Instructions for use

Action OncoKitDx (Automatic)

Ref. IMG-365

CE IVD

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Code: HIC-PT-KIT 03-F-03 V.01

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Our products are intended for *in vitro* diagnostic use. Health in Code S.L. 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 S.L. 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 S.L. products undergo strict quality control. Action OncoKitDx (Automatic) 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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Instructions for Use (IFU) modifications		
Version 17	FEB 2023	Update of section 8.7 on how variant filtering works
Version 16	NOV 2022	Change of the manufacturer's address: Health in Code S.L., Calle de la Travesía s/n, 15E Base 5, Valencia 46024, Spain.
Version 15	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 14	SEP 2022	Update of the quality criteria for the analysis of SVs and the acceptance criteria for the analysis of MSI (sections 8.4 and 8.5).
Version 13	JUL 2022	Change of manufacturer's identification from Imegen to Health in Code.
Version 12	FEB 2022	Update of section 7.2.2 number 7 of Enzymatic Fragmentation.
Version 11	NOV 2021	Update of section 2, 7.7 and 8.2.
Version 10	OCT 2021	Update of analyzed regions included in the kit.
Version 09	SEP 2021	Update of section 7, 8 and 10.
Version 08	SEP 2021	Update of the protocol for the Magnis system, applicable from batch 36521C007.
Version 07	SEP 2021	Update of sections 5, 8, and 10.

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01 General information

The term 'cancer' refers to a wide and very variable group of diseases characterized by uncontrolled growth of certain body cells which spread to other tissues in the body. There is a wide range of causes that trigger the development of cancer, which often results from the interaction of a great number of risk factors. These risk factors generate variations in genes and in the genome, which result in loss of control of certain biological processes and, in turn, lead to uncontrolled cell growth.

Traditionally, different types of tumors have been classified depending on the affected organ. However, there is a recent trend to categorize tumors based on their genetic profile, which can provide valuable and more accurate diagnostic and prognostic information, such as establishing personalized therapies that can be more efficient and less toxic.

References

- *J Mol Diagn. 2017 May;19(3):341–365. doi: 10.1016/j.jmoldx.2017.01.011. Epub 2017 Mar 21.*
- *NCCN Clinical Practice Guidelines in Oncology. Breast Cancer, Version 4.2017.*
- *NCCN Clinical Practice Guidelines in Oncology. Lung Cancer Screening, Version 3.2018.*
- *NCCN Clinical Practice Guidelines in Oncology. Anal Carcinoma, Version 3.2018.*
- *NCCN Clinical Practice Guidelines in Oncology. Rectal Cancer, Version 2.2018.*
- *NCCN Clinical Practice Guidelines in Oncology. Uterine Neoplasms, Version 1.2018.*
- *NCCN Clinical Practice Guidelines in Oncology. Bladder Cancer, Version 5.2017.*
- *NCCN Clinical Practice Guidelines in Oncology. Pancreatic Adenocarcinoma, Version 2.2017*

02 Intended use

Action OncoKitDx (Automatic) has been designed to test simultaneously all the biomarkers for treatment that are currently available on the market and approved by international agencies, such as the FDA (*Food & Drug Administration*) or the EMA (*European Medicines Agency*). The panel also includes the analysis of those genetic alterations for which there are clinical trials under development and that, not having actionable therapy recommended, could open a second treatment pathway for the patient. Action OncoKitDx (Automatic) also tests for alterations in other genes that have genetic and prognostic value and that have experimental evidence as a therapy target.

To achieve this comprehensive vision of the tumor, Action OncoKitDx (Automatic) is the first test on the market that via next generation sequencing (NGS) integrates:

- Complete sequencing of the exonic regions of the following **55 genes**:

ALK, ARID1A, ATM, ATRX, BAP1, BRAF, BRCA1, BRCA2, CHEK2, CDH1, CTNNB1, EGFR, ERBB2, ESR1, FGFR1, FGFR2, FGFR3, FGFR4, GNA11, GNAQ, H3F3A, HIST1H3B, HIST1H3H, HRAS, IDH1, IDH2, KIT, KRAS, MAP2K1, MET, MLH1, MSH2, MSH6, MTOR, MYC, NRAS, NTRK1, NTRK2, NTRK3, PALB2, PBRM1, PDGFRA, PIK3CA, PMS2 + 5'UTR, PTEN, POLD1, POLE, RET, ROS1, SDHA, SDHB, SDHD, TERT + 5'UTR, TP53 and VHL.

- Sequencing of **hotspot regions** of the *TSC1* and *TSC2* genes, in a total of 36 regions, as well as of the E17K variant in *AKT1*.

- Capture of **11 fusion genes** with all possible rearrangements covered:

  To this end, Action OncoKitDx (Automatic) includes those intronic regions where breakpoints have commonly been identified in the literature. Said genes and covered regions are the following: ***ALK*** (intron 19), ***ATP1B1*** (introns 3 and 4), ***BRAF*** (introns 7, 8, 9, and 10), ***EGFR*** (introns 7, 23, 24, and 25), ***ETV6*** (introns 4 and 5), ***FGFR2*** (intron 17 and the 3'UTR region), ***FGFR3*** (intron 17 and the 3'UTR region), ***NTRK1*** (introns 8, 9, 10, 11, and 12), ***NTRK2*** (introns 12 and 15), ***RET*** (introns 9, 10, and 11), and ***ROS1*** (introns 31, 32, 33, 34, and 35)

- Microsatellite instability testing (**MSI**) through a 110-microsatellite panel that allows detecting MSI based on NGS results. MSI testing with this panel corresponds with the Bethesda panel in > 99% of cases.

- Detection of copy number variations (**CNVs**) in the whole genome, allowing for the detection of alterations such as 1p/19q or 1q/16p. Moreover, this analysis has been improved using a low-density SNP array through the capture of >500 SNPs distributed across the genome. This allows both validating the obtained results and detecting alterations where loss of heterozygosity has occurred, but the copy number has been neutralized by a duplication (**copy-neutral LOH**).

Action OncoKitDx (Automatic) also includes the detection of **pharmacogenetic** variants directly involved in response to chemotherapy treatments, which provides optional guidance for dose adjustment in each particular case. Different alterations that may affect response to treatment of tumors of different origins are tested for in 20 genes.

The different variants included in the pharmacogenetic testing of Action OncoKItDx (Automatic) are: ***DPYD*** (rs3918290, rs67376798, rs55886062, rs115232898, and rs75017182), ***UGT1A1*** (rs4148323), ***CYP2D6*** (rs5030655), ***MTHFR*** (rs1801133), ***TPMT*** (rs1142345 and rs1800460), and ***CYP2C9*** (rs1799853 and rs1057910).

Action OncoKitDx (Automatic) offers the most comprehensive solution on the market for guiding the treatment of patients with solid tumors, as it can be adjusted to all currently approved targeted treatments and those clinical trials that are in advanced stage.

Action OncoKitDx (Automatic) analyzes clinical cancer samples via a protocol that integrates high-sensitivity capture of the regions of interest with hybridization probes, with molecular barcoding of each DNA fragment with a unique adapter and subsequent 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.

Action OncoKitDx (Automatic) is intended solely for *in vitro* diagnostics and is aimed at professionals in the sector of molecular biology.

03 Technical characteristics

Action OncoKitDx (Automatic) has been validated on the **Illumina**'s NextSeq 500/550 System platform via the analysis of DNA reference samples from the Coriell Institute and Horizon Dx, as well as of patient samples that had been previously genotyped with other techniques. In this validation, the specific detection of the variants present in the selected genes (see above) and the repeatability and reproducibility of the technique were verified.

The protocol employs 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: DNA from peripheral blood or fresh, frozen, or paraffin-embedded tissue.
- Necessary amount of DNA: 50-200 ng.
- Detection of microsatellite instability when at least 85 of the 110 markers included are analyzable.
- Limits of detection:
  - The detection limit of the analysis of structural and point variants, small insertions, and deletions is 5%.
  - The detection limit of CNV analysis is three copies for duplications and one copy for deletions, as long as CNV is present in 30% of the cells in the sample.
- Mean coverage: 2500X
- Mean coverage after UMI analysis: 1600X.
- Coverage: 99.3% of bases covered at a depth of 100X.
- Uniformity: 98.9% of the covered bases at >20% of the mean coverage.
- Specificity: > 99%
- Sensitivity: > 99%
- Repeatability: > 99.9%
- Reproducibility: > 99.9%

Health in Code S.L. is certified against the norm **UNE-EN ISO 13485:2018 Medical devices: Quality management systems - Requirements for regulatory purposes** by the SPANISH AGENCY OF MEDICINES AND MEDICAL DEVICES (AEMPS) for the design, development, and production of medical devices for *in vitro* diagnostic use:

- Genetic testing kits
- Software for the bioinformatics analysis of genetic data

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 legally approved waste containers established by the legal norm and manage their treatment through an authorized waste management facility.
- In the case 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 S.L.
- The manufacturer does not guarantee the assay's reproducibility when the user uses reagents that have not been validated by Health in Code S.L. 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:

- **Fragmentation Buffer**: Buffer required for DNA fragmentation prior to NGS library preparation.
- **Fragmentation Enzyme**: Enzyme required for DNA fragmentation and preparation prior to adaptor binding.
- **Elution Buffer**: Buffer to elute DNA.
- **Reagents 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/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 48 indexes distributed in single-use strips.
- **Action Probes 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.
- **Sample Input Strips**: 8-well strips for sample DNA.
- **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 the 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.

Kit components are listed below:

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

Table 1. Reagents of box 1 of Action OncoKitDx (Automatic)

Box 2 of 4			
Reagents	Color indicator	Quantity	Conservation
Fragmentation Buffer	Green cap	54 µL	-20°C
Fragmentation Enzyme	White cap	27 µL	-20°C
Reagents Plate	Black/White	3 plates	-20°C
Index strip*	Black	3 strips	-20°C

Table 2. Reagents of box 2 of Action OncoKitDx (Automatic).

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

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

Table 3. Reagents of box 3 of Action OncoKitDx (Automatic).

"Magnis Empty Consumables" box. 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 of box 4 of Action OncoKitDx (Automatic).

NOTE: Each kit includes three *"Magnis Empty Consumables"* boxes, one for each 8-sample run on the Magnis system.

06 Equipment, reagents and material not included in the kit

Equipment:

- **Optional**: Sonicator (recommended: ME220 Focused-ultrasonicator™; Covaris) or thermal cycler with adjustable temperature lid
- 10 µL, 20 µL, 200 µL, and 1000 µL micropipettes
- Vortex (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)

Reagents:

- Extraction kit (recommended: QIAamp DNA Investigator Kit; cat. no. 56504; Qiagen)
- 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), and Genomic DNA ScreenTape (cat. no. 5067-5365).*
  - *DNA 1K/ 12K/ High Sensitivity Reagent Kit (cat. no. CLS760672; PerkinElmer).*

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

Materials:

- Filter pipette tips (10 µL, 20 µL, 200 µL, and 1000µL)
- 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).*
  - *DNA 1K/ 12K/ Hi Sensitivity Assay LabChip (cat. no. 760517; PerkinElmer)*

NOTE

Action OncoKitDx (Automatic) is prepared to be used in combination with the *Health in Code-Sample tracking components* (REF: IMG-340) kits, which allow for the tracking of each sample from DNA dilution to bioinformatics analysis of the results via an integrated system for sample identification. This way, the traceability of samples during the whole protocol can be guaranteed. These references are available upon request..

Action OncoKitDx (Automatic) is prepared to be used in combination with the *Health in Code - gDNA Reference Samples* (REF: IMG-368) kit, which includes good-quality germline DNA, both male and female, used for normalization necessary for CNV analysis.

07 Assay protocol

The reagents included in Action OncoKitDx (Automatic) that are to be used by the *Magnis NGS Prep System* come pre-dosed for 24 libraries, used in 3 assays with 8 libraries each, thus optimizing the performance of the equipment.

Action OncoKitDx (Automatic) can analyze samples of different qualities, from good-quality DNA to DNA from paraffin-embedded tissue samples, which often have their quality compromised. However, the quality and quantity must be similar across samples within a single Magnis assay.

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 have fastq files of germline reference samples of both sexes available, Health in Code S.L. provides the user with the IMG-368 kit, which contains one female and one male sample, which should be processed with Action OncoKitDx (Automatic).

The steps necessary to carry out the preparation of 8 libraries using Action OncoKitDx (Automatic) are outlined below.

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

01 Ensure that there is no material from previous assays on the instrument unit, as it could interfere with the initial steps and configuration of the instrument.

02 Close the equipment's door.

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

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

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

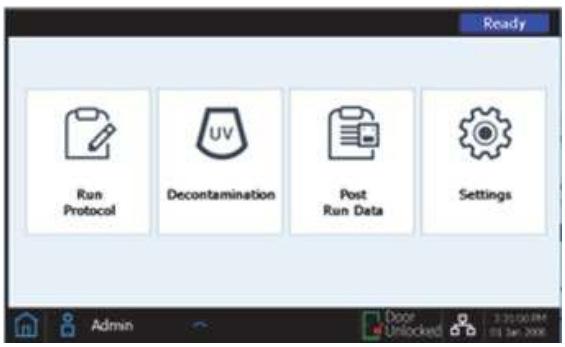


Figure 1. Magnis NGS Prep system Home screen.

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

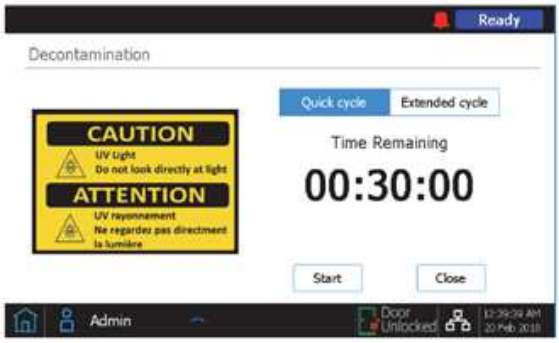


Figure 2. Magnis NGS Prep Decontamination screen.

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

NOTE: During the 30 minutes it takes for the decontamination process to end, 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 and fragmentation of the DNA template strand

The steps necessary for the preparation of 8 samples using Action OncoKitDx (Automatic) 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.

07.2.1 | Quantification of DNA samples

01 Thaw DNA samples at room temperature.

02 Mix on a vortex mixer and quantify DNA samples on a fluorometer, such as Qubit.

03 Use any of the following methods for DNA quality control:

- ↘ Quality analysis using Agilent's Genomic DNA ScreenTape together with the accompanying reagents, following the manufacturer's instructions for the use of the instrument and the kit.
  This analysis allows determining the DNA integrity of the sample and provides a DNA integrity number (DIN) for each sample.
- ↘ Quality analysis using PerkinElmer's LabChip GX Touch/GXII Touch together with the accompanying reagents, following the manufacturer's instructions for the use of the instrument and the kit.

This analysis allows determining the DNA integrity of the sample and provides a quality value (DQS) for each sample.

> NOTE: DNA samples that are too degraded may result in decreased specificity and sensitivity of the analysis. This is why Health in Code S.L. recommends the use of DNA with DIN greater than 3 if TapeStation is used, and DQS greater than 1.5 if LabChip is used.

07.2.2 | DNA fragmentation and dilution

OPTION A. MECHANICAL FRAGMENTATION

Requires a sonicator (recommended: ME220 Focused-ultrasonicator™; Covaris).

In this step, DNA is enzymatically fragmented to obtain DNA fragments of between 150 and 200 bp in size.

01 Dilute each DNA sample with *Elution Buffer* to obtain a concentration of a total of 50, 100, or 200 ng, to a final volume of 50 µL.

Optional: If Health in Code's integrated tracking system used (*Sample tracking components*; Ref. IMG-340), carry out this step by substituting 2 µL of *Elution Buffer* for the same quantity of one of the twelve sample tracking reagents.

Optional: If the reference samples included in the IMG-368 kit are processed, proceed with step 2.

> NOTE: It is recommended to use the highest amount possible of DNA within the 50-200 ng range. Using DNA samples at lower concentrations may result in decreased specificity and sensitivity of the analysis.

02 **Optional**: For the processing of the reference samples included in the IMG-368 kit, for a concentration of 25 ng/µL, add in a 1.5 mL tube:

- 8 µL of the reference sample.
- 2 µL of the tracking reagent used (or *Elution Buffer* if the integrated tracking system is not used).
- 40 µL of *Elution Buffer*.

03 Transfer the 50 µL of the DNA sample to a 130 µL microTUBE (Covaris sonicator consumables) through the lid's partitions. Verify that there are no bubbles.

04 Place the 130 µL microTUBE in the sonicator.

05 In order to obtain a size between 150 and 250 bp, following configurations as in Table 5, at a temperature between 2-8 °C.

Configuration	Value
Time (in seconds)	430
Peak Power	50
Duty Factor (%)	20
Cycles per burst	200
Avg Power	10

Table 5. Optimal fragmentation program for Covaris ME220.

NOTE: If using other Covaris instruments, follow the manufacturer's recommendations to obtain the same fragment size.

06 Once the program has ended, spin the tube.

07 Immediately transfer the whole volume from each 130 µL microTUBE to a corresponding *Sample Input Strip* well, seal with the provided aluminum seals, and keep cold until use in the next step.

NOTE: The sample must be placed in the *Magnis NGS Prep System* device as shown in Figure 3, with Sample 1 loaded in the well located farthest from the barcode.

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

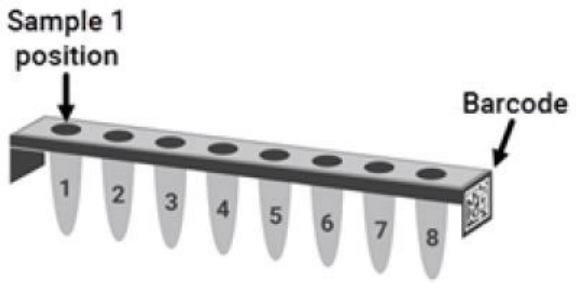


Figure 3. Required placement of samples in the Sample Input Strip.

08 **Optional**: Health in Code S.L. recommends verifying the size of the obtained libraries using Tapestation 2200 and the commercial kits High Sensitivity D1000 Reagents (cat. no. 5067-5585) and High Sensitivity D1000 ScreenTape (cat. no. 5067-5584) by Agilent Technologies. After the analysis of the samples, the expected size of the library is approximately 150-250 bp. In the case of obtaining an unexpected size, review the protocol or contact Health in Code's technical support team.

OPTION B. ENZYMATIC FRAGMENTATION

Thermal cycler with adjustable temperature lid.

Reagents to use in this step:

Reagent	Color	Conservation
Fragmentation Buffer	Green cap	-20°C
Fragmentation Enzyme	White cap	-20°C

01 Thaw and keep cold the *Fragmentation Buffer*. Keep the *Fragmentation Enzyme* at -20 °C until its use.

02 Dilute each DNA sample with nuclease-free water to obtain a total concentration of 50, 100, or 200 ng, to a final volume of 7 µL, in a 0.2 mL tube strip.

If it is not possible to obtain the total ng necessary in a volume of 7 µL, increase the final volume to 12 µL..

03 **Optional**: For the processing of the reference samples included in the IMG-368 kit, for a concentration of 25 ng/µL, add to the corresponding 0.2 mL tube on the strip:

- 8 µL of the reference sample.
- 1 µL of the used tracking reagent.

04 Mix all dilutions on the vortex mixer, spin, and keep cold until their use.

05 Prepare the required volume of the fragmentation mix in cold, as described below, mixing each reagent before use. The *Fragmentation Buffer* should be mixed vigorously on a vortex while the *Fragmentation Enzyme* is mixed by inversion several times. When processing several samples, we recommend preparing the reagent mixes with an excess of 12%.

Reagent	Volume per reaction	Volume (8 samples)
Fragmentation Buffer	2 µL	18 µL
Fragmentation Enzyme	1 µL	9 µL

06 Mix vigorously on a vortex mixer.

07 Add 3 µL of the fragmentation mix to each 0.2 mL tube with the sample. Mix by pipetting 20 times.

08 Seal the strip, spin the samples, immediately after place tubes in the thermal cycler and execute the fragmentation program to obtain a size between 150 and 200 bp.

- Pre-heat lid to 100 °C.
- Reaction volume 10 µL.

Temperature	Time	Cycles
37°C	15 minutes	1
65°C	5 minutes	1
4°C	∞	

Table 6. Optimal fragmentation program.

NOTE: This program requires that the lid be pre-heated to 100 °C. When using thermal cyclers with high ramp rates, such as the one used during the validation of this protocol, GeneAmp PCR System 9700 (ThermoFisher), it is not necessary to pre-heat the lid. If this is not your case, pre-heat the lid for a few minutes before commencing the protocol.

09 Once the fragmentation program ends, remove samples from the thermal cycler, spin, add 40 µL of nuclease-free water to each sample, transfer all volume to a *Sample Input Strip*, seal with included aluminum seals, and keep cold until its use in the next step.

NOTE: The sample must be placed in the *Magnis NGS Prep System* device as shown in Figure 3, with Sample 1 loaded in the well located farthest from the barcode.

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

07.3 | Preparation of the reagents and plastic materials used by the Magnis system

Reagents to use in this step:

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

01 Preparation of the *Reagents plate* reagent:

- Thaw the plate at room temperature while keeping its white packaging.
- Once the contents of all wells are thawed, mix the plate on the vortex while keeping it in its white carton. Begin by pressing the long side of the plate against the vortex head for 10 seconds. Afterwards, rotate the plate 90° and press the short side of the plate against the vortex head for another 10 seconds. Repeat the sequence on all four sides of the plate.
- Centrifuge the plate in the box at 250 x g for 1 minute.
- Ensure that there are no bubbles at the bottom of the plate wells. If there were, centrifuge again.
- Keep plate in its packaging and in cold for its use on the same day.

02 Preparation of the *Beads and Buffer plate*:

- To ensure room temperature, leave it out in its white carton for about 30 minutes.
- Mix the plate on a vortex mixer while keeping it in its packaging. Start pressing the long side of the plate against the vortex head for 10 seconds. Afterwards, rotate the plate 90° and press the short side of the plate against the vortex head for another 10 seconds. Repeat the sequence on all four sides of the plate.
- Centrifuge the plate in the box at 150 x g for 10 seconds. Do not exceed recommended centrifugation times to prevent sedimentation of magnetic particles.
- Keep plate in its packaging at room temperature for its use on the same day.

03 Preparation of the *Index strip*:

- Determine and record the index combination that will be used in the assay. The provided strips have their combination, A1, A2, A3, or A4 marked on the end opposite 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 7. Sequences of the indexes included in the kit.

- Thaw the chosen Index strip in cold, mix for 5 seconds on a vortex mixer, and spin.
- Check the wells of the strip to verify that liquid accumulates at the bottom of the wells and that there are no bubbles.

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 the screen, open the *Labware Info* tab and search for the *Index Strip* row. The strip number is a value between 1 and 12 in the *Index Strip* column found 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 8. Correlation between the Index on the Post-Run Data screen and the inscription on the strip.

04 Immediately before it is used, thaw the *Action Probe Strip* in cold. Mix on a vortex mixer 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 legible labels that show the specific identity of the probe design. It is recommended that special care be exercised to guarantee the traceability of this reagent in both storage and during the protocol.

05 Finally, prepare a box of *Magnis Empty Consumables* to use when configuring the unit.

07.4 | Execution of the library preparation protocol

07.4.1 | Start of the protocol

01 On the Home screen, press *Run Protocol*. The system will block the instrument gate and carry out an *Instrument Health Check (IHC)*, which may last a few minutes.

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

03 **Recommended**: Check the *Aliquot sample* for QC verification box so that the equipment may take an aliquot of each pre-capture library. This will allow for its quality control later.

> NOTE: Quality control of the pre-capture libraries will only be available once the whole assay has concluded.

Figure 4. Magnis NGS Prep Enter Run Info screen.

04 Advance to the next screen.

05 Select appropriate sample type: FFPE DNA.

06 Select the starting DNA amount in the Input *Amount* menu. Even though the 10 ng, 50 ng, 100 ng, and 200 ng options appear, it is recommended that 200 ng be used (and at least 50 ng) for library preparation using Action OncoKitDx (Automatic).

> NOTE: The quality and quantity adjustment of the template DNA will determine the number of cycles of the amplifications that the equipment will later carry out, which is why it is important to enter this information correctly and to ensure that all samples have the same identical amount of starting DNA.

07.4.2 | Unit configuration

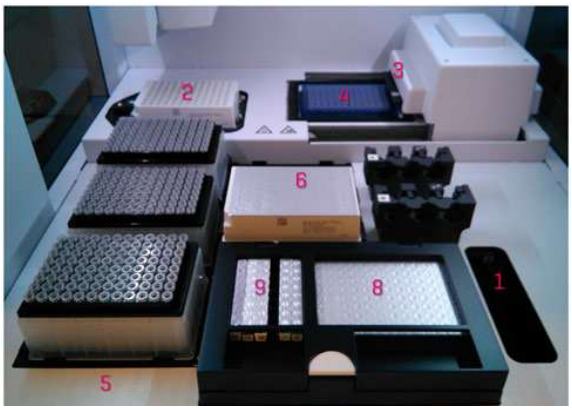
The unit configuration can be done very easily in two steps, which are indicated in the Magnis touch screen.

For each loading step of the unit, the position that has to be loaded will appear highlighted in blue on the touch screen. Once each step has been completed, advance to the next screen.

In order to guarantee the correct placement of the reagents and fungible materials in the Magnis unit, check that the barcode of each element is facing the user, i.e., towards the front part of the instrument, except in the case of the *Magnis Thermal Cycler Seal*, whose barcode is facing upward, and of the three necessary boxes of tips not included in the kit, which do not have a barcode.

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

The following figure shows a completely loaded unit, numbering each material from 1 to 10, following the steps that the Magnis equipment requests.



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

The setup steps shown on the Magnis touchscreen are outlined below:

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 drawer.

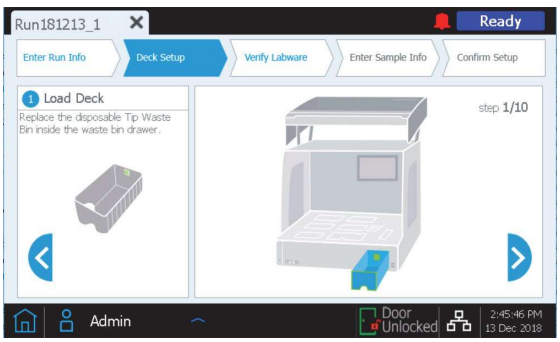


Figure 6. Step 1 of 10 on the Deck Setup screen of the Magnis NGS Prep System.

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.

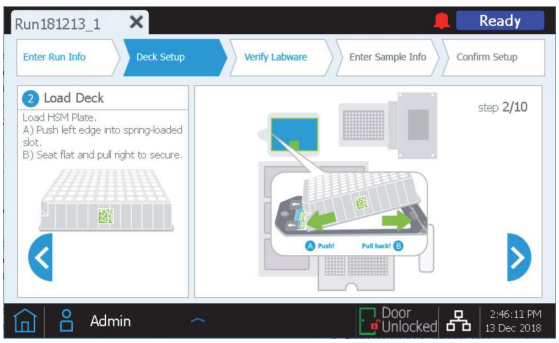


Figure 7. Step 2 of 10 of the Deck Setup screen of the Magnis NGS Prep System.

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.

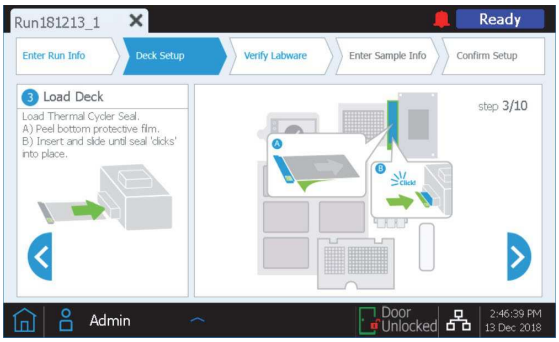


Figure 8. Step 3 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument.

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.

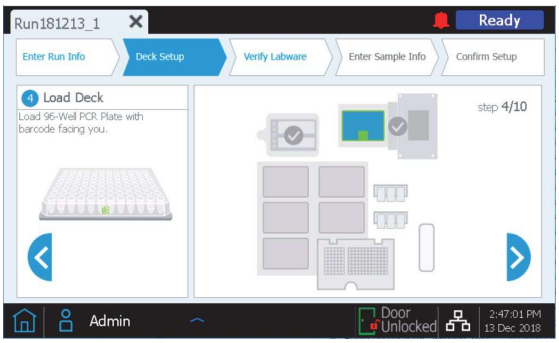


Figure 9. Step 4 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument.

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

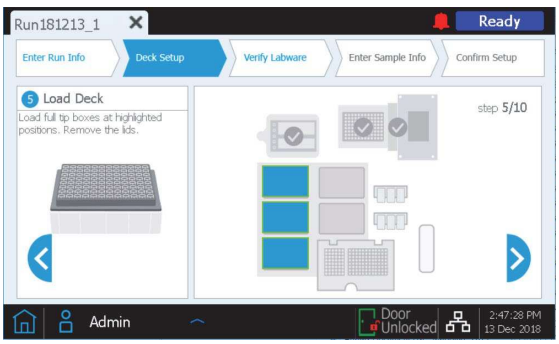


Figure 10. Step 5 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument.

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.

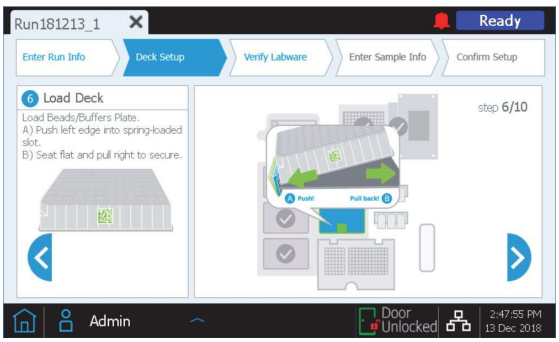


Figure 11. Step 6 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

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 12. However, if the chiller has already reached the necessary temperature, this screen will not appear.

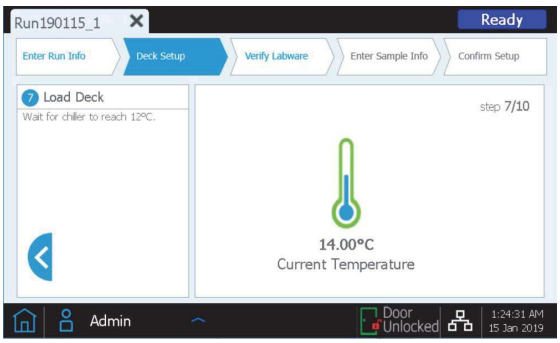


Figure 12. Step 7 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument.

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.

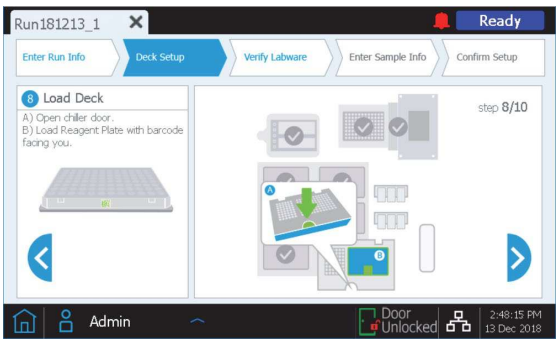


Figure 13. Step 8 of 10 of the Deck Setup screen of the Magnis NGS Prep equipment.

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. Make sure all the strip tubes have their 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 *Hereditary 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 S.L., load the *QC Strip* (blue strip), included in the "*Magnis Empty Consumables*" box, into the chiller holder position labeled with **Q**.

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

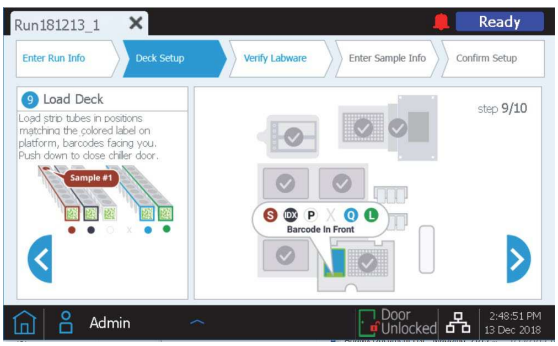


Figure 14. Step 9 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument.

10 Close the instrument door.

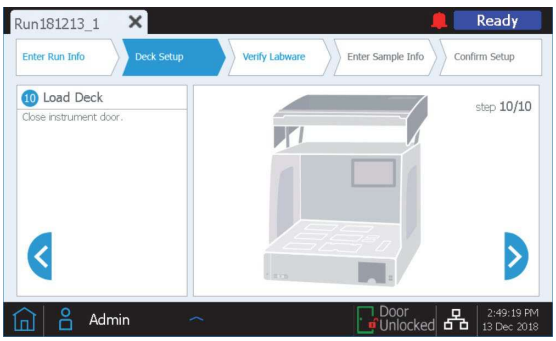


Figure 15. Step 10 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument.

07.4.3 | Laboratory material verification

Once that the loading of the equipment has concluded, proceed to the *Verify Labware* phase, in which the equipment scans the barcode of each of the components that is present in the unit.

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 material verification.

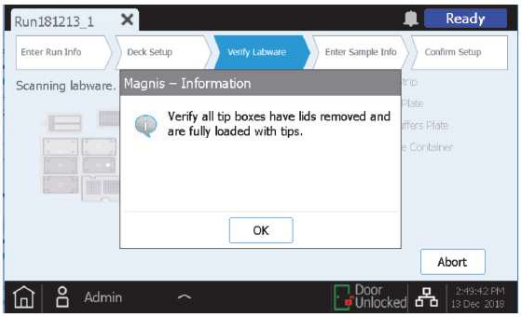


Figure 16. Verify Labware pop-up window of the Magnis NGS Prep equipment..

During the verification of the material, the instrument will verify that all the necessary components for the assay are present, are placed in the correct position and facing the right way, and that they have not exceeded their expiration date.

The verification results will be shown on the Magnis touch screen; if everything is correct (Figure 17), proceed to the following screen. Otherwise, please see section 9 of this document.

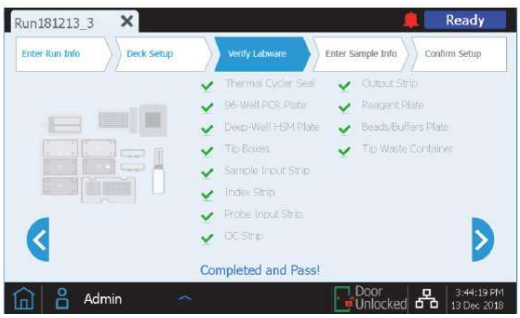


Figure 17. Verify Labware window of the Magnis NGS Prep equipment, after a correct verification of the material.

The final screen in the *Verify Labware* section allows checking the details of the probeand moving on to the next screen.

07.4.4 | Assigning sample information

The Magnis software automatically assigns a predetermined *Sample ID* for the position of each sample, which can be replaced with a sample name chosen by the user by following any of the two methods described below:

01 Manual assignment of samples:

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

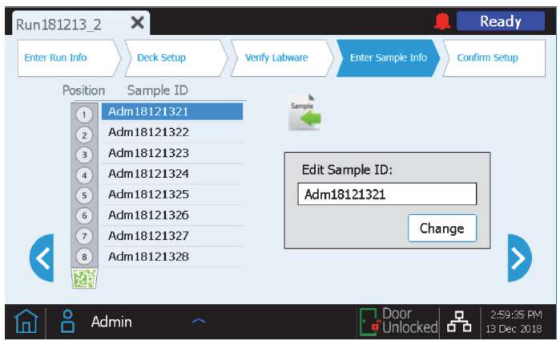


Figure 18. Edit Sample Info screen of the Magnis NGS Prep equipment; the load samples button is highlighted with a circle.

02 Importing sample assignments using a .csv file:

- Create a .csv (comma-separated values) file containing the names of the ordered samples. To type the sample name, you may use Microsoft Excel and later save it in .csv format.
- Write the header text "sample_id" in cell A1, as shown in Figure 19.

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

Figure 19. Example of .csv file content (shown in spreadsheet format) to load sample assignments.

- Type the name of each sample from cell A2 to A9. The entry file of the sample must contain 8 unique sample IDs. If you wish to run the protocol with fewer than 8 samples, you must fill in the remaining positions in the file as shown in Figure 19 (empty1 and empty 2)
- Save file in.csv format.
- Download the .csv file to an unencrypted USB drive and introduce said USB in one of the Magnis ports.
- When configuring the assay, on the *Enter Sample* Info screen, press the download samples button (highlighted with a circle in Figure 18).
- Follow the instructions of the protocol configuration assistant to transfer the sample IDs from the USB drive.

07.4.5 | Confirming the configuration and starting the assay

01 Verify the general characteristics of the assay. Once everything has been confirmed to be is correct, press the arrow forward to move on to the final configuration screen.

02 Verify the details of the assay related to DNA sample characteristics of the. After confirming that all configuration details are correct, click the Start button to begin the assay.

IMPORTANT: The number of pre- and post-capture PCR cycles are based on the amount and quality of DNA. Using different values would affect the sensitivity, specificity, and LOD of Action OncoKitDx (Automatic).

Once the assay has started, the LED indicator will be green and the touch screen will show the status of the assay, including the estimated remaining time to completion.

The *SSEL XTHS-RevB-ILM* protocol takes approximately 9 hours to complete and can work overnight for greater convenience. Once the protocol is completed, the prepared libraries will be automatically preserved at 12 °C. Recover the libraries from the instrument within a maximum period of 24 hours.

If necessary, the assay can be aborted by clicking on the *Stop* red box of the *Running* screen. A warning message will ask you to confirm whether you wish to abort the assay. Once the assay has stopped, it cannot be resumed, and the laboratory material that has been used cannot be used again for subsequent assays.

The *Running* screen must remain open during the entire assay, and the close-window button (x) and other navigation buttons will be inactive while the assay is ongoing. The touch screen cannot be used for any other actions while an assay is running.

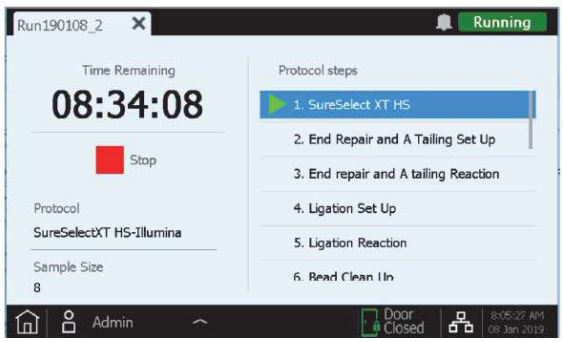


Figure 20. The Running screen during the assay.

07.4.6 | Library collection

After the assay is completed, the touch screen will appear as shown in the following figure. By clicking OK, the equipment transfers the libraries from the thermal cycler, where they have been kept since the conclusion of the protocol, to the green *Library Output Strip*, located in the cooling module.

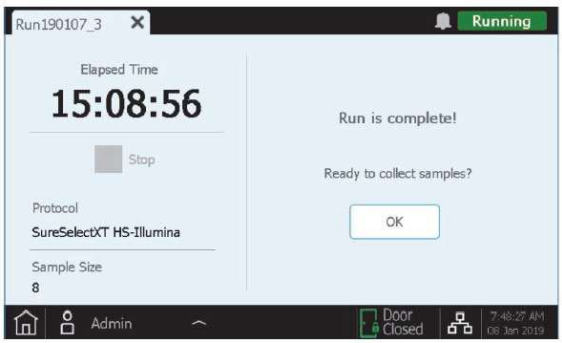


Figure 21. The Running screen after the assay.

Before opening the door, wait for the LED lights to turn blue, indicating that all the processing steps of the sample performed by the equipment have finished.

The cooling module will remain at 12 °C for a maximum of 2 hours since the libraries are placed in the green *Library Output Strip*, as long as the equipment door remains closed.

Open the equipment door (until the LED turns white) to collect and seal the libraries on the green *Library Output Strip*.

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

If the optional samples for quality control of pre-capture libraries were collected, remove the blue *QC Strip* from the cooling module and leave at room temperature to dry unsealed, if the protocol is to be continued within the next 24 hours, or sealed, for longer storage periods.

Once the door is open for the collection of the libraries, the touch screen of the equipment will appear as shown below.

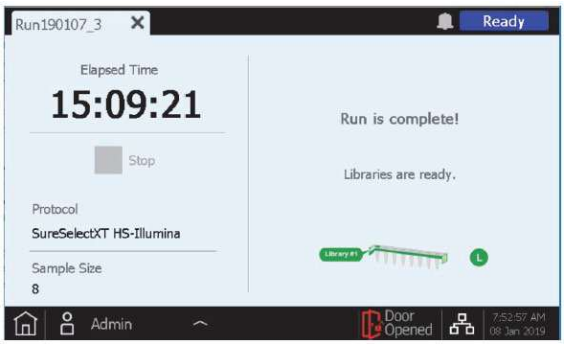


Figure 22. The Running screen after an assay with libraries already removed.

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

07.5 | Equipment cleaning after an assay

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

- Disposable container 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 and Buffer Plate*.
- *Reagent Plate*.
- Red, black, and white strips used during the assay.

If any material spills or leaks are observed on the instrument unit, it is recommended to follow the *Extended Cycle UV* decontamination 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* and 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. Lastly, mix vigorously on a vortex mixer to ensure full resuspension.

After the analysis of the samples with *TapeStation*, a library with a size between 200-400 bp should be obtained (Figure 23). In the case of obtaining an unexpected size, review the protocol or contact Health in Code's technical support team.

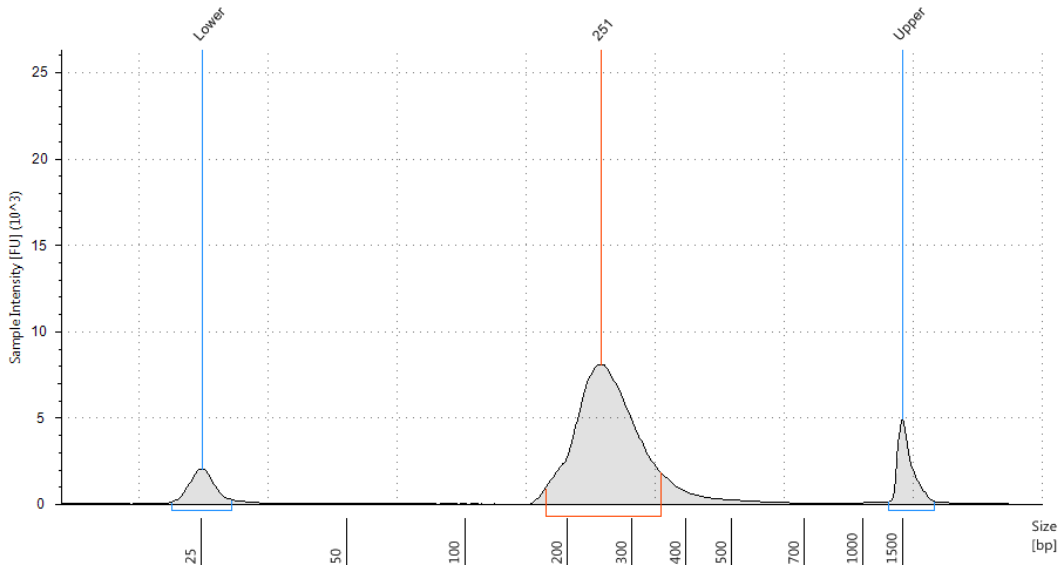


Figure 23. Expected result after analyzing the size of the pre-capture libraries with TapeStation 2200.

To determine DNA concentration, the corresponding peak area should be integrated with the expected library size. The amount of obtained library DNA will vary depending on the initial DNA concentration, ranging from **30 to 160 ng/µL**. The overall yield of the pre-capture library 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 | Optional quality control of the post-capture library

Before grouping the libraries for multiplexed sequencing, it is necessary to analyze the quantity and quality of each of them.

To measure the concentration of DNA it is recommended to use a *Qubit® 2.0 fluorometer*, the *Qubit ds DNA HS Assay* commercial kit (cat. no. Q32854), and *Qubit™ assay tubes* (cat. no. Q32856) by Invitrogen.

The concentration of post-capture libraries will vary between 5 and 20 ng/µL.

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

The mean size expected for the fragments is between **250 and 350 bp**. In the case of obtaining an unexpected size, review the protocol and the pre-capture library quality control, carefully read section 9, or contact Health in Code's technical support team.

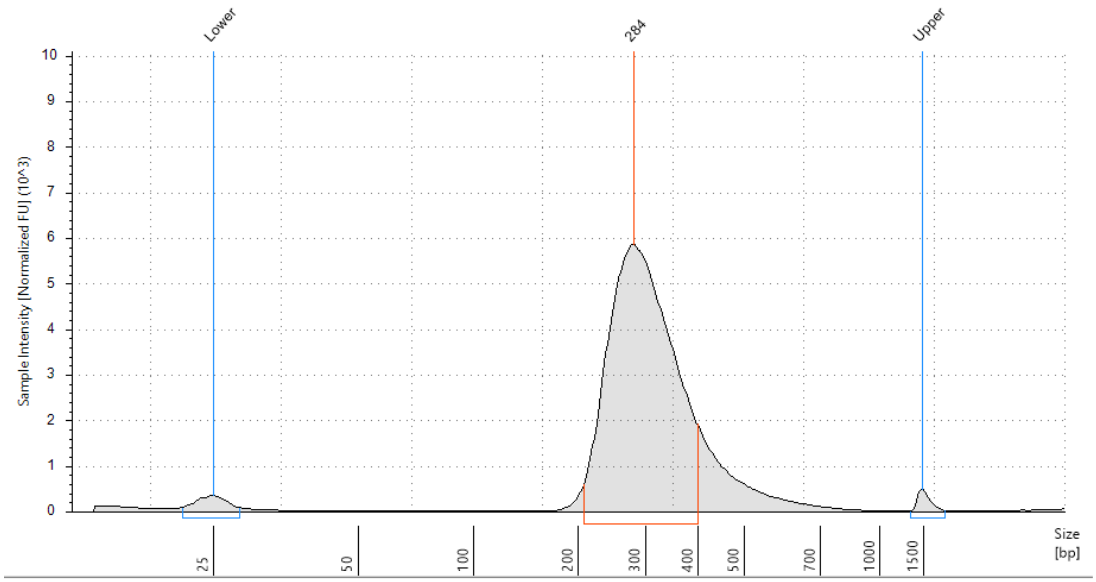


Figure 24. Expected result after the analysis of the post-capture libraries' size with TapeStation 2200.

From DNA concentration and the peak of captured library fragments size, 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 make 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 next 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 an Illumina *NextSeq* sequencer, following these steps:

01 Thaw the HT1 reagent (including the reagents kit from Illumina, with which the sequencing will be carried out) and keep cold until it is used.

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

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

03 Add 5 µL of the libraries pool, previously diluted to 4 nM, to a 1.5 mL tube and 5 5 µL of NaOH 0.2N. Mix on a vortex and spin.

04 Incubate at room temperature for 5 minutes.

05 Add 5 µL of Tris-HCl 200 mM pH 7. Mix on a vortex and spin.

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

07 Transfer 97.5 µL of the 20 pM library to a new 1.5 mL tube.

08 Add 1202.5 µL of HT1.

09 To this mix, add 1.2 5 µL of denatured PhiX control diluted to 20 pM. At this stage the library should be diluted to 1.5 pM.

10 Load all the volume that the 1.5 mL tube has inside in the cartridge.

The following table specifies the maximum number of samples per run, according to the sequencing kit that is used, to guarantee a minimum number of PF clusters of approximately 13 million per sample:

NextSeq Reagents Kit	Maximum no. of samples
NextSeq 500/550 Mid Output v2.5 kit (150 cycles). Ref: 20024904	13
NextSeq 500/550 High Output v2.5 kit (150 cycles). Ref: 20024907	32*

Table 9. NextSeq Illumina kit and maximum number of samples to be analyzed with Action OncoKitDx (Automatic).

(*) The kit includes an index to analyze 24 samples; should you need to analyze 32 samples in the same sequencing assay, contact Health in Code's technical support team.

If fewer samples are sequenced than suggested, the *FastQ* files will be bioinformatically pre-processed to generate files with a maximum of 40 million reads per library.

07.8 | *NextSeq* configuration

01 Configure the platform by executing the standalone 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 Once 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 *Sample Sheet*

The *Sample Sheet*, which is necessary for sequencing, can be generated by using the *Illumina Experiment Manager Program* (version 1.14.0), following these steps:

01 Open the program and select the option: *Create Sample Sheet*.

02 Select the option *NextSeq>Next* (or other available equipment).

03 Select *FASTQ Only>Next*.

04 For each assay, fill in the fields: *Reagent Cartridge Barcode, Library Prepr Workflow (Nextera XT), Index Adapters* (you can select any one of these, since it will be changed manually later on), *Index Reads* (2, dual) *Experiment Name, Investigator Name, Description, Read Type (Paired End),* and *Cycles* (*Read1: 75, Read2: 75*).

05 Select *Next*.

06 On the next screen, select *Add Blank Row* to add a line for each sample that is included in the assay. Fill in the fields for each sample. In the columns I7 *Sequence* and I5 *Sequence*, assign any Illumina index, as these will be modified later.

07 Select *Finish*.

08 The option to save the created *Sample Sheet* will appear; save as .csv.

NOTA: The name of the document must be SampleSheet.csv.

09 Open the generated file with a text editor and modify the following information:

- In the column I7_Index_ID, type the name of the selected index, for example: A01 for the first index.
- In the index column, enter the corresponding nucleotide sequence (see table 7), GTCTGTCA for the A01 Index.
- In the I5_Index_ID, type MolBC (molecular barcode) in all the samples.
- In the column index2, type the text NNNNNNNNNN (10 in total) for all the samples.

10 Save changes.

07.10 | Generation of *FastQ* files necessary for bioinformatics analysis

After the sequencing of Action OncoKitDx (Automatic) libraries, several .bcl files are generated, which must be de-multiplexed to generate the 12 *FastQ* files necessary for bioinformatics analysis.

For transformation of 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 the **Data Genomics** platform, using an analysis pipeline designed especially for Action OncoKitDx (Automatic). To access this tool, visit: www.datagenomics.es.

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

In samples with DIN <3 and tumor infiltration with non-tumorous cells higher than 30%, the detection limit for the different types of events (CNVs, SVs, MSI, point mutations, and small indels) can be affected and, therefore, may not be the same as established in the technical specifications.

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

08.1 | Request for testing

01 Select the "*Import Samples*" on the main screen (*Orders* tab) to begin the analysis of the sequenced samples. This way, you will access the file import screen (Figure 25). On this screen, the 12 fastq files associated with each sample must be imported; optionally, the *Sample Sheet* file, which will allow us to import simultaneously all the files from the same sequencing run, can also be imported.

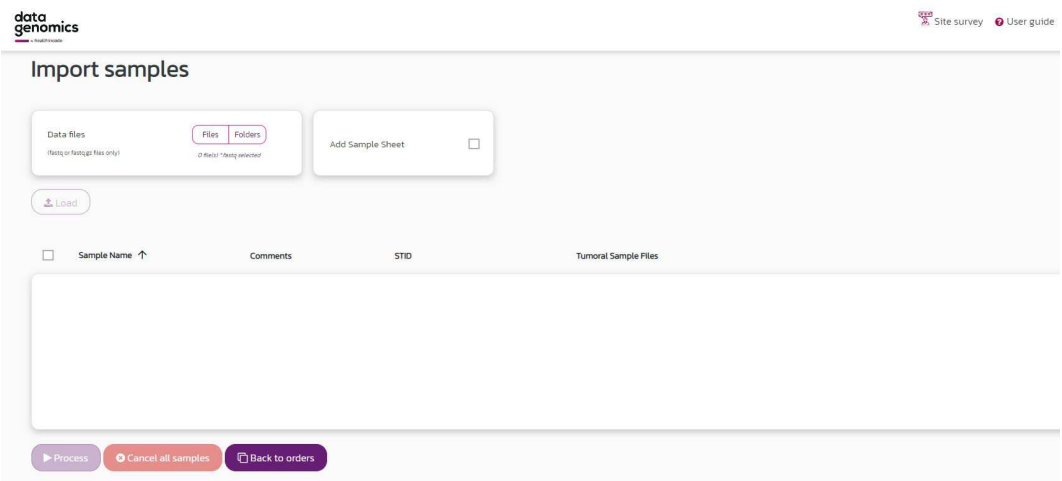


Figure 25. Screen for importing the fastq files and the sample sheet and commence the analysis request.

02 Once the files are uploaded, the sequencing run name must be indicated, and the study modality (Action OncoKitDx) and the STID (Sample Tracking ID) used for each sample (or "no stid" if none was used) must be selected.

03 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 . In addition to the required fields, there are additional fields that the user may find useful (Figure 26). Once filled in, press "*Accept*".

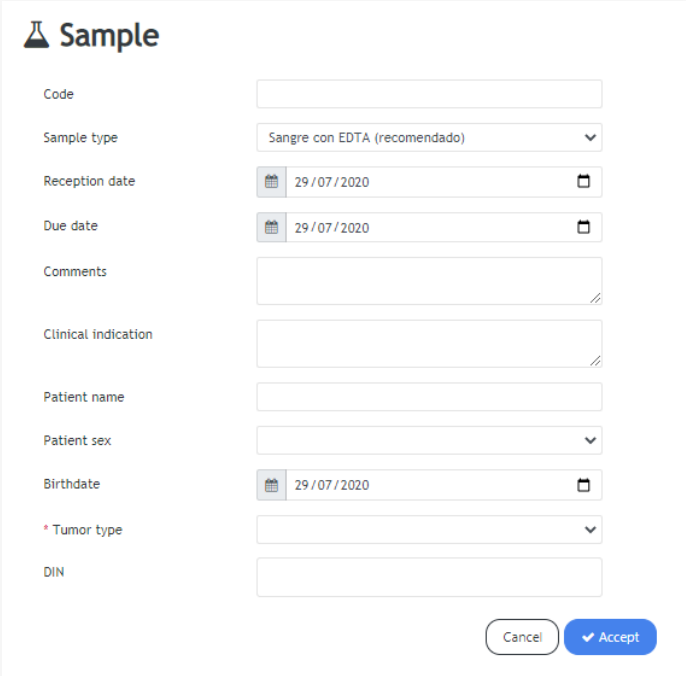


Figure 26. Pop-up screen with the required fields for each sample

> NOTE: In case that the DIN value is not optimal (DIN <3), the results report will show a warning indicating that, due to the low DIN value, the technique's LOD may be higher than the one specified in this document.

04 To carry out the analysis request, select the samples that you want to be analyzed and click the "*Process*" button. Once the process has finished successfully, the following message will appear: **√ The import has been carried out correctly**.

05 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*, *Cancelled*). The request will show the sample's name and the modality and status of the analysis.

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

To access the results of the bioinformatics analysis, select "*Show results*" in the "*bioinformatics*" order and the "*Workspace*" window will open. This screen allows the user to access the files resulting from the bioinformatics analysis: files resulting from the pharmacogenetic analysis (.pgx.tsv), microsatellite instability (.msi.txt), analysis of CNVs

(.CNVs.png and CNVs.txt) and structural variants (.SV.txt), alignment files (bam and bai), a list of variants (vcf), and other files with coverage information and the sequencing quality report after the bioinformatics analysis.

For a sample to pass the bioinformatics quality control established for the Action OncoKitDx (Automatic) assay, the parameters that are taken into account in the different files generated during sequencing are:

- *FASTQ*: The established acceptance criteria are detailed in the instructions for use of **Data Genomics**, available at www.datagenomics.es.
- BAMs:
  - *Mapped Reads* (%):
    - Fail: < 95
    - Warn: 95-98
    - Pass: > 98
  - DP100 (%):
    - Fail: < 80
    - Warn: 80-90
    - Pass: > 90
  - *On-target* (%):
    - Fail: < 30
    - Warn: 30-35
    - Pass: > 35
- STIDs: Verification that the tracking reagent obtained matches the expected one (if it has been used), as shown in figure 27.

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

Quality control for the Action OncoKitDx (Automatic) assay does not take into account the VCF files or sample heterozygosity, as this is a somatic analysis where the frequency of the identified variants can widely vary.

Feature	Obtained	Expected	Status
STID	1011	1011	PASS
Gender	Mujer	Mujer	PASS

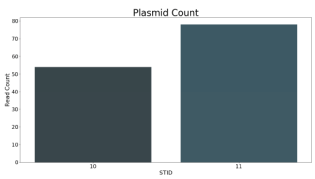


Figure 27. Quality control of the integrated tracking system

To access the variants filter, the "*Filtering*" request will apply the *Action OncoKitDx Default* filter, which is characterized by:

- Quality variants: ***PASS; d200; pseudogenic homology*** (*Fault summary*).
- Depth: **≥ 20X** (Clean total count).
- Readings with the balanced variant: **≥ 2 forward / 2 reverse**, to filter artifacts.

- Allele frequency: **≥ 5%** (*Variant Freq*). Limit of detection established for samples with DIN >3 and tumor cellularity ≥30%.
- Low population frequency: **GnomAD Freq <2%** to filter out frequent variants in the population that are not relevant for tumor formation.
- Exclusion of variants in non-coding regions and synonymous variants, except for those found in potential splice regions.
- Filtering out variants described as benign, likely benign, or non-assessable (*Clinical significance*) in our own database and/or in ClinVar.

Including in the filter, the ***HotSpot*** label (*Fault summary*) will also be shown the hot spot variants of TSC1 and TSC2, covered by Action OncoKitDx (Automatic).

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 readings in a region and the number of DNA copies of the same region.

Since the number of readings must be normalized between different readings, variability between samples will hinder CNV identification and, therefore, it is very important to homogenize as much as possible the experimental conditions among different samples and among different genomic regions from the same sample. To reduce variability and guarantee a correct CNV analysis, we suggest following these recommendations:

- The conditions for the preparation of the libraries and the capture process must be homogeneous; to this end, the different steps must be performed simultaneously for samples from the same sequencing assay, simultaneously using the same equipment and following the indications specified in section 7 of this document.
- The starting 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, which must have been processed with Action OncoKitDx (Automatic).

Action OncoKitDx (Automatic), in addition to CNV analysis of genes included in the panel, has an SNP array distributed through the genome, which can be used for CNV analysis, such as the study of copy-neutral loss of heterozygosity (*Copy-neutral LOH*).

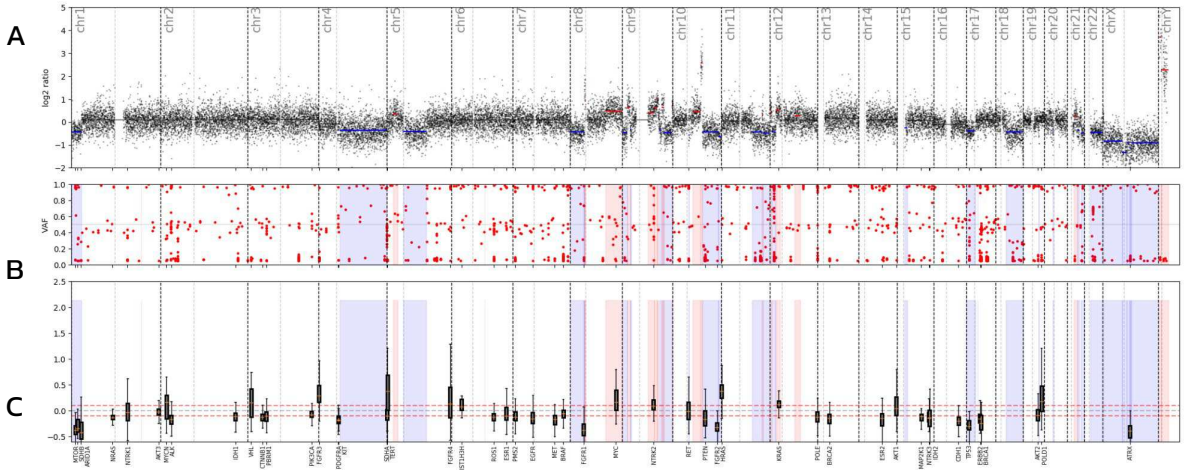


Figure 28. The log2_ratio value (A), allele frequency of the variant (B), and deviation of the normalized coverage of a gene in the tumor with respect to the coverage of that same gene in germline DNA (C) are shown on the Y-axis; and the 24 different chromosomes present in human cells and the genes included in the Action OncoKitDx (Automatic) according to their location in the genome are shown on the X-axis.
A) Results 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.
C) Analysis of CNVs 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 detection limit for CNVs is 3 copies for amplifications and ≤1 copy for deletions in samples with a tumor infiltration percentage with non-tumorous cells up to 30%.

To analyze the results of CNVs with **Data Genomics**, access the results of the "*Filtering*" request and, specifically, 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 will calculate a *Score* by taking into account parameters such as CNV size, nearby calls (<3 Mb), the value of the log2 ratio, and whether the gene or genes affected by the CNV are included in this panel; this will classify 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 28). If an excessive number of CNVs are observed, the user can select the "*Extensive chromosomal abnormalities*" option, and the increased number of copy number variations will be reflected in the report.

The calculation of the number of copies will be adjusted according to the degree of infiltration of the tumor with non-tumorous cells, a value that 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 onc	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 29. CNV analysis results.

08.4 | Analysis of structural variants (SVs)

Data Genomics analyzes structural variants based on the BAM alignment file, which is obtained after mapping the reads of the reference genome. Two types of reads are identified after mapping:

- **Split reads**: Reads whose sequence maps to two different locations of the genome. These reads also provide us with information on the exact location of the event (see Figure 30).

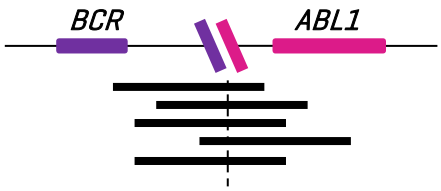


Figure 30. Reads that include the fusion point, represented as a non-continuous vertical line, and whose bases map in different regions of the genome (Split reads).

- **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 and reverse) is used to detect these types of events (see Figure 31).

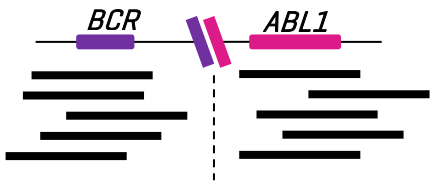


Figure 31. Reads that do not include the fusion point, represented as a non-continuous vertical line, and whose search for the sequencing pair (Discordant read pairs) allows for the translocation's identification.

The compatibility of these two detection types allows identifying structural variants with a high degree of reliability, as well as other types of rearrangements, such as deletions or duplicates, while removing possible artifacts produced during sequencing.

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

The results screen (Figure 32) shows all the detected events, classified within three quality ranges: *High, Mid,* and *Low* quality. The quality category assigned to each event will depend on the reads obtained, as shown below.

- **High quality**: if Split coverage and Mate split coverage ≥ 5, or total is ≥ 15.
- **Medium quality**: if Split coverage and Mate split coverage ≥ 1 and < 5.
- **Low quality**: if Split coverage and/or Mate split coverage = 0.

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

It is recommended to initially filter variants to display only those with *High Quality* (of greater reliability), 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 32. Structural variant results

08.5 | Microsatellite instability analysis (MSI)

Traditionally, microsatellite instability has been analyzed by immunohistochemistry or by analyzing fragments of at least five microsatellites. However, a panel of 110 microsatellite markers that allows determining the instability condition in the same sequencing reaction has been developed for Action OncoKitDx (Automatic).

To view the results of MSI analysis using **Data Genomics**, the user must access them by the "*Filtering*" request on the "*Microsatellites*" tab.

As an acceptance criterion for the MSI analysis in a sample, it has been established that there must be at least 99 valid markers for study. If a sample has the minimum number of valid markers, instability can be assigned according to Table 10.

MSS	Inconclusive results	MSI-L	MSI-H
0 – 0.17	0.18 – 0.20	0.21 – 0.30	0.31 – 1

Table 10. Instability range using Action OncoKitDx (Automatic).

In the opposite scenario (< 99 valid markers for analysis), a warning will indicate that the MSI analysis could not be carried out.

The analysis results that shall appear in the results report must be previously selected by the user.

Inestability fraction	Clinical interpretation	Unstable Markers
0.12	El tumor presenta inestabilidad de microsatélites negativa.	13

Figure 33. MSI results

08.6 | Pharmacogenetic testing

To view the results of pharmacogenetic testing using **Data Genomics**, the user must access them by the "*Filtering*" request on the "*Pharmacogenetics*" tab.

Pharmacogenetics of chemotherapeutic treatments is a constantly evolving field; therefore, it is recommended:

- Verifying that the version of the available IFUs contains information on the pharmacogenetics employed in the kit batch used (see page 2).
- Bearing in mind that genotypes with clinical implications identified in any of the analyzed regions (see section 2) are shown by default, among which only those that are to appear in the results report can subsequently be selected.
- Verifying the interpretation of the variants found using the *PharmaGKB* database.

08.7 | Variant filtering

By pressing the *Request*: *Filtering* button, a pop-up screen with the different tests on variants generated so far appears (see figure 34).

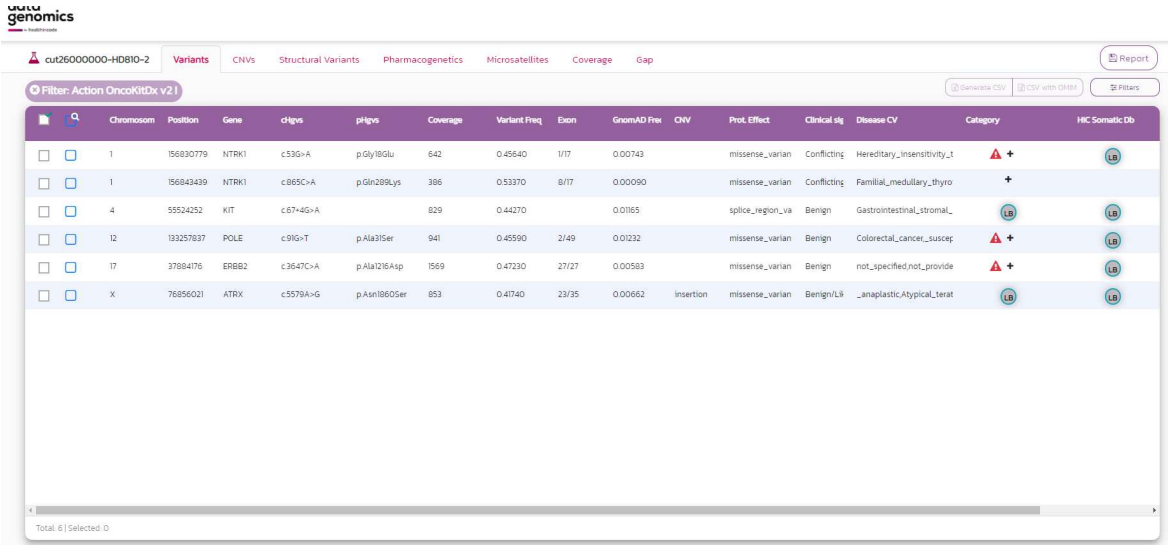


Figure 34. Variant filtering with Data Genomics.

Once variant analysis is opened, the variants that have met the *Action OncoKitDx Default* filter criteria are shown.

Variant filtering is meant to show the best-quality variants identified within the target regions of Action OncoKitDx (Automatic).

Once all variants shown after the first variant filtering step have been reviewed and categorized, it is recommended to apply a second filter, *Action OncoKitDx II*, which filters all variants classified by ClinVar as pathogenic or possibly pathogenic even if they do not meet the set criteria of the *Action OncoKitDx Default* filter. This way the user will have a list of variants that, regardless of their quality, have clinical implications. If any variant of interest is identified by this low-quality variant filtering, Health in Code S.L. recommends confirming its presence with a complementary technique.

New filters can be created by clicking the "*Filters*" button on the "*Variants*" page. A pop-up window where the new filter can be created appears. The user shall adjust the different options as desired. Once the filter characteristics 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 instructions for use of **Data Genomics**, available at www.datagenomics.es.

Below are shown the quality labels exclusive to Action OncoKitDx (Automatic):

- d200: Read depth covering the position lower than 200.
- f0.03: Allele frequency lower than 0.03.

In samples with a DIN <3, 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.

08.7.1 | 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 menu appears for each variant (see Figure 35), showing the different categories to which the variant can be assigned.

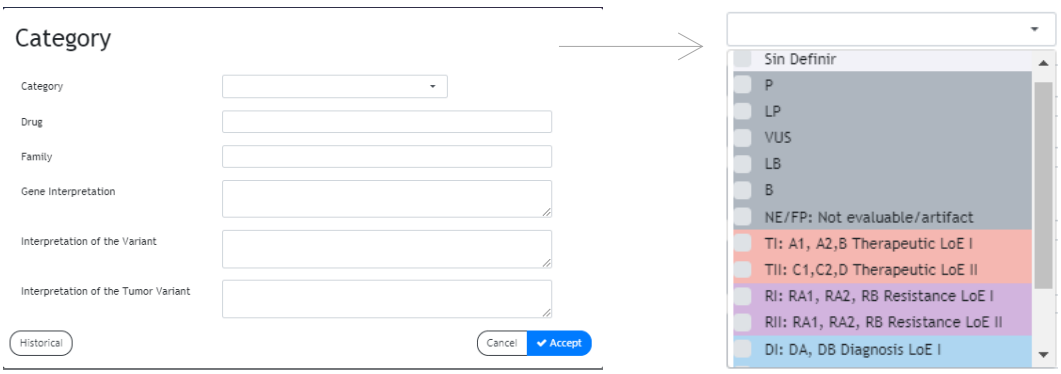


Figure 35. Drop-down menu of the "Category" column

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 necessary to evaluate the variant, or if it is suspected to be a false positive, it is recommended categorizing it 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. Therefore, the tool allows assigning the same variant a relevance level in each of the different categories: *therapeutic* (red color), *therapeutic resistance* (purple color), *diagnostic* (blue color), and *prognostic* (green color).

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

- **Gene**: Information about 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 S.L. 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.

By selecting the category recommended by Health in Code S.L. in this column (for example DI PII), you can access a pop-up window that allows you to view 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 categorization 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 the Health in Code S.L. database, the user will be alerted with the following alert icon:

Figure 36. An example of when the category assigned by the user does not match the one recommended by Health in Code S.L.

After the analysis of the samples, it is possible to generate a file of the selected variants, either as a csv file or 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 enter information about the clinical context of the sample, the interpretation of the results, indications regarding the presence of pertinent negatives, and data of the signees of the report. Once the desired fields have been completed, the automatic report can be downloaded.

Should you have any doubt about the analysis of the results, 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 Action OncoKitDx (Automatic) and the steps to follow to solve them can be found below. For other general issues relating to the Magnis equipment not listed in this section, consult the system's user guide.

Using the touchscreen for run setup presents usability issues:

As an alternative to the touchscreen controls, you can 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, the system must be rebooted.

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* reachpoint 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 completed, begin run setup 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 decline initiation of diagnostic testing.

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 completed, 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 labware 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 DNA from paraffin-embedded tissue, it is possible to obtain smaller than expected fragment sizes. This is due to the fact that the starting DNA is highly fragmented (characteristic of highly degraded DNA, such as those extracted from paraffin-embedded tissue), 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.

- Errors in STIDs:

  If sample tracking reagents provided by Health in Code S.L. are not used, the STID may not match the expected result. In this case, it is recommended ensuring that the STIDs specified in the *Sample Sheet* or those listed in the **Data Genomics** analysis platform are the correct ones.

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 is shown in Table 11. In the analysis of results, the label "*Pseudogenic_homology*" will appear in the column "*Fault summary*" when a variant is detected in a region of homology with pseudogenes.

Chromosome	Initial position	End position	Gene	Exon	Reference sequence
1	226259048	226259190	H3-3A	EX4	NM_002107.6
3	178937031	178937075	PIK3CA	EX11	NM_006218.4
3	178937348	178937423	PIK3CA	EX12	NM_006218.4
5	223586	223622	SDHA	EX2	NM_004168.4
5	236532	236608	SDHA	EX10	NM_004168.4
5	240462	240554	SDHA	EX11	NM_004168.4
5	254497	254504	SDHA	IN	NM_004168.4
5	254517	254631	SDHA	EX14	NM_004168.4
7	6013019	6013183	PMS2	EX15	NM_000535.7
7	6017208	6017306	PMS2	EX14	NM_000535.7
7	6017345	6017431	PMS2	EX14	NM_000535.7
7	6018216	6018243	PMS2	EX13	NM_000535.7
7	6018335	6018337	PMS2	IN	NM_000535.7
7	6022444	6022632	PMS2	EX12	NM_000535.7
7	6027042	6027134	PMS2	EX11	NM_000535.7
10	89725112	89725239	PTEN	EX9	NM_000314.8
22	29083960	29083984	CHEK2	EX16	NM_001005735.2
22	29085154	29085213	CHEK2	EX15	NM_001005735.2
22	29091820	29091871	CHEK2	EX12	NM_001005735.2

*Table 11. List of pseudogenic regions. *IN=Intron.*

- If results below the established quality parameters are obtained, we cannot guarantee their correctness.
- NGS is not yet considered the "*Gold Standard*" for some types of mutation, so it is recommended, whenever possible, to confirm positive results using a complementary and standardized technique.
- With Action OncoKitDx (Automatic), it is possible to detect germline variants of potential clinical interest. If a germline origin is suspected, a 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 in each individual 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 other results of complementary analytical or imaging tests.

10.2 | Equipment

Action OncoKitDx (Automatic) has been validated using the fragmentation platform:

- *ME220 Focused-ultrasonicator (Covaris)*

Action OncoKitDx (Automatic) has been validated using the following automated library preparation system:

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

Action OncoKitDx (Automatic) has been validated using the following massive sequencing platform:

- *NextSeq 500/550 System* (Illumina)

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

10.3 | Reagents

Action OncoKitDx (Automatic) has been validated using the reagents included in the kit and those recommended in section 6 of this manual (Necessary equipment and materials not included in the kit).

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

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

10.4 | Bioinformatics analysis platform

Action OncoKitDx (Automatic) has been validated using **Data Genomics**, which is a platform for in vitro bioinformatics analysis. This platform includes a pipeline tailored specifically for Action OncoKitDx (Automatic), 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 S.L. cannot guarantee the correctness of the results.

10.5 | Product stability

The optimal performance of this product is achieved when the specified recommended storage conditions are applied, within the product due date associated with each batch.

For any questions about the applications of this product or its protocols, please contact our Technical Department:

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