white strip), prepared in section 7.3 of this document, into the chiller holder position labeled with **P**.
- Load the Magnis Library Output Strip (green strip), included in the "Magnis Empty Consumables" box, into the chiller holder position labeled with **L**.
- **Optional**: If the run will include the collection of aliquots of the pre-capture libraries for quality control, as recommended by Health in Code, load the *QC Strip* (blue strip), included in the "*Magnis Empty Consumables*" box, into the chiller holder position labeled with **Q**.

Once all the strips have been loaded, close the chiller door.

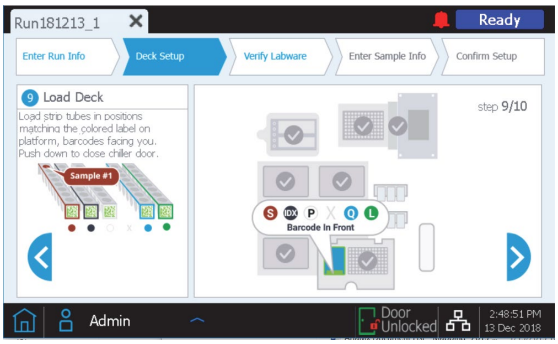


Figure 17. Step 9 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

10 Close the instrument door.

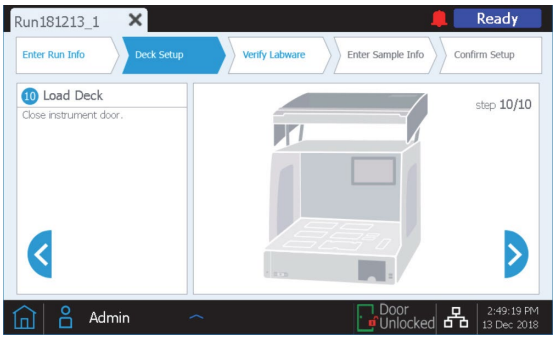


Figure 18. Step 10 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

07.4.3 | Labware verification

Once loading has been completed, the instrument performs the *Verify Labware* phase, in which the instrument scans the barcode of each of the labware components present on the deck.

Before initiating the automated verification, make sure that all the lids of all the tip boxes have been removed and that the tips are full, as indicated in the following figure. Once this has been verified, click *OK* to perform the labware verification.

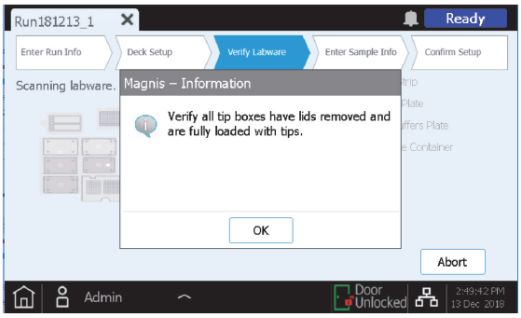


Figure 19. Verify Labware pop-up window of the Magnis NGS Prep System

During the labware verification, the instrument will verify that all the necessary components for the run are present, in the correct position and facing the right way, and that they are not past their expiration date.

The verification results will be shown on the Magnis touch screen, if everything is correct (Figure 20), proceed to the following screen. On the contrary, check with section 9 of this document.

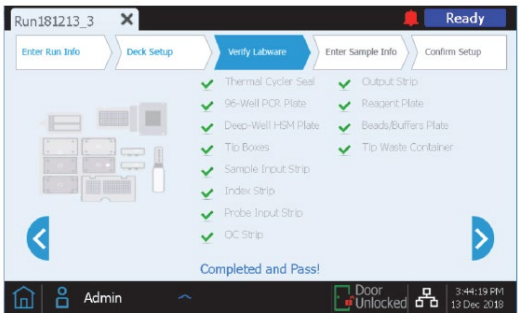


Figure 20. Verify Labwarescreen of the Magnis NGS Prep System after correct labware verification

The final screen of *Verify Labware* shows details of the probe. Move on to the next screen.

07.4.4 | Entering sample information

The Magnis software automatically assigns a default *Sample ID* to each sample position, which can be replaced with a sample name chosen by the user using either of the two methods below.

01 Manual sample assignment:

- On the *Enter Sample Info* screen, select a specific sample position shown on the touchscreen.
- Use the *Edit Sample ID* tool to enter the desired text.
- Press *Change* to save the new text for the selected sample position.

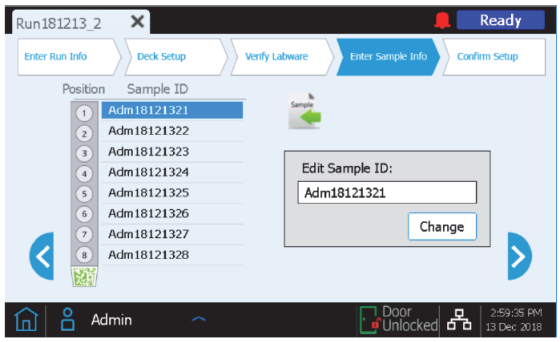


Figure 21. Edit Sample Info screen of the Magnis NGS Prep System; the Load samples button is highlighted with a circle.

02 Import of sample assignments using a .csv file:

- Create a .csv (comma-separated value) file containing sample names in the correct order. To enter the new name of the sample, you may use Microsoft Excel and later save the file in .csv format.
- Write the header sample_id in cell A1, as shown in the following figure.

	A
1	sample_id
2	HD18060701
3	HD18060702
4	HD18060703
5	HD18060704
6	HD18060705
7	HD18060706
8	empty1
9	empty2

Figure 22. Example of .csv file content (shown in spreadsheet format) to load sample assignment.

- Type the name of each sample in cells A2 to A9. The entry file of the sample must contain 8 unique sample IDs. If the protocol is to be carried out with less than 8 samples, you must fill in these positions in the file as shown in Figure 22 (empty1 and empty 2).
- Save file in .csv format.
- Transfer the .csv file to an unencrypted USB disk and connect the disk to one of the USB ports of the Magnis instrument.
- When setting up the run, on the *Enter Sample Info* screen, press the *Load* samples button (highlighted with a circle in Figure 21).
- Follow the instructions of the protocol setup assistant to transfer sample IDs from the USB disk.

07.4.5 | Confirm setup and start the run

01 Confirm the run setup details. Once entries are confirmed to be correct, press the forward arrow to move on to the final setup screen.

02 Verifying run setup details related to characteristics of the DNA sample. After confirming that the run setup details are correct, press the Start button to start the run.

IMPORTANT: The number of pre- and post-capture PCR cycles has been set according to DNA quantity and quality, as well as the size of the panel. Modifying them could affect the sensitivity, specificity, and LOD of Targeted Liquid Biopsy OncoKit.

Once the run starts, the LED indicator will glow green and the touchscreen will display the status of the run, along with an estimate of the time remaining prior to run completion.

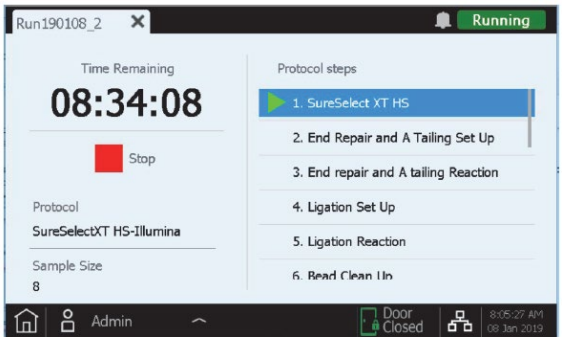


Figure 23. Running screen during a run

The SSEL XTHS-RevB-ILM protocol takes approximately 9 hours to complete and may be done overnight for convenience. Once the protocol is complete, the prepared libraries are automatically held at 12 °C. Collect the libraries from the instrument within a maximum of 24 hours.

If necessary, the run can be aborted by clicking on the red *Stop* button on the *Running* screen. A warning message will appear, asking you for confirmation to abort the run. Once the run has stopped, it cannot be resumed, and the labware used cannot be reloaded for a future run.

The *Running* screen must remain open at all times during the run, and the screen close button (x) and other navigation buttons are inactive while the run is in progress. The touchscreen cannot be used for other actions during a run.

07.4.6 | Collecting libraries from the instrument

After the run is completed, the touchscreen displays the prompt below. By pressing *OK*, the instrument transfers the libraries from the thermal cycler, where they have been held since the end of the protocol, to the green *Library Output Strip*, located in the chiller module.

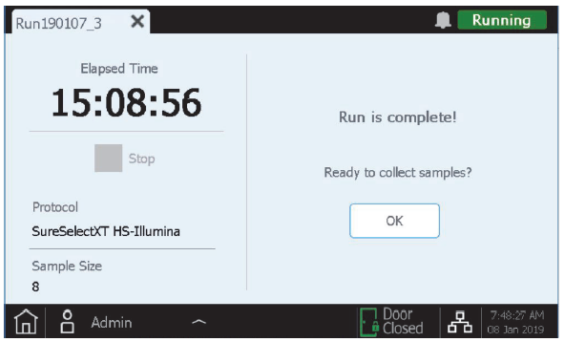


Figure 24. Running screen after a run

Before opening the instrument door, wait for the LED indicator lights to turn blue, indicating that all the instrument-mediated sample processing steps are complete.

The chiller module will be held at 12°C for up to 2 hours from the moment the libraries are placed in the green *Library Output Strip*, as long as the instrument's door remains closed.

Open the instrument door (until the LED indicator lights turn white), collect the libraries in the green *Library Output Strip* and seal them.

> It is possible to stop the protocol at this point, storing the libraries at 4 °C if they are to be used within the following 24 hours or at -20 °C for longer storage periods.

If the optional library quality control samples were collected for the run, remove the blue *QC Strip* from the chiller module and leave at room temperature until dry, either unsealed if the protocol is to be continued within the next 24 hours or sealed for longer storage.

Once the door is open for library sample collection, the touchscreen of the instrument will appear as shown below.

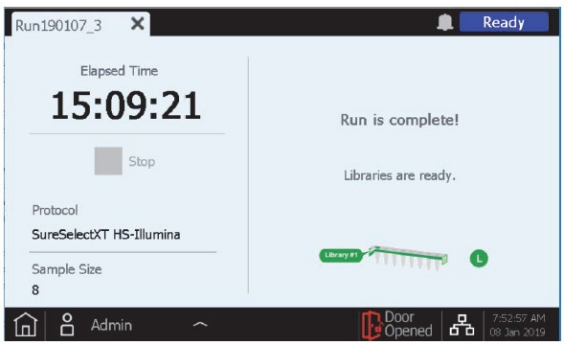


Figure 25. The Running screen after a run and after removing the libraries

To close the run screen and return to the *Home* screen, press *X* on the tab. This may take a few seconds.

07.5 | Instrument cleaning after a run

Remove and dispose of all used consumables left in the instrument unit:

- Tip waste bin filled with the tips used throughout the test.
- *Magnis Deep-Well HSM.*
- *Magnis Thermal Cycler Seal.*
- *Magnis 96-Well PCR Plate.*
- All tip boxes, including partially filled ones.
- *Beads/Buffers Plate.*
- *Reagent Plate.*
- Red, black, and white strips used during the run.

If any spilled or leaked materials are observed on the instrument deck, it is recommended to run the UV decontamination *Extended Cycle* procedure. Clean up the spill following the instructions provided in the *Instrument User Guide*.

07.6 | Library validation and quantification

07.6.1 | Optional quality control of the pre-capture library

If analysis of pre-capture libraries is necessary, resuspend the dry libraries in 6 µL of nuclease-free water to obtain a suitable concentration for analysis, following the recommended use of Tapestation 2200 and the commercial kits D1000 Reagents (cat. no. 5067-5583) and D1000 ScreenTape (cat. no. 5067-5582) by Agilent Technologies.

After adding 6 µL of nuclease-free water, incubate at room temperature for 10 minutes. Finally, vortex vigorously to ensure complete resuspension.

After the analysis of the samples with TapeStation, a library with a size between 200-400 bp should be obtained (Figure 26). In case unexpected sizes are obtained, please review the protocol or contact Health in Code's technical support team.

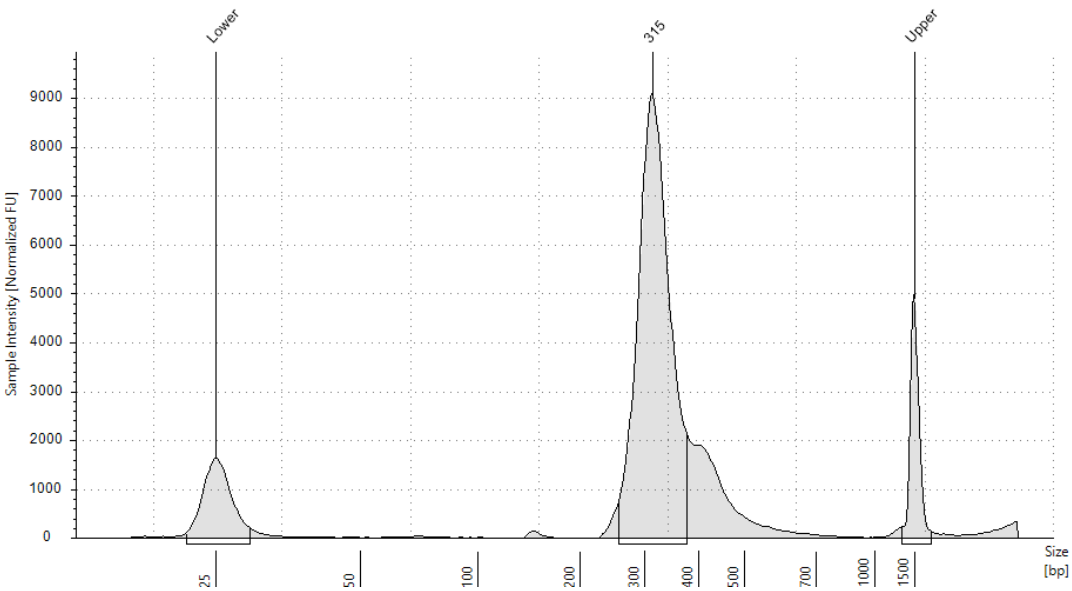


Figure 26. Expected result for a pre-capture library from ctDNA, obtained with Tapestation 2200

To determine DNA concentration, the peak area corresponding to the expected library size must be integrated. The amount of library DNA obtained will vary depending on the concentration of input DNA, ranging from **30 to 160 ng/µL**. The overall pre-capture library yield can be calculated as the amount of DNA in 1 µL of the reconstituted QC sample x 36 (this value includes dilution adjustments).

07.6.2 | Quality control of the post-capture library

Prior to pooling the libraries for multiplexed sequencing, it is necessary to analyze the quantity and quality of each one of them.

To measure DNA concentration, it is recommended to use a Qubit® 2.0 fluorometer, the Qubit ds DNA HS Assay commercial kit (cat. no. Q32854), and the Qubit™ assay tubes (cat. no. Q32856) by Invitrogen. The concentration of post-capture libraries will vary between 2 and 20 ng/µL.

To analyze the quality of the captured fragments, Health in Code recommends the use of TapeStation and of the commercial kits High Sensitivity D1000 Reagents (cat. no. 5067-5585) and High Sensitivity D1000 ScreenTape (cat. no. 5067-5584) by Agilent Technologies.

The expected average size of the fragments is between **250 and 350 bp**, but ctDNA, due to its low quality, frequently results in libraries with a second peak. In these cases, it is recommended to integrate the area of the corresponding peak with the expected library size and obtain from it the concentration of the libraries.

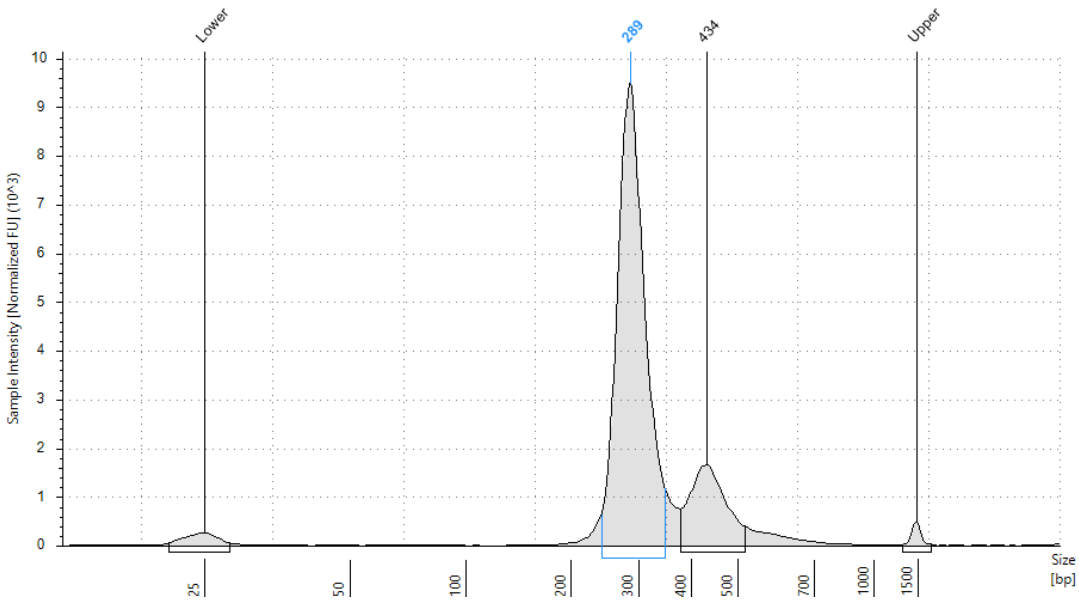


Figure 27. Expected result for a post-capture library from ctDNA, obtained with Tapestation 2200

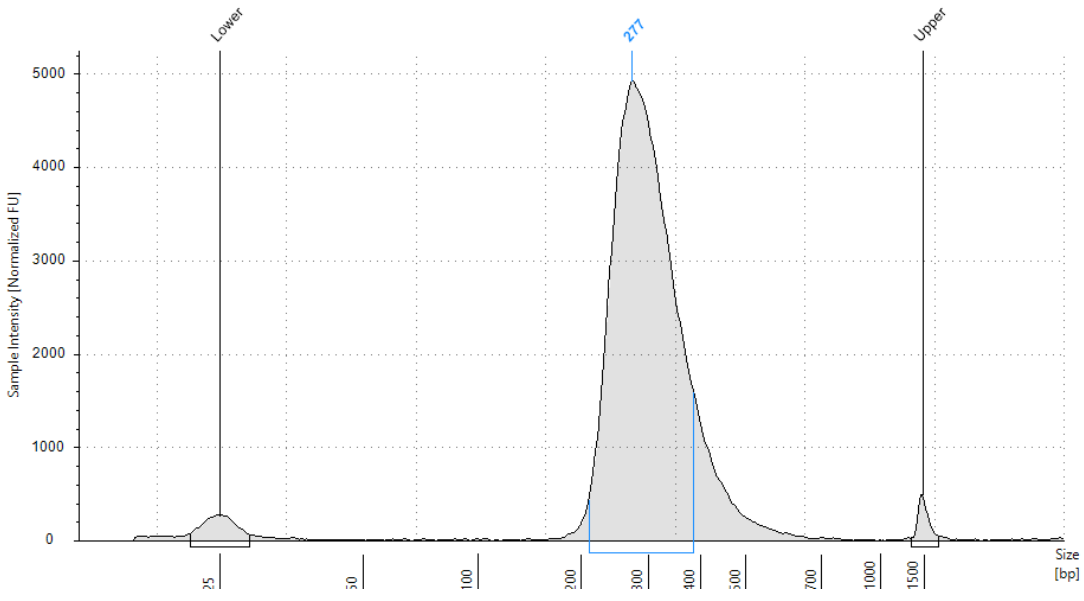


Figure 28. Expected result for a post-capture library from ctDNA, obtained with Tapestation 2200

From DNA concentration and the peak size of the libraries, we can calculate their concentration by applying the following formula:

$$Library\ concentration\ (nM) = \left[Concentration\ (^{ng}/_{\mu L}) \cdot \frac{1500}{Size\ (pb)}\right]$$

Finally, dilute each library to 4 nM with *Elution Buffer* and create an equimolar pool of all the libraries that will be included in the run.

↘ It is possible to stop the protocol at this point, storing the libraries at 4 °C if they are to be used within the following 24 hours or at -20 °C for longer storage periods.

07.7 | Library denaturation

A denaturation protocol must be carried out before the libraries are placed in a Illumina NextSeq 500/550 sequencer following these steps:

01 Thaw the HT1 reagent (included in the Illumina reagent kit to be used during sequencing) and keep cold until its use.

02 Thaw the Phix control and keep cold until its use. The PhiX control must be denatured and diluted to 20 pM.

NOTE: For the denaturation of the PhiX control this same denaturation protocol for libraries must be followed.

03 Add 5 µL of the library pool, previously diluted to 4 nM, and 5 µL of NaOH 0.2N to a 1.5 mL tube. Vortex and spin.

04 Incubate at room temperature for 5 minutes.

05 Add 5 µL of Tris-HCl 200 mM pH 7. Vortex and spin.

06 Add 985 µL of HT1 and vortex. The library should now be at 20 pM.

07 Transfer 78 µL of the 20 pM library to a new 1.5 mL tube.

08 Add 1,222 µL of HT1.

09 To this mix add 1.2 µL of denatured PhiX control diluted to 20 pM. At this point, the library should be diluted to 1.2 pM.

10 Load the entire volume contained in the 1.5 mL tube into the cartridge.

The following tables specify the maximum number of samples per run, depending on the sequencing kit used, to guarantee a minimum threshold of PF clusters:

↘ **Circulating tumor DNA:** 50.000.000 PF clusters per sample.

NextSeq Reagents Kit	Maximum no. of samples
NextSeq 500/550 High Output v2.5 kit (150 cycles). Ref: 20024907	32*

Table 7. NextSeq Illumina kit and maximum number of ctDNA samples to be analyzed with Targeted Liquid Biopsy OncoKit

07.8 | Configuration of the *NextSeq platform*

01 Configure the platform by executing the independent mode ("*Standalone*"), since *BaseSpace* currently does not admit the sequencing of the "molecular barcode" as an index.

02 Follow the equipment's loading instructions.

03 When the loading has finished, the run configuration will appear on the screen. Select the following parameters:

- *Read Type: Paired End.*
- *Cycles:*
  - *Read* 1: 75
  - *Read* 2: 75
  - *Index* 1 (i7): 8
  - *Index* 2 (i5): 10

07.9 | Generation of *FASTQ* files necessary for bioinformatics analysis

After the sequencing of Targeted Liquid Biopsy OncoKit libraries, several .bcl files are generated, which must be de-multiplexed to generate the 12 *FastQ* files necessary for the bioinformatics analysis.

To convert the .bcl files to *FastQ* files, contact Health in Code's support team.

08 Analysis of results

Bioinformatics analysis of the results is done through an analysis pipeline designed especially for Targeted Liquid Biopsy OncoKit, through the **Data Genomics** platform. To access this tool, visit www.datagenomics.es.

The tool can be used for the analysis of the different samples and collect all the generated files after their bioinformatics analysis.

As NGS is not yet considered the *Gold Standard* for some types of mutation, it is recommended, whenever possible, to confirm positive results using a complementary standardized technique.

08.1 | Request for analysis

01 Seleccionar *"Import Samples"* en la pantalla principal (pestaña de *Orders*) para iniciar el análisis de las muestras secuenciadas. De esta forma se accede a la pantalla de importación de ficheros (Figura 29). En dicha pantalla se deben importar los 12 ficheros *FastQ* asociados a cada muestra y opcionalmente, el fichero de la *SampleSheet*, que permitiría importar todos los ficheros del mismo run de secuenciación simultáneamente.

02 Select the "Import Samples" on the main screen (Orders tab) to begin the analysis of the sequenced samples. This will take you to the file import screen (Figure 29). On this screen, the two fastq files associated with each sample must be imported. Optionally, the SampleSheet file could be used as well; this file allows simultaneously importing all files from the same sequencing run.

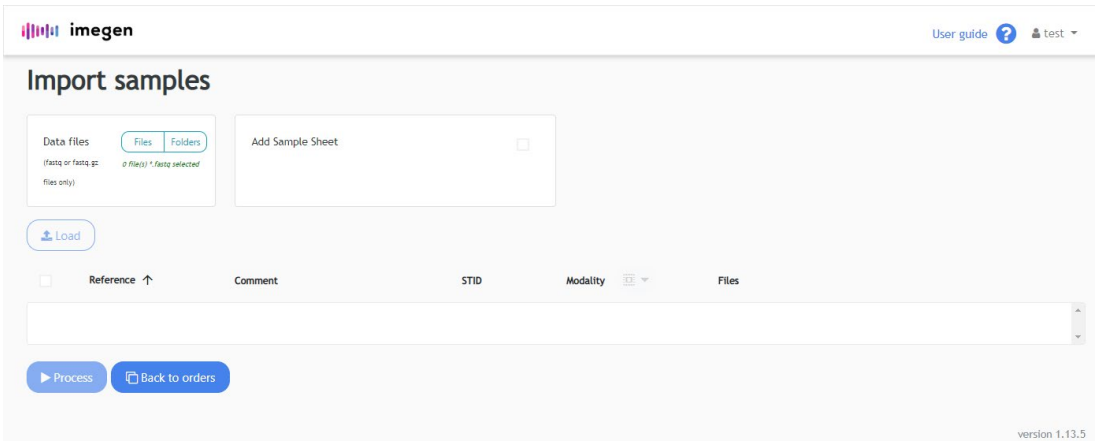


Figure 29. Screen for importing the fastq files and the sample sheet and commence the analysis request

03 Once the files are uploaded, the sequencing run name must be indicated, and the study modality (*Targeted Liquid Biopsy OncoKit*) and the *STID (Sample Tracking ID)* used for each sample (or "no stid" if none was used) must be selected.

04 Before processing the sequencing files, it is necessary to fill in some required fields for each sample: tumor type and percentage of tumor cells. To access the pop-up screen with these fields, click the pencil icon . Apart from the required fields, there are additional fields that the user may find useful (Figure 30). Once filled in, click "Accept".

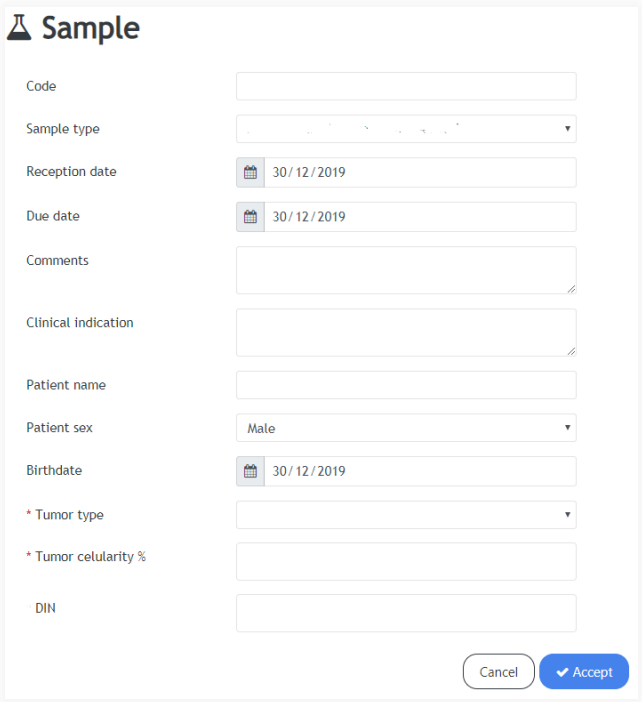


Figure 30. Pop-up screen with the required fields for each sample

05 To carry out the analysis request, select the samples to be analyzed and click the "*Process*" button. When the process has finished successfully, the following message will appear: ✓ **The import has been performed correctly**.

06 Select "*Back to orders*" to return to the main screen.

08.2 | Management of orders

All the created requests will appear in the *Orders* tab in the corresponding section according to their status (*In bioinformatic process, Pending, In review, Finished,* or *Cancelled*). The request will display the sample's name and the analysis modality and status.

By clicking on the sample, you will access a window where you can enter and save certain characteristics of each sample, such as received date, clinical indications, etc.

To access the results of the bioinformatics analysis, in the "*bioinformatics*" order, you must select "*Show results*," and the "*Workspace*" window will open. This screen allows the user to access the files resulting from the bioinformatics analysis: files resulting from CNV analysis (CNVs.png and CNVs.txt) and structural variants (.SV.txt), alignment files (bam and bai), list of variants (vcf), as well as other files with information on coverages and the quality report of the sequencing after the bioinformatics analysis.

The parameters that are taken into account in the different files that are generated from the sequencing, for a sample to pass the bioinformatic quality control established for Targeted Liquid Biopsy OncoKit are:

- FASTQ: The established acceptance criteria are detailed in the **Data Genomics** instructions for use, available at: www.datagenomics.es.
- BAMs:
  - *Mapped Reads* (%):
    - Fail: < 97
    - Warn: 97 – 99
    - Pass: > 99
  - *Duplicates* (%):
    - Fail: > 57.4
    - Warn: 51.2 – 57.4
    - Pass: < 51.2
  - DP100 (%)
    - Fail: < 92.2
    - Warn: 92.2 – 93.9
    - Pass: > 93.9
  - *On-target* (%):
    - Fail: < 28.3
    - Warn: 28.3 – 32.4
    - Pass: > 32.4

In the event of not meeting any of the parameters mentioned, the icon will appear on the main screen, next to the sample in question.

In the quality control of the Targeted Liquid Biopsy OncoKit assay, neither the VCF files nor the sample's heterozygosity are taken into account, because it is a somatic analysis and the frequency of the identified variants can vary greatly.

To access the variants filter, press "*Filtering*" and the filter to be used will be chosen according to the introduced DNA type:

The ctDNA filter, Targeted Liquid Biopsy OncoKit Default, is characterized by:

- Quality variants: ***PASS; d100; bias; pseudogenic homology*** (Fault summary).
- Depth: **≥ 20X** (Clean total count).
- Allele frequency: **≥ 1%** (*Variant Freq*). Limit of detection established for samples with DIN >3 and tumor cellularity ≥30%.
- Low population frequency: ***gnomAD Freq <2%***. To filter out variants frequent in the population that will not be relevant in tumor formation.
- Exclusion of variants in non-coding regions and synonymous variants, except those found in possible splicing regions.

Adding the ***HotSpot*** (*Fault summary*) tag in the filter will show hot spot ***NTRK1*** and ***NTRK3*** variants covered by Targeted Liquid Biopsy OncoKit.

Once the variants that appear after applying the previous filter have been reviewed and categorized, it is recommended that a second filter showing all the variants listed in *ClinVar* as pathogenic or possibly pathogenic be used, the *Targeted Liquid Biopsy OncoKit II*.

08.3 | Analysis of large rearrangements (CNVs)

The analysis of large rearrangements or CNVs from NGS sequencing data consists of a correlation between the number of normalized reads in a region and the number of DNA copies for the same region.

Since the number of reads must be normalized among different samples, the variability among samples will result in poorer CNV identification and, therefore, it is very important to homogenize the experimental conditions among different samples and among different genomic regions from the same sample to the extent possible. To reduce variability and guarantee a correct CNV analysis, the following recommendations are suggested:

- The conditions for library preparation and capture process need to be homogeneous and, for this, the different steps must be taken simultaneously for all the samples within the same sequencing run, using the same equipment simultaneously and following the indications specified in section 7 of this document.
- Input DNA is another source of variability. Therefore, it is recommended that all analyzed DNA be extracted following the same extraction protocols.

In addition, for coverage normalization in CNV analysis, it is necessary to have a germline reference sample of the same sex as the test sample and that has been processed with Targeted Liquid Biopsy OncoKit.

Targeted Liquid Biopsy OncoKit, apart from the analysis of CNVs in the genes included in the panel, also counts with a SNP array distributed through the genome, which allows for the analysis of CNVs and for the study of the loss of heterozygosity with a neutral copy number (*Copy-neutral LOH*).

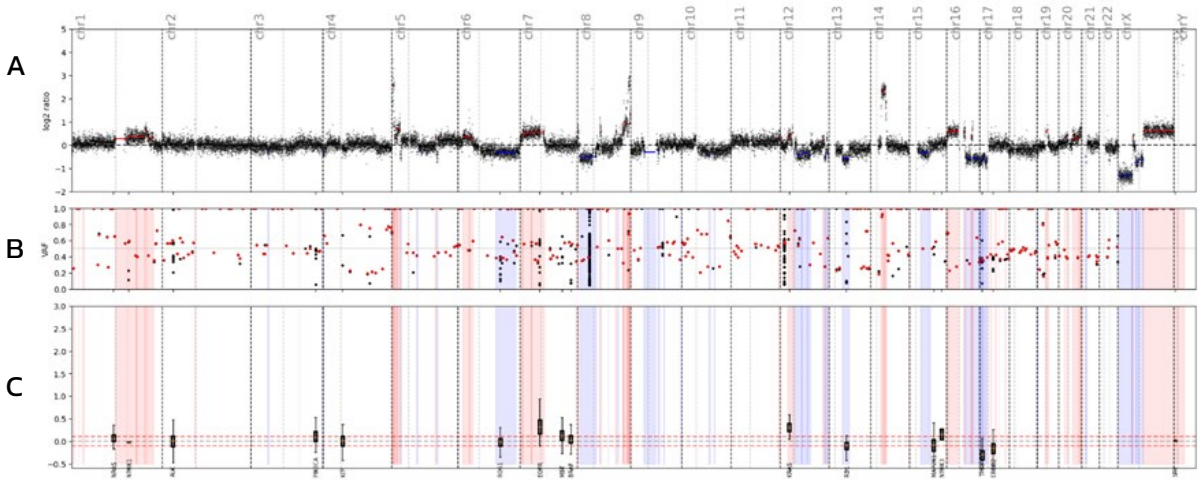


Figure 31. The ordinate axis shows the log2_ratio value (A), the allele frequency of the variant (B), and deviation of the normalized coverage of a gene in the tumor with respect to the coverage of that same gene in germline DNA (C); and on the abscissa axis marks the 24 chromosomes present in human cells and the genes included in the Targeted Liquid Biopsy OncoKit panel according to their location in the genome.

A) Result of CNV testing of the entire genome by means of off-target analysis. Each point represents 100 Kb of sequence. Deleted regions appear in blue and amplified regions in red.

B) Result of CNV testing of the entire genome by means of SNP array analysis. Each red dot is one of the 500 SNPs tested for, and the black dots correspond to variants in the rest of the panel.

C) CNV analysis per gene in which the difference between gene coverage in tumor DNA and germline DNA is represented, as well as the location of the gene in the genome.

The limit of detection for CNVs is 2.9 total copies for gains, i.e. 4 copies at 50% tumor representation.

To analyze CNV results with **Data Genomics**, access the results in "*Filtering*", specifically in the CNVs tab. On this screen, you can apply different filters and select the variants that you wish to include in the results report.

For each CNV call, the software calculates a Score, by taking into account parameters such as CNV size, nearby calls (<3 Mb), the value of the log2 ratio, and if the gene or genes affected by the CNV are included in this panel; this information is used to classify the variants as *High-Score, Medium-Score*, and *Low-Score*.

It is recommended to filter out variants that do not have a *High Score* (greater reliability) and carefully examine the *CNV plot* (Figure 31). If an excessive number of CNVs are found, the user can select the "*Extensive chromosomal abnormalities*" option, and this way the increased number of copy number variants will be reflected in the report.

If working with DNA extracted from a paraffin-embedded tissue sample, the calculation of the number of copies will be adjusted according to the degree of infiltration of the tumor with non-tumorous cells, whose value will be indicated to the user in the "*Tumor cellularity*" field.

Variants that are to be included in the results report should be selected.

		Event	log2Ratio	Region	Size (Mb)	Total copies	Tumoral copies	Score	Genes in Panel	Genes related to oncc	Category
☐	☐	Gain	1.0374	14:106350000-1069500	0.60	4.11	4.11	Medium			+
☐	☐	Gain	0.266	16:6650000-7650000	1.00	2.40	2.40	Low			+
☐	☐	Loss	-0.8604	4:14350000-16250000	1.90	1.10	1.10	High		PROM1	+

Figure 32. CNV results.

08.4 | Analysis of structural variants (SVs)

Data Genomics does the analysis of structural variants using as its basis the BAM alignment file, which is obtained after the mapping of the readings of the referred genome. Two types of reads are identified after the mapping:

- ***Split reads***: Reads whose sequence maps to different locations of the genome. These reads also provide us information on where exactly the event occurs (see Figure 33).

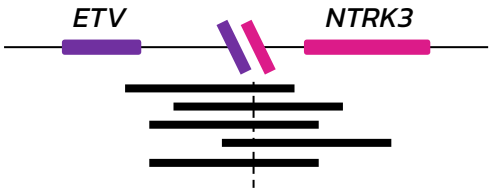


Figure 33. Representation of the readings that include the fusion point, represented as a non-continuous vertical line, and whose bases map in different regions of the genome (Split reads).

- *Discordant read pairs o mate reads:* Reads surrounding the event. Since sequencing is done via paired-ends, the distance or mapping position difference between these (forward y reverse) is used to detect these types of events. (See Figure 34).

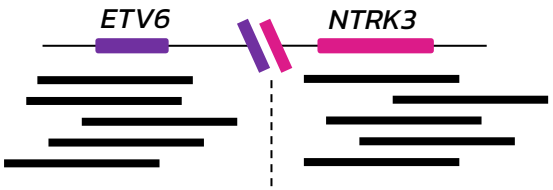


Figure 34. Representation of the readings that do not include the fusion point represented as a non-continuous vertical line, and whose search for the sequencing pair (Discordant read pairs) allows for the translocation's identification.

The compatibility of these two detection types makes the identification of structural variants with high trust possible, apart from other types of rearrangements, such as deletions or duplicates, also eliminating possible artefacts produced during sequencing.

For an interactive analysis of the results of structural variants with **Data Genomics**, access the petition's results at "*Filtering*" and, specifically, the "*Structural Variants*" tab.

In the results screen (Figure 35), you can see all the detected events, classified within three quality ranges: *High*, *Mid*, and *Low*. The quality range given to each event will depend primarily on the reads obtained relative to the average coverage of the region.

On this screen, you can select the variants that you want to include in the results report.

It is recommended to filter variants so that only those with *High Quality* (of greater reliability) are shown, as well as to examine the sequences of each variant in the IGV viewer. Subsequently, it is recommended to review the lower quality variants, as they could be real variants with a frequency close to the detection limit.

Event type	Region 1	Gene 1	Region 2	Gene 2	Split coverage	Mate split coverage	Total coverage	Quality	Category
Translocation	12:12028120	ETV6	4:773154	RP11-440L14.1	73	19	92	High	+
Translocation	12:12028117	ETV6	15:88512713	NTRK3	47	18	65	High	+

Figure 35. Structural variants results

08.5 | Variant filtering

By pressing the *Request*: "*Filtering*" button, a pop-up screen with the different tests on variants generated so far appears (see Figure 36).

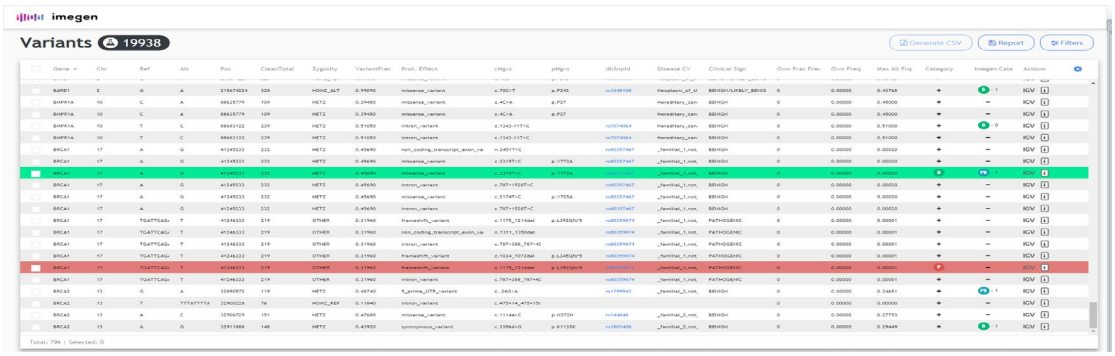


Figure 36. Variant filtering with Data Genomics

Once variant analysis is opened, the variants that have met *Targeted Liquid Biopsy OncoKit Default* criteria appear. For a user to evaluate whether a variant shall be validated as pathogenic even if it has not met *Targeted Liquid Biopsy OncoKit Default* conditions, it is recommended to clean the previous filter and set up a new one to show pathogenic variants in a proprietary database and/or *ClinVar* (Clinical significance).

New filters can be created by clicking the "*Filters*" button on the "*Variants*" page. A pop-up window where a new filter can be created appears. The user must adjust the different options as desired. Once the filtering parameters have been selected, they can be saved (*Save*) and used on the current sample by clicking "*Apply*".

Each found variant will be assigned a quality label in the column "*Fault summary*". All possible labels, as well as their description and all information obtained from variant filtering, are summarized in the **Data Genomics** instructions for use, available at www.datagenomics.es.

Below are shown the quality labels exclusive to Targeted Liquid Biopsy OncoKit:

- d100: Read depth covering the position less than 100
- f0.01: Allele frequency lower than 0.01

In gDNA samples with a DIN <3, or ctDNA with a percentage of circulating free DNA below 50%, it is recommended that variants with allele frequencies lower than 10% be not reported, since the degradation of the sample may have affected the detection limit of the technique.

08.5.1 | Categorization of SNV, INDEL, CNV, and SV variants

Once the desired filters have been set up by the user, each variant found, be it point variants, small deletions and insertions, CNVs, or SVs, can be categorized.

By clicking on the "*Category*" column, a drop-down list appears for each variant (see Figure 37), with the different categories that can be assigned to the variant.

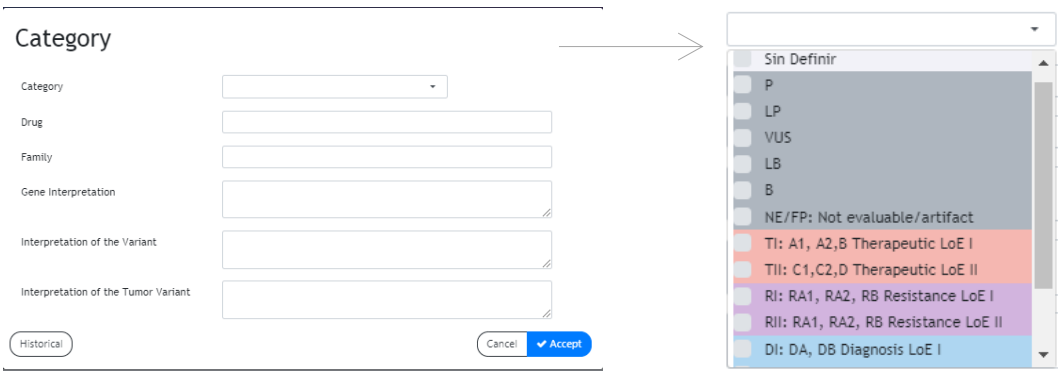


Figure 37. Drop-down list in the "Category" column

There are different categorization groups, distinguished in the tool by different colors, and each variant may only belong to one of the different groups.

First, it is recommended assigning the variant a functional category (gray color), which can be: pathogenic (P), probably pathogenic (PP), variant of uncertain significance (VUS), probably benign (PB), or benign (B).

If it is not needed to evaluate the variant, or if it is suspected that it is a false positive, it is recommended that it be categorized as "*not evaluable/artifact*", which, if selected, would prevent any further selection.

If a variant is considered benign or probably benign at the functional level, it is recommended against categorizing it in any of the groups of clinical relevance. In the case of variants of uncertain clinical significance, it is generally recommended to report them if they are not described in population databases and predictors indicate that they are pathogenic or that they could have a deleterious effect on the function of the protein; although it is recommended to assess each case in particular, taking into account the alteration and the type of tumor studied.

In the event of classifying a variant as pathogenic or probably pathogenic, the same variant may have a different level of therapeutic relevance, drug resistance, diagnostic relevance, or prognostic relevance, so the tool allows assigning the same variant a relevance level in each of the different categories: therapeutic (red), therapeutic resistance (purple), diagnostic (blue), and prognostic (green).

The classification of each variant can also be accompanied by one, two, or three explanatory texts referring to:

- **Gene**: Information on the role of the gene in cancer development.
- **Variant**: Information on and relevance of the variant type in this gene.
- **Tumor type**: Information on and clinical implications of the variant in the tumor type studied.

Furthermore, in the case of variants with clinical relevance at the therapeutic level, they can be associated with drugs or drug families.

In the *Hic Somatic Db*, Health in Code provides the categorization of somatic variants considering both the functional impact that the variant would cause at the biological level and its clinical relevance in the tumor type that the patient presents. Since genetic oncology is a field in continuous development, the information contained in this database is continually updated.

Selecting the category recommended by Health in Code in this column (DI PII for example), you access a pop-up window that allows you to consult the information registered in relation to the categorization of said variant. After reviewing this information, the user will be able to accept the recommended categorization by clicking the button "*Accept HIC category*". Automatically, the accepted category will become part of the *Category* column, corresponding to the category registered in the user's database.

When the category specified in the *Category* column does not match the one recommended by Health in Code's database, the user will be alerted with the following alert icon ⚠ :

Figure 38. An example of when the category assigned by the user does not match the one recommended by Health in Code

After analyzing the samples, it is possible to generate a file containing the selected variants, either as a .csv file or as an automatic .pdf report. In order to do so, after pressing the "*Report*" button, the analysis will end, after a final review of the variants to be included in the report. Next, the "*Interpretation*" tab will be enabled, in which the user can fill in information about the clinical context of the sample, the interpretation of the results, indications regarding the presence of pertinent negatives, and data of the signers of the report. Once the desired fields have been completed, the automatic report can be downloaded.

Should you have any doubt about the results analysis, please contact Health in Code's technical support, and your issue will be responded to within 24 hours.

09 Troubleshooting

A list of possible unexpected results throughout the library preparation and sequencing protocol using Targeted Liquid Biopsy OncoKit and the steps to follow for their solution are outlined below. For all other general issues related to the Magnis instrument and not listed in this section, please see the instrument user guide for the instrument:

Using the touchscreen for run setup presents usability issues:

As an alternative to the touchscreen controls, it is possible to use a mouse connected to either of the two USB ports located on the front of the instrument. Once connected, it can be used to make selections on the interface displayed on the touchscreen.

To reset touchscreen functionality, it is necessary to reboot the system.

The instrument's LED indicator lights turn red and the touchscreen displays the error message "*Teach points are shifted. Please perform auto teaching from the Settings screen*":

This error message appears when the Instrument Health Check (IHC) has not passed one of its teachpoints, indicating that the teachpoint markers may be obscured or that the instrument needs to perform an Auto Teaching teachpoint routine before setting up a run. To prepare the instrument for a run, do the following:

01 Verify that all deck positions are cleared of kit consumables and other debris. The presence of any materials on the instrument can prevent the successful detection of all verified teachpoint markers.

02 Clean the barcode scanner window according to the cleaning instructions in the *Magnis Instrument User Guide*. Debris or fingerprints on the scanner can obscure the teachpoints, consequently causing verification failure.

03 Reboot the system. After logging in, the instrument will perform another IHC. If this health check is successful, you can resume the setup process without performing the *Auto Teaching* routine.

If the IHC is unsuccessful, an *Auto Teaching* routine should be completed following the steps below:

01 On the *Home* screen, open *Settings* and press *Auto Teaching*. Follow the instructions displayed on the touchscreen. The *Auto Teaching* process takes approximately 30 minutes and requires the presence of the user to place the labware on the instrument.

02 Once the *Auto Teaching* process is finished, start configuration of the assay by pressing "*Run Protocol*" on the *Home* screen.

The instrument's LED indicator lights turn red and the touchscreen displays an *Instrument Health Check (IHC)* failure message:

The instrument should be restarted after a failed IHC, following the steps below:

01 In the error dialog box, press "*Cancel*" to reject the start of the diagnostic test.

02 Press the error icon at the bottom of the screen and record the error code for potential use in troubleshooting with *Agilent Technical Support*.

03 Turn off the instrument by pressing the power button on the front of the instrument.

04 Verify that all deck positions are cleared of kit consumables and other debris. The presence of any materials on the instrument may interfere with IHC after restarting.

05 Turn on the instrument by pressing the power button.

06 After logging in, the instrument will perform another *IHC*. If this check is successful, begin run setup. If the *IHC* fails again, contact *Agilent Technical Support* for assistance.

The *Verify Labware* screen reports an issue with one or more labware components after automated labware verification:

If all or most of the labware failed verification, the scanner window may need to be cleaned. See the *Instrument User Guide* for cleaning instructions. Once cleaning is finished, repeat the *Verify Labware* step.

If only one or a few labware components failed verification, then press the error icon at the bottom of the screen and expand the information for the failed position to view the reason for the failure.

- ***If the barcode scanner failed to scan a specific labware component:***

  Verify that the labware is present at the required deck position and oriented correctly (review section 7 of this document for complete deck loading steps). Should there be any positioning errors, correct them and repeat the *Verify Labware* step. If the failed components are present and correctly positioned, visually inspect the barcode for integrity. For successful scanning, barcodes must be free of scratches, smudges, condensation, obstruction by foil seals, and writing, or other marks on the plasticware. Should any barcode be damaged, replace the component and repeat the *Verify Labware* step.

- ***The scanned labware is past its expiration date:***

  Replace any expired components with unexpired components and repeat the *Verify Labware* step.

- ***The scanned labware is incorrectly positioned:***

  Replace the wrong laboratory material with the correct component and repeat the *Verify Labware* step.

Touchscreen displays *Time Remaining* of 0:00 at end of run for a period of time and does not proceed to completed run/sample collection screens:

The *Time Remaining* value displayed on the touchscreen is only an estimate of the remaining time, and this counter may remain at 0:00 for several minutes before the system is ready to proceed with sample collection. This is not indicative of an issue with the run or the instrument.

- **Sizes greater than expected after DNA fragmentation:**

  Any bubble on the filament of the microTUBEs of the sonicator may interfere with fragmentation. Make sure there are no bubbles before proceeding with fragmentation, shortly centrifuging the microTUBEs if necessary.

- **Pre-capture library fragment size greater than expected:**

  In libraries prepared from highly degraded DNA from paraffin-embedded tissue samples, and particularly ctDNA, it is possible to obtain smaller than expected fragment sizes. This is due to the fact that the starting DNA is highly fragmented, and the size of these fragments is smaller than the final size of the libraries.

- **Low yield of post-capture libraries:**

  Verify that the input DNA sample meets the specified guidelines for quality and concentration range.

  Verify that the run has been set up for the appropriate input DNA concentration and quality. On the *Run Setup* tab of the *Post-Run Data* screen, you can review the different settings for the runs performed.

  Ensure that runs are completed in humidity conditions between 30% to 70% (non-condensing). Operating the system at humidity levels outside of this range can impact performance.

  Very low or even zero yield for one or more samples in the run may indicate an issue with the pipette tips used in the run. To perform the protocol correctly, all tip boxes must be completely filled, seated flat and within the raised-tab frames of the platforms.

10 Limitations

10.1 | Analytical

- The employed technique cannot distinguish between regions with high sequence homology, such as homologous genes, pseudogenes, etc., which can lead to false positives or negatives. A list of pseudogenic regions can be found in Table 8. In the analysis of results, the label "*Pseudogenic_homology*" will appear in the column "*Fault summary*" whenever a variant is detected in a region of homology with pseudogenes.

Chromosome	Initial position	Final position	Gene	Exon	Reference sequence
3	178935987	178936081	*PIK3CA*	EX10	NM_006218.2
3	178937348	178937446	*PIK3CA*	EX12	NM_006218.2
3	178937728	178937850	*PIK3CA*	EX13	NM_006218.2
3	178938830	178938880	*PIK3CA*	EX14	NM_006218.2
7	140439611	140439722	*BRAF*	EX17	NM_00433.4
7	140481374	140481467	*BRAF*	EX11	NM_00433.4

Table 8. List of pseudogenic regions IN=Intron

- The correctness of any obtained result with quality parameters below the established criteria cannot be ensured.
- NGS is not yet considered the *Gold Standard* for some types of mutation; therefore, it is recommended, whenever possible, to confirm positive results using a complementary standardized technique.
- With Targeted Liquid Biopsy OncoKit, it is possible to detect germline variants of potential clinical interest. If a germline origin is suspected, validation of the result in the patient's blood and specific genetic counseling for hereditary cancer to evaluate the need of family study are recommended.
- All the information on drugs or clinical trials should be taken as a reference for clinical evaluation, and in no case is there evidence that its use would result in appropriate treatment for each specific patient.
- All the obtained data and information must be clinically evaluated and interpreted by the oncologist in an integrated way, together with the rest of the patient's clinical information and any other results of complementary analytical or imaging tests.

10.2 | Equipment

Targeted Liquid Biopsy OncoKit has been validated using the following automated library preparation system:

- Magnis NGS Prep System by Agilent Technologies (cat. no. G9710AA)

Targeted Liquid Biopsy OncoKit has been validated using the following massive sequencing platform:

- NextSeq 500/550 Dx System (Illumina)

  This kit is compatible with **Illumina**'s massive sequencing platforms only. If massive sequencing equipment other than the *NextSeq 500/550Dx System* is used, the final concentration of the libraries must be adjusted according to the instructions of the specific protocols of these platforms.

10.3 | Reagents

Targeted Liquid Biopsy OncoKit has been validated using the reagents included in the kit and those recommended in section 6 of this manual (*Equipment, reagents and materials not included in the kit*).

For NGS sequencing, it is advised to use the reagents recommended by the sequencer manufacturer: **Illumina**.

Should you have any questions, please contact Health in Code's technical support team.

10.4 | Bioinformatics analysis platform

Targeted Liquid Biopsy OncoKit has been validated using **Data Genomics**, which is a platform for *in vitro* bioinformatics analysis. This platform includes a pipeline tailored specifically for Targeted Liquid Biopsy OncoKit, which enables the detection of all the targets specified in section 2 of this document.

If a different analysis platform is used, Health in Code cannot guarantee the correctness of the results.

10.5 | Product stability

Optimal performance of this product is achieved provided that the specified recommended storage conditions are applied, within the product's expiration date associated with each production batch.