Instructions for use

Haematology OncoKitDx

Ref. IMG-363

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 other 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 products or reimburse the price thereof, at the client's choice, provided that, however, materials or workmanship prove to be defective. Health in Code S.L. shall not be liable for any loss or damage, whether direct or indirect, resulting in economic loss or harm incurred as a result of use of the product by the buyer or user.

All Health in Code S.L. products undergo strict quality control. Haematology OncoKitDx 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, please contact our Technical Department:

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Instructions for Use (IFU) modifications		
Version 19	FEB 2023	Update of sections 8.2 and 8.6 on how variant filtering works
Version 18	JAN 2023	Content review, sections 7.2.
Version 17	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 16.2	OCT 2022	Update of sections 3, 5 and 10. Update of quality QC of pre-capture and post-capture libraries (sections 7.6.1 and 7.6.2)
Version 16.1	JUN 2022	Change of the manufacturer's identification, going from Imegen to Health in Code S.L. Specification in section 3 of the amount of DNA required to carry out the analysis. Pharmacogenetics and sections 3, 7.6.2, and 7.9. updated.
Version 16	NOV 2021	Sections 2, 7.7 and 8.4 updated.
Version 15	SEP 2021	Section 7 updated
Version 14	AUG 2021	Magnis equipment protocol updated, applicable from batch 36321C005 onwards.
Version 13	JUL 2021	Sections 5 and 10 updated
Version 12	JUN 2021	Sections 3 and 8 updated.

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01 General information

The term oncohematology encompasses a wide and heterogeneous group of diseases with very different natural histories, but with excessive and uncontrolled proliferation of at least one hematopoietic cell line as a common feature. A number of molecular events are known to be involved in each one of these diseases.

This improvement in biological knowledge has allowed to better classify the different entities both at the diagnostic, prognostic, and therapeutical levels. The high number of mutations, deletions, and gene fusions involved in the latest classification by the WHO (2016) emphasizes the need for comprehensive and integrated testing, NGS (*Next-Generation Sequencing*) being the most efficient technology for this purpose.

References

- *Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127(20):2391-405.*

02 Intended use

Haematology OncoKitDx has been designed to test for all biomarkers with recognized diagnostic, prognostic, and therapeutical value among the different oncohematological diseases, according to the current classification by the *World Health Organization*, the ELN (*European Leukemia Network*) working group recommendations, and the NCCN (*National Comprehensive Cancer Network*) guidelines. Moreover, it also includes molecular alterations that allow for inclusion in early clinical trials on drugs aimed at specific targets. Haematology OncoKitDx has been designed for the adequate molecular testing of patients with ALL, AML, CML, MDS, MPN, and other hematologic neoplasms both in adults and in children, within the internationally accepted turnaround times and based on a single DNA sample.

Reaching a correct molecular diagnosis, providing valuable prognostic information and making the best therapeutical biology-based decisions for patients can now be achieved with a single test.

To offer this complete vision of the tumor, Haematology OncoKitDx is the first test on the market that via next generation sequencing (NGS) integrates in a single protocol:

- Sequencing of the whole exonic regions of **76 genes**:

ARID5B, ASXL1, ASXL2, ATRX, BCOR, BCORL1, BLNK, BRAF, CALR, CBL, CDKN2A, CDKN2B, CEBPA, CHIC2, CREBBP, CSF3R, CSNK1A1, CUX1, DDX3X, DDX41, DNMT3A, EP300, ETNK1, ETV6, EZH2, FBXW7, FLT3, GATA1, GATA2 (including intron 4), GATA3, HAVCR2, IDH1, IDH2, IKZF1, IL7R, JAK1, JAK2, JAK3, KIT, KMT2A, KMT2C, KRAS, MPL, NF1, NFE2, NOTCH1, NPM1, NR3C1, NRAS, P2RY8, PAX5, PHF6, PIGA, PPM1D, PTEN, PTK2B, PTPN11, RAD21, RB1, RUNX1, SETBP1, SF3B1, SH2B3, SMC1A, SMC3, SRP72, SRSF2, STAG1, STAG2, STAT5B, TET2, TP53, TYK2, U2AF1, WT1 and ZRSR2.

- Capture of **27 fusion genes** with all possible rearrangements.

  To this end, Haematology OncoKitDx includes those intronic regions where breakpoints have commonly been identified in the literature. These genes and the covered regions within them are the following: ***ABL1*** (NM_005157; 5'UTR region, introns 1, 2, and 3), ***ABL2*** (NM_001136000.2; introns 3, 4, and 5), ***BCR*** (NM_004327.4; introns 6, 13, 14, 15, and 19), ***CBFA2T3*** (NM_005187; introns 10 and 11 and the 3'UTR region), ***CBFB*** (NM_001755.2; intron 5), ***CSF1R*** (NM_005211; introns 11 and 13), ***EPOR*** (NM_000121; intron 7 and CDS exon 8), ***ETV6*** (NM_001987; introns 2, 3, 4, and 5), ***FGFR1*** (NM_023110.3; introns 7, 8, 9, and 10), ***FUS*** (NM_001170937; introns 5, 6, 7, 8, 9, 11, and 14), ***JAK2*** (NM_004972; introns 8, 9, 10, 11, 15, 16, 17, 18, and 19), ***KMT2A*** (NM_001197104.2; introns 6, 7, 8, 9, 10, 11, 15, 22, and 29), ***MEF2D*** (NM_001271629; introns 5 and 6), ***MNX1*** (NM_005515; introns 1 and 2), ***MYH11*** (NM_002474; intron 7), ***NPM1*** (NM_002520.6; intron 4), ***NUP214*** (NM_005085; introns 1, 9, 16, and 17), ***NUP98*** (NM_016320.5; introns 10, 11, 12, 13, and 14), ***PDGFRA*** (NM_006206; introns 11 and 12), ***PDGFRB*** (NM_002609; introns 9, 10, 11, and 12), ***RARA*** (NM_000964; intron 2), ***RBM15*** (NM_001201545; intron 1), ***RUNX1*** (NM_001754; intron 6), ***SET*** (NM_001122821.1; intron 7), ***STIL*** (NM_001048166.1; 5'UTR region), ***TAL1*** (NM_001287347; intron 3), and ***TCF3*** (NM_001136139.2; introns 13, 14, 15, 16, and 17).

- Detection of copy number variations (**CNVs**) throughout the whole genome, allowing for the detection of alterations such as hypo- and hyperploidias, gain or loss of whole chromofomes (+8, –7, –17) or chromosomic regions (5q, 7q, 11q), por ejemplo. for example. It can also detect copy number variations both of whole genes or of parts of genes included in the panel. Moreover, this analysis has been improved using a low-density SNP array through the capture of 2734 SNPs distributed throughout the whole 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*).

Haematology OncoKitDx also includes the detection of variants related to patient **pharmacogenetics**, which is directly involved in the response to chemotherapy treatments and which provides optional guidance for dose adjustment in each particular case. Pharmacogenetic-related variants included in Haematology OncoKItDx are: ***CYP2C9*** (rs1799853 y rs1057910), ***MTHFR*** (rs1801133), ***NUDT15*** (rs116855232), y ***TMPT*** (rs1800462, rs1800460, rs1142345 y rs1800584).

Haematology OncoKitDx offers the most comprehensive and efficient solution on the market that allows reaching a correct molecular diagnosis, providing prognostic information, and detecting actionable alterations with specific drugs in patients with haematologic neoplasms, all while complying with international standards and considering the availability of clinical trials. To achieve this, germline and somatic alterations are analyzed in the included genes. Moreover, copy number variations in different chromosomal regions and gene fusions with clinical relevance are detected. All this information is obtained using a single DNA sample.

Haematology OncoKitDx analyzes clinical samples from haematologic neoplasms via a protocol that integrates high sensitivity capture of regions of interest with hybridization probes using a molecular barcoding technique on each DNA fragment with a unique adapter and subsequently performing 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. Thus, the results obtained significantly increase sensitivity, therefore improving variant detection in low-quality DNA samples.

Haematology OncoKitDx is solely intended for *in vitro* diagnostic use and us aimed at professionals in the molecular biology sector.

03 Technical characteristics

Haematology OncoKitDx has been validated on the Illumina's *NextSeq* 500/550 *System* latform via the analysis of DNA reference samples from the *Coriell Institute and Horizon Dx*, and from clinically relevant samples that have been previously genotyped with other technologies. This validation has confirmed the specific detection of the variants present in the selected genes (see above), as well as the repeatability, reproducibility, and limit of detection of the technique.

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

- The **reduction of sequencing errors** introduced by 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 and bone marrow.
- Necessary amount of DNA: 50–100 ng.
- Limits of detection:
  - The limit of detection of the analysis of structural and point variants, small insertions and deletions is 2%.
  - The limit of detection for CNVs with respect to the total number of copies in a sample has been established at 3 copies for gain of copies and at 1 copy for loss of copies.
- Mean coverage: 1400X.
- Mean coverage after UMI analysis: 990X.
- Coverage: 99.1% of bases covered at 100X depth.
- Uniformity: 98.0% of bases covered at >20% of mean coverage.
- Specificity: > 99 %
- Sensitivity: > 99 %
- Repeatability: > 99.99 %
- Reproducibility: > 99.9 %

Health in Code S.L. is certified under **UNE-EN ISO 13485:2018 Medical Devices: Quality Management Systems - Requirements for regulatory purposes** standard 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

- Strictly follow the instructions of this manual, especially regarding the handling and storage conditions of the reagents.
- Do not mouth-pipette.
- Do not smoke, eat, drink, or apply cosmetics in areas where kits and samples are handled.
- Any cuts, abrasions, and other skin injuries must be properly protected.
- Do not pour the remains of reagents down the drain. It is recommended to use waste containers established by the legal norm and manage their treatment through an authorized waste management facility.
- In the event of an accidental spill of any of the reagents, avoid contact with the skin, eyes, and mucous membranes and rinse with abundant water.
- Safety data-sheets (MSDS) of all hazardous components contained in this kit are available on request.
- This product requires the handling of samples and materials of human origin. You should consider all materials of human origin as potentially infectious and handle 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 using specific equipment and under specific conditions that may vary widely among laboratories. Therefore, each laboratory should 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 the malfunction of the assay when the reagents included in the kit are replaced with other reagents not supplied 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 the following:

- **Fragmentation Buffer**: Buffer required for DNA fragmentation prior to NGS library preparation.
- **Fragmentation Enzyme**: Enzyme required for DNA fragmentation and preparation prior to adapter binding.
- **Elution Buffer**: Buffer to elute DNA.
- **Reagents Plate**: Plate containing all the necessary reagents for DNA end repair reactions and Illumina adapter 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 adapters. They are necessary to label libraries for each sample, leading to a unique combination that will allow for their analysis after sequencing. The kit includes 24 different indexes distributed in single-use strips.
- **Haematology Probe Strips**: Synthetic biotinylated oligonucleotides complementary to the kit's target regions, which allow for the hybridization with said regions and which are later captured via streptavidin magnetic particles due to the biological property of bonding between biotin and streptavidin molecules.
- **Input Strips**: Empty 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 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 to collect tip waste generated during the protocol.

The components of the kit are listed below:

Box 1 of 4			
Reagents	Color indicator	Quantity	Conservation
Sample input strips	Red	3 strips	15–25°C
Beads 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 Haematology OncoKitDx

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

Table2. Reagents of box 2 of Haematology OncoKitDx

NOTE: Each kit includes 3 of four possible index combinations: A1, A2, A3, and A4.

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

Table 3. Reagents of box 3 of Haematology OncoKitDx

"*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 Haematology OncoKitDx

NOTE: Each kit includes 3 "*Magnis Empty Consumables*" boxes, one for each 8-sample run on the Magnis instrument.

06 Equipment, reagents and material not included in the kit

Equipment:

- 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 lest 1,000 rpm)
- Plate centrifuge
- 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 Technologiesn (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)
    - *DNA 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 1000 µL)
- Sterile filter tips compatible with *Magnis NGS Prep System* (Ref: 19477-022; Agilent)
- Sterile 1.5 mL tubes
- Sterile 0.2 mL tubes or strips.
- 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

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

Haematology OncoKitDx 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 the CNV analysis.

07 Assay protocol

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

The steps necessary to carry out the preparation of 8 libraries with Haematology OncoKitDx are outlined below.

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, the germline reference samples need to 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, containing one female and one male sample, which should be processed with Haematology OncoKitDx.

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

01 Ensure that there are no materials from previous runs on the instrument unit, as they could interfere with the initial steps and instrument setup.

02 Close the instrument door.

03 Turn on by pressing the power button on the front of the instrument (LED indicator lights will turn on). Wait while the system launches, which 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*".

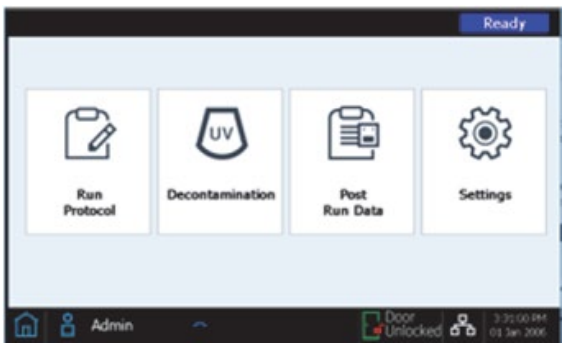


Figure 1. Magnis NGS Prep system Home screen

◊ 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).

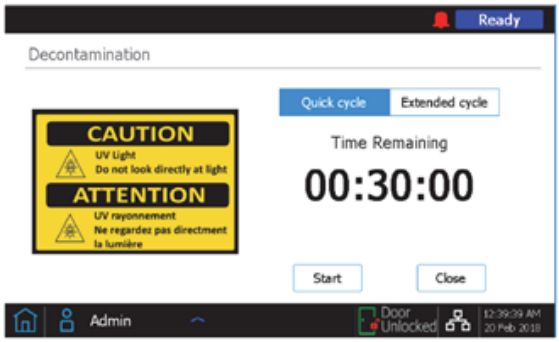


Figure 2. Magnis NGS Prep system Decontamination screen

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 completed (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 to carry out the preparation and fragmentation of 8 libraries with Haematology OncoKitDx 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 and dilution 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 Dilute DNA sample to 25 ng/µL with nuclease-free water to a total of 20 µL.

Optional: If Health in Code's integrated tracking system (*Sample tracking components*; Ref. IMG-340) is to be used, this step must be carried out by substituting 1.5 µL of nuclease-free water for the same amount of a single tracking reagent per sample.

Optional: If the reference samples included in kit IMG-368 are processed, proceed with step 6 of this section.

04 Mix on a vortex mixer and quantify each sample again on a fluorometer, such as Qubit.

05 Dilute each DNA sample with nuclease-free water to obtain a total concentration of 100 ng, to a final volume of 7 µL, on a 0.2 mL tube strip.

If a total of 100 ng cannot be obtained in a volume of 7µL:

- Increase the final volumen to 12 µL
- The total DNA amount can also be reduced to 50 ng. If this method is selected, it must be applied to all 8 samples in the run

06 **Optional**: To process the reference samples included in the IMG-368 kit, for a concentration of 25 ng/µL, add to the corresponding position on the 0.2 mL well strip:

- 4 µL of the reference sample.
- 1 µL of the tracking reagent used (or nuclease-free wáter if the integrated tracking system is not used).
- 2 µL of nuclease-free water.

07 Mix all dilutions on a vortex mixer, spin, and keep cold until their use in the next step.

07.2.2 | DNA fragmentation

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

Reagents to be used in this step:

Reagent	Color	Conservation
Fragmentation Buffer	Green cap	-20°C
Fragmentation Enzyme	White cap	-20°C
Sample Input Strip	Red strip	15-25°C

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

02 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 mixer, while the *Fragmentation Enzyme* must be mixed by inversion several times. When processing several samples, we recommend preparing an extra 12% of reagent mixes.

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

03 Mix vigorously on a vortex mixer.

04 Add 3 µL of the fragmentation mix to each 0.2 mL well with the fragmented sample. Mix by pipetting 20 times.

05 Seal the strip, spin the samples, place tubes immediately after in the thermal cycler and execute the fragmentation program.

- 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 5. Optimal fragmentation program

NOTE: This protocol requires the lid to be preheated 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.

06 Once the fragmentation program ends, remove samples from the thermal cycler, spin, add nuclease-free water to each sample to a final volume of 50 µL, 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 simple 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*.

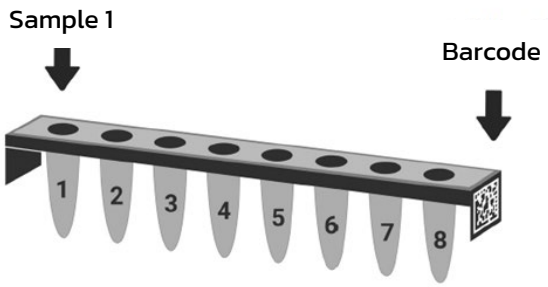


Figure 3. Required placement of samples in the Sample Input Strip

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

Reagents to be used in this step:

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

01 Preparation of the *Reagents plate*:

- Thaw the plate at room temperature while keeping its white carboard packaging.
- Once the contents of all wells are thawed, mix the plate on a vortex mixer while keeping it in its cardboard package. 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 rotation and spinning 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/Buffers plate*:

- To ensure room temperature, leave it out in its white cardboard package 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 magnetic particles from sedimenting.
- Keep plate in its packaging at room temperature for its use on the same day.

03 Preparation of the *Index strip*:

- D 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 6. 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 its use, thaw the *Haematology Probe Strip* at cold temperature. Vortex for 5 seconds and spin. It is important to check that bubbles have not formed at the bottom of the well.

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

05 Last, prepare a *Magnis Empty Consumables* box to use during unit setup.

07.4 | Running 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 door 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**: Tick the *Aliquot sample* for QC checkbox for the equipment to collect an aliquot from each pre-capture library for subsequent quality control.

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

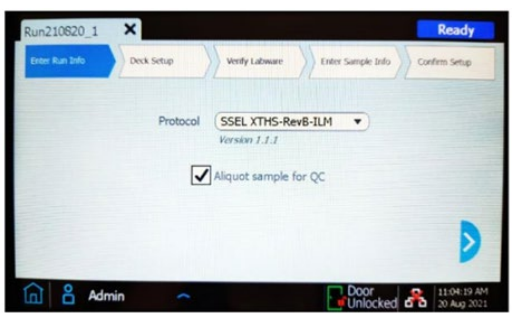


Figure 4. Pantalla Enter Run Info del sistema Magnis NGS Prep

04 Move on to the next screen.

05 Select the appropriate sample type: *High Quality DNA*.

06 Select the starting DNA amount in the *Input Amount* menu. Even though options 10 ng, 50 ng, 100 ng, and 200 ng are available, for library preparation using Haematology OncoKitDx, using an amount of 100 ng is recommended. Change the amount of DNA for any other initial amount (minimum 50 ng).

> NOTE: Quality and quantity adjustments for template DNA will determine the number of amplification cycles that the equipment will subsequently carry out; for this reason, it is important to enter the correct information and to ensure that all samples have the same amount of starting DNA.

07.4.2 | Deck setup

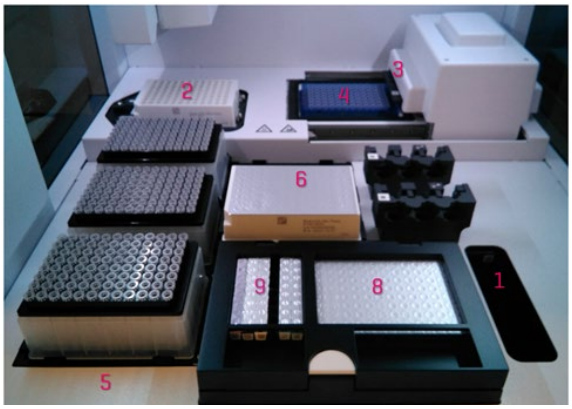
The deck can be very easily set up by following the steps on the Magnis touchscreen.

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

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

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

The following figure shows a completely loaded deck with each material numbered from 1 to 10, following the steps displayed by the Magnis instrument. As can be observed, both reagent plates, as well as the five necessary strips, must be sealed when placed inside the instrument.



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

Figure 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 drawer in the lower-left corner. The barcode must be facing the user, as shown on the touchscreen. Close the waste bin drawer.

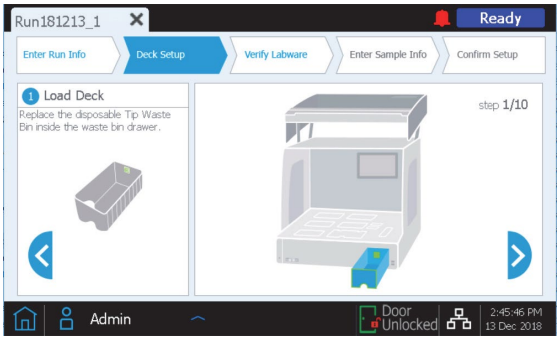


Figure 6. Step 1 of 10 on the Deck Setup screen of the Magnis NGS Prep System

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.

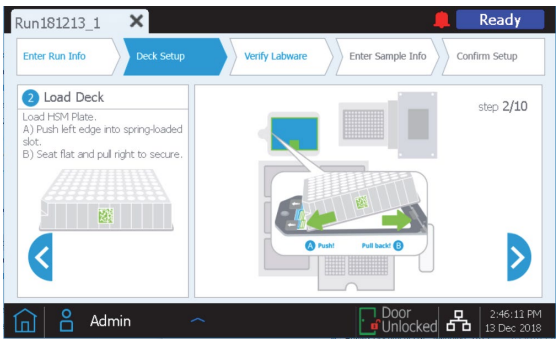


Figure 7. Step 2 of 10 of the Deck Setup screen of the Magnis NGS Prep System

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.

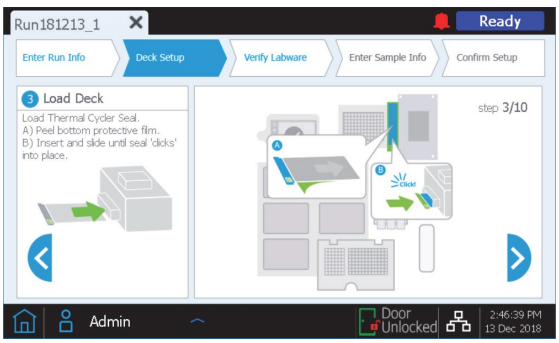


Figure 8. Step 3 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

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.

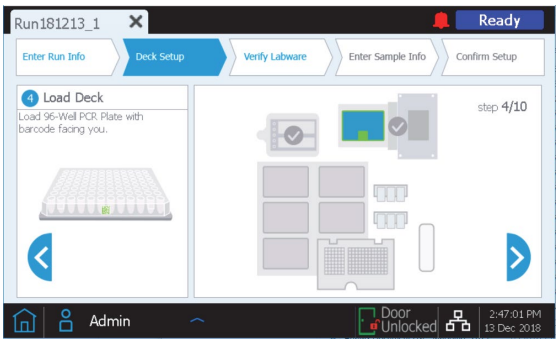


Figure 9. Step 4 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

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.

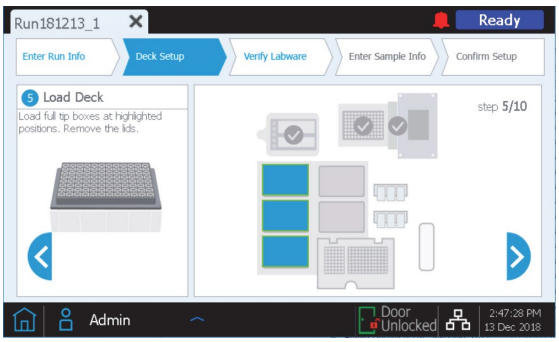


Figure 10. Step 5 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

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.

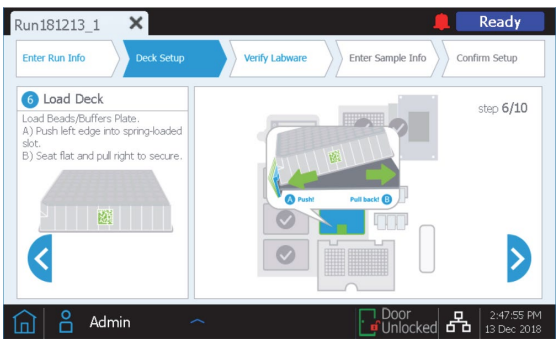


Figure 11. Step 6 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

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.

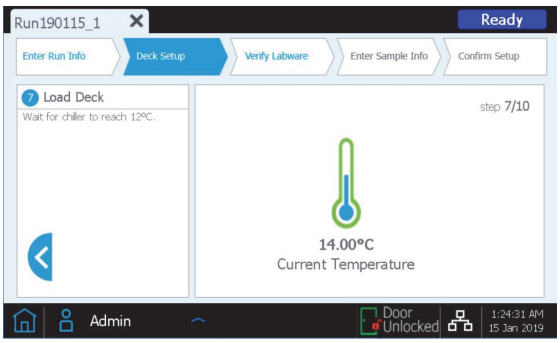


Figure 12. Step 7 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument.

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.

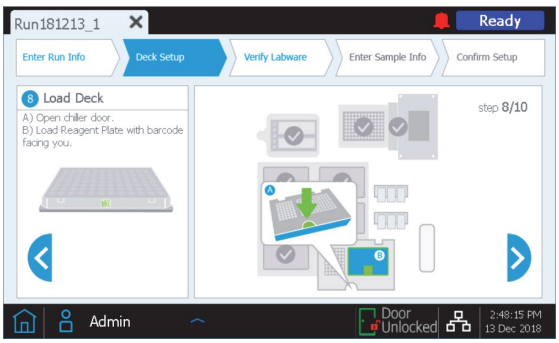


Figure 13. Step 8 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

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

- Load the *Sample Input Strip* (red strip) containing the DNA samples prepared in section 7.2 of this document into the chiller holder position labeled with **S**.
- Load the *Index Strip* (black strip), prepared in section 7.3 of this document, into the chiller holder position labeled with **IDX**.
- Load the *Haematology 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.

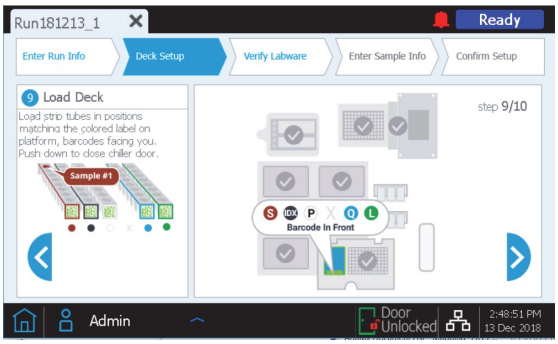


Figure 14. Step 9 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument.

10 Close the instrument door.

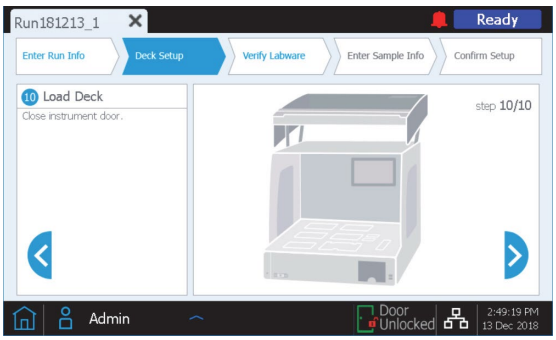


Figure 15. Step 10 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

07.4.3 | Labware verification

Once that the loading of the equipment has concluded, proceed to the *Verify Ladware* 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 the labware verification.

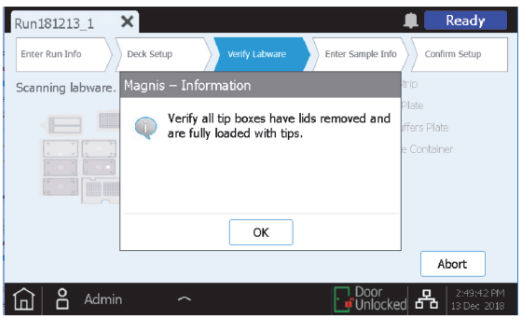


Figure 16. Verify Labware pop-up window of the Magnis NGS Prep System

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

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

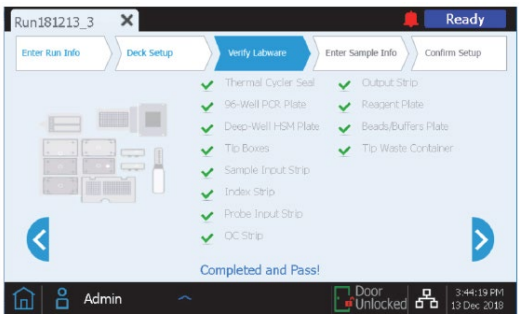


Figure 17. Verify Labwarescreen of the Magnis NGS Prep System after correct labware verification

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

07.4.4 | Entering sample information

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

01 Manual sample assignment:

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

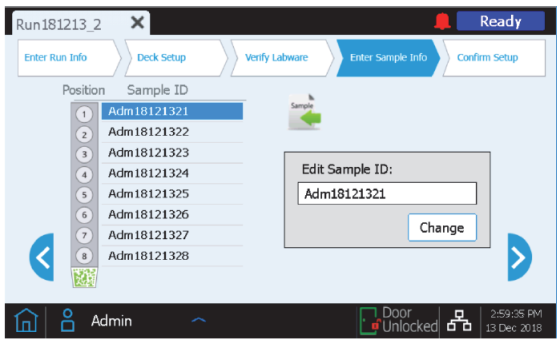
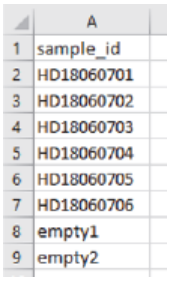


Figure 18. Edit Sample Info screen of the Magnis NGS Prep System; the Load samples button is highlighted with a circle.

02 Import of sample assignments using a .csv file:

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

- Type the name of each sample in cells A2 to A9. The entry file of the sample must contain 8 unique sample IDs. If the protocol is to be carried out with less than 8 samples, you must fill in these positions in the file as shown in Figure 19 (empty1 and empty 2).
- Save file in .csv format.
- Download the .csv file to an unencrypted USB disk and connect the disk to one of the USB ports of the Magnis instrument.
- When setting up the run, on the *Enter Sample Info* screen, press the Load samples button (highlighted with a circle in Figure 18).
- Follow the instructions of the protocol setup assistant to transfer sample IDs from the USB disk.

07.4.5 | Confirm setup and start the run

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

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

> IMPORTANT: **The number of pre- and post-capture PCR cycles have been set according to DNA quantity and quality. Modifying them would affect the sensitivity, specificity, and LOD of Haematology OncoKitDx.**

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

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

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

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

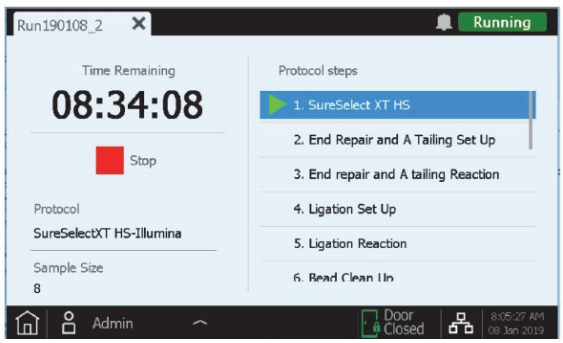


Figure 20. Running screen during a run.

07.4.6 | Collecting libraries from the instrument

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

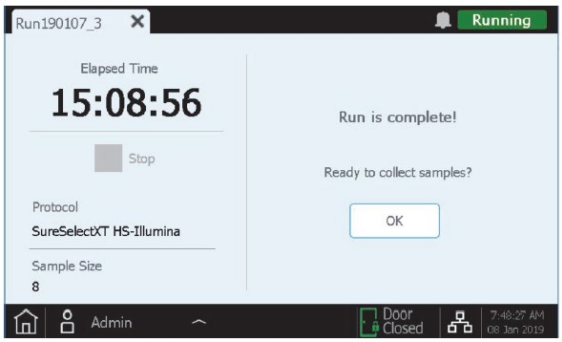


Figure 21. Running screen after a run

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

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

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

The protocol can be stopped at this point, storing the libraries at 4 °C if they are to be used within the following 24 hours or at -20°C for longer storage periods.

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

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

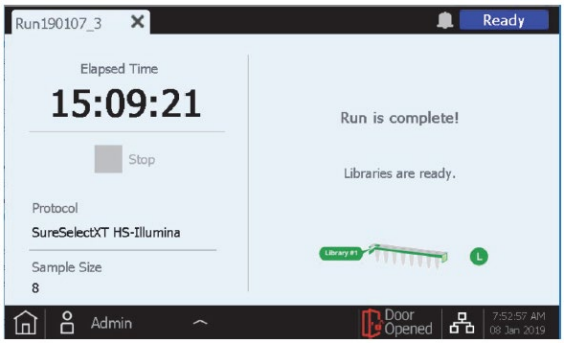


Figure 22. Running screen after a run and after removing the libraries

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

07.5 | Instrument cleaning after a run

Remove and dispose of all used consumables remaining on the instrument deck:

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

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

07.6 | Library validation and quantification

07.6.1 | Optional quality control of the pre-capture library

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

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

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

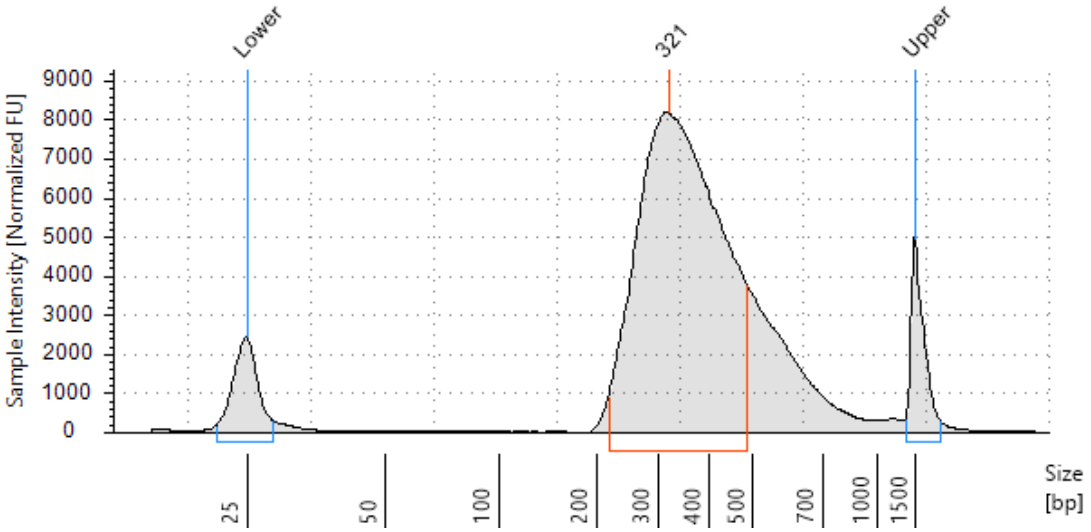


Figure 23. Expected result after analyzing pre-capture library size with TapeStation 2200

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

07.6.2 | Optional quality control of the post-capture library

Prior to pooling the libraries for multiplexed sequencing, the quantity and quality of each of them should be analyzed.

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

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

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

The expected mean fragment size ranges from 250 to 350 bp. If unexpected sizes are obtained, review the protocol and pre-capture library quality control, carefully read section 9, or contact Health in Code's technical support team.

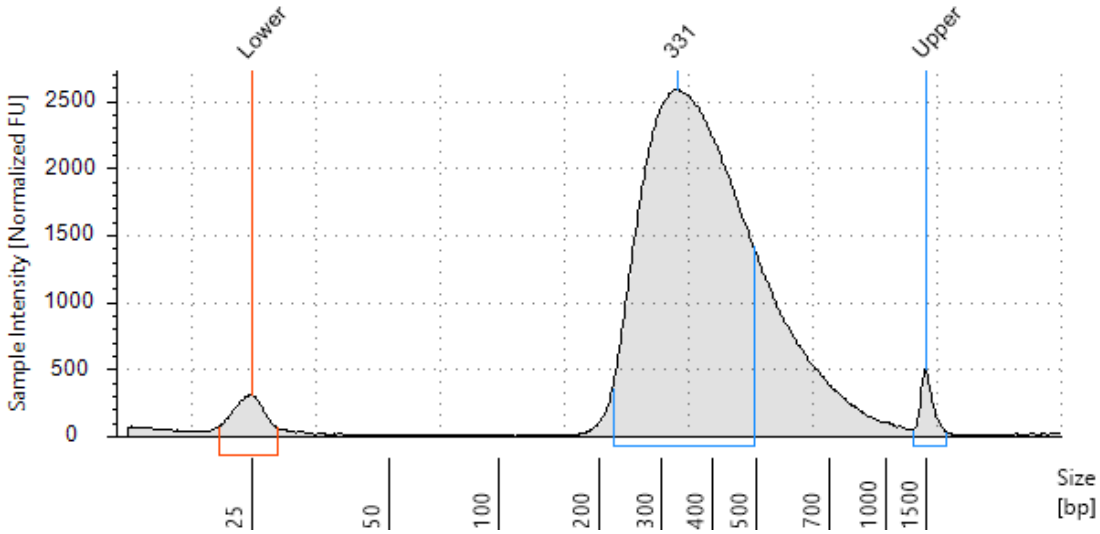


Figure 24. Expected result after the analysis of post-capture library size with TapeStation 2200

With the DNA concentration and the peak of captured library fragments size, we obtain their concentration, applying the following formula:

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

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

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

07.7 | Library denaturation

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

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

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

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

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

04 Incubate at room temperature for 5 minutes.

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

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

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

08 Add 1,222 µL of HT1.

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

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

The table below specifies the maximum number of samples per run, depending on the sequencing kit used, to guarantee a minimum number of PF clusters of approximately 17.5 millions per sample:

NextSeq Reagents Kit	Maximum no. of samples
NextSeq 500/550 Mid Output v2.5 kit (150 cycles). Ref: 20024904	8
NextSeq 500/550 High Output v2.5 kit (150 cycles). Ref: 20024907	24

Table 9. Kit de NextSeq Illumina kit and maximum number of samples to be analyzed with Haematology OncoKitDx

If a smaller number of samples are to be sequenced, the generated fastq files will undergo bioinformatic pre-processing to generate tiles with a maximum number of 40 million reads per library.

07.8 | *NextSeq* platform setup

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

02 Follow the equipment's loading instructions.

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

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

07.9 | Generation of the fastq files necessary for bioinformatic analysis

After the sequencing of Haematology OncoKitDx libraries a series of .bcl files are generated, which must be de-multiplexed to generate the 12 fastq files that are necessary for the bioinformatics analysis of each library.

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

08 Analysis of results

Bioinformatic analysis of the results is done through an analysis pipeline tailored specifically for Haematology OncoKitDx, through the **Data Genomics** platform. To access this tool, visit www.datagenomics.es.

The tool allows analyzing the different samples and obtaining all the folders generated after their bioinformatic analysis.

In samples of hematologic neoplasms, the detection limit of the different types of variants that are specified in the technical documents will depend on the tumor cellularity of each of the tumor clones that the sample has, which is generally unknown. Likewise, calculating the number of tumor copies will also not be possible if tumor cellularity of the analyzed sample is unknown, which is why only the number of total copies in the sample can be determined.

08.1 | Request for analysis

01 Select the "*Import Samples*" on the main screen (*Orders* tab) to begin the analysis of the sequenced samples. This will take you to the file import screen (Figure 25). On this screen, the 12 fastq files associated with each sample must be imported. Optionally, the *SampleSheet* file could be used as well; this file allows simultaneously importing all files from the same sequencing run.

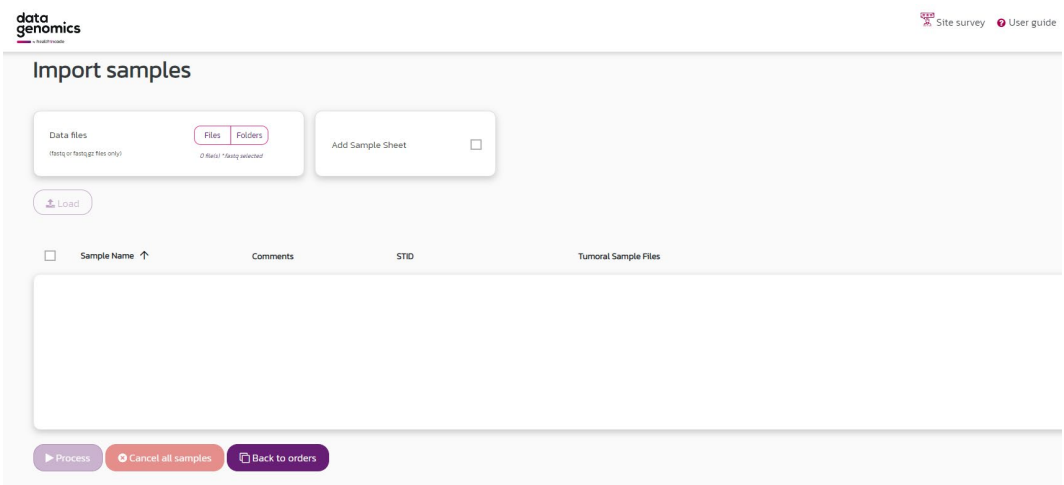


Figure 25. Screen for importing the fastq files and the sample sheet and commence the analysis request

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

03 Before processing the sequencing files, the tumor type for each sample must be filled in.

To access the pop-up screen with this field, 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*".

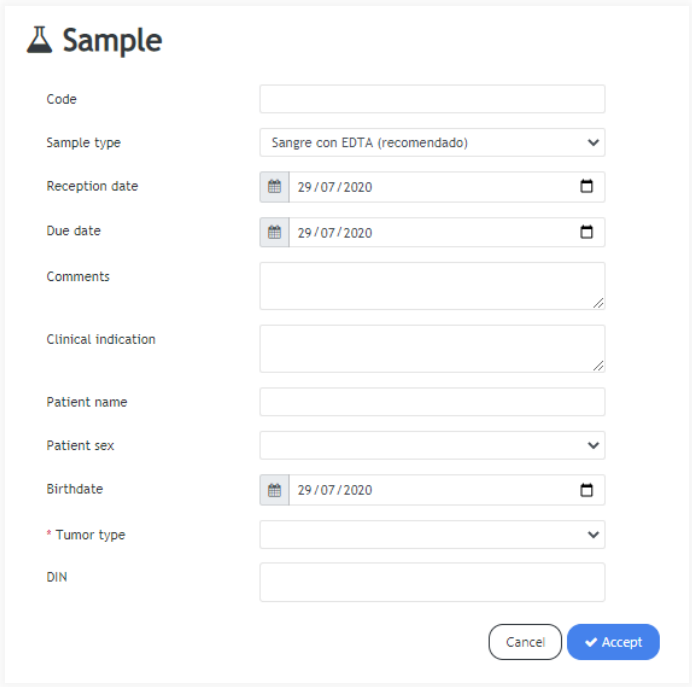


Figure 26. Pop-up screen with the required fields for each sample

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

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

08.2 | Management of orders

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

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

To access the results of the bioinformatics analysis, in the "*bioinformatics*" order, you must select "*Show results*," and the "*Workspace*" window will open. This screen allows the user to access the files resulting from the bioinformatics analysis: files resulting from the pharmacogenetic analysis (.pgx.tsv), analysis of CNVs per genome (.CNVs.png and CNVs.txt) and structural variants (.SV.txt), the alignment files (bam and bai), and the list of variants (vcf), and also other files with information on coverages and the quality report of the sequencing after the bioinformatics analysis. In the "CNV" order, by selecting "*Show results*" you can access the files generated from the CNV analysis by gene (_calls.tsv, images_cnv.zip, and _sample_QC.tsv).

The parameters that are taken into account in the different files that are generated from the sequencing, for a sample to pass the bioinformatic quality control established for the Haematology OncoKitDx assay are:

- FASTQ: The established acceptance criteria are detailed in the **Data Genomics** instructions for use, available at: www.datagenomics.es.
- BAMs:
  - *Mapped Reads* (%):
    - Fail: < 97
    - Warn: 97-99
    - Pass: > 99
  - DP200 (%):
    - Fail: < 97
    - Warn: 96-97
    - Pass: > 97
  - *On-target* (%):
    - Fail: < 44.5
    - Warn: 44.5-47
    - Pass: > 47
- STIDs: Verification that the tracking reagent obtained matches the expected one (if it has been used), as shown in Figure 27.

If any of the parameters mentioned is not met, the icon will appear on the main screen, next to the sample in question.

In the Haematology OncoKitDx assay, the VCF files are not taken into account for quality control, nor is the sample's heterozygosity, since it can be a somatic analysis, where the frequency of the identified variants can be very variable.

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

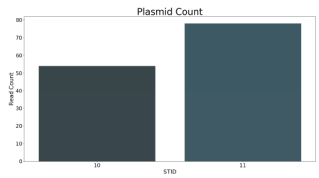


Figura 27. Quality control of the sample tracking system

To access the variants filter, in the "*Filtering*" request the *Haematology OncoKitDx Default* filter will be applied, which is characterized by

- Quality variants: ***PASS; d200; pseudogenic homology*** (*Fault summary*).
- Depth: ***≥ 20X*** (Clean total count).
- Allele frequency: ***≥ 2%*** (*Variant Freq*).
- Distance to the exon: ***10*** (*Exon distance*).
- Low population frequency: ***gnomAD Freq <2%***. To filter out variants frequent in the population that will not be relevant in tumor formation.

- Exclusion of variants in non-coding regions and synonymous variants, except those found in possible splicing regions.

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 reading in a region and the number of DNA copies for the same region.

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

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

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

Haematology OncoKitDx offers two different analysis algorithms to approach CNV analysis. One of them allows for the analysis of smaller CNVs that can affect one or several exons in a gene or an entire gene included in the panel (CNVs per gene) and the other allows for the visualizing of large CNVs that affect one or several genes, including chromosome regions or entire chromosomes along the entire genome (CNVs per genome).

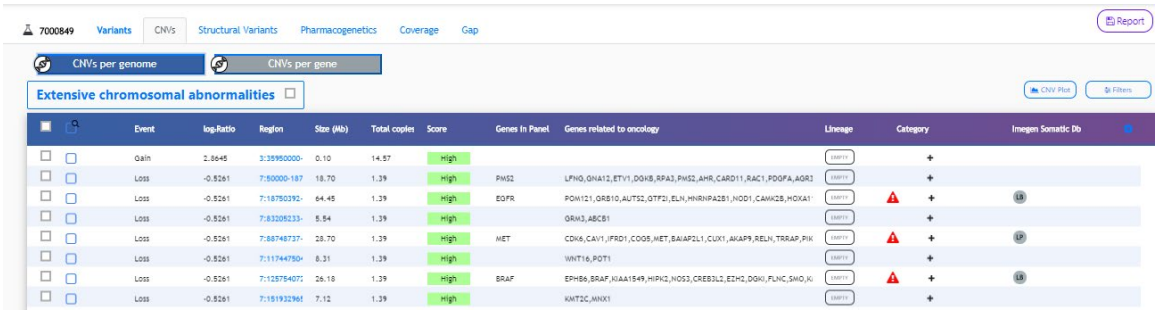


*Figure 28. CNV results shown by the **Data Genomics** platform*

To analyze CNV results with **DataGenomics**, you must access the results of the "*Filtering*" request and, specifically, the CNVs tab. This screen will have two sections: CNVs per gene and CNVs per genome, to access the results of the different CNVs analysis types. On each tab, you can apply different filters and select the variants that you wish to include in the results report.

08.3.1 | CNVs per genome

In addition to CNV analysis in the genes included in the panel, it has an SNP array distributed through the genome, which allows for CNV analysis and loss-of-heterozygosity study with a neutral copy number (*copy-neutral LOH*).

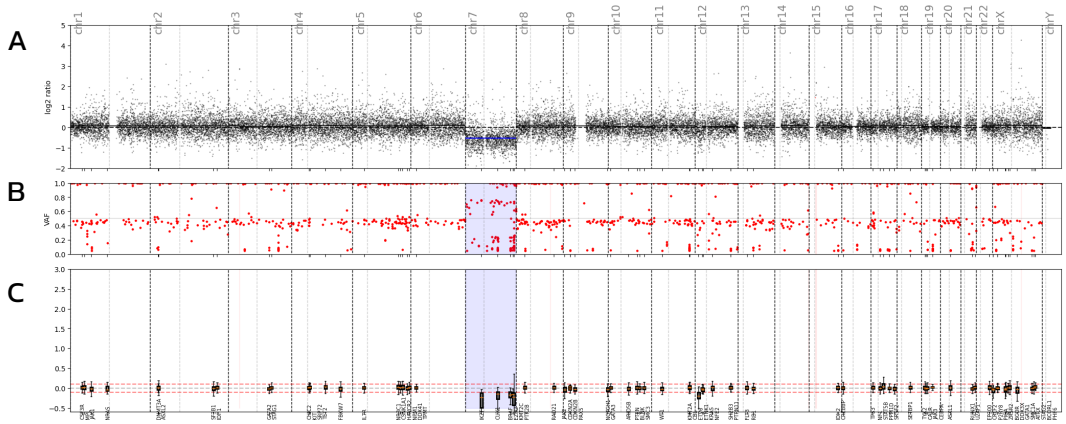


Figure 29. The Y axis represents the log2_ratio value (A), the allelic frequency of the variant (B), and the deviation of the normalized coverage of a gene in the tumor with respect to the coverage of that same gene in germline DNA (C), while the X axis represents the 24 chromosomes present in human cells and the genes included in the Haemotology OncoKitDx panel according to their location in the genome.
A) Results from whole-genome CNV testing by means of off-target analysis. Each point represents 100 Kb of sequence. Deleted regions appear in blue and amplified regions in red.
B) Results from whole-genome CNV testing by means of SNP array analysis. Each red dot represents one of the 2734 SNPs tested for.
C) Analysis of CNVs per gene, representing the difference between gene coverage in tumor DNA and germline DNA, as well as the location of the gene in the genoma

The limit of detection for CNVs is 3 copies for duplications and ≤1 copy for deletions.

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

It is recommended to filter out variants that do not have a *High Score* (greater reliability) and carefully examine the CNV plot (Figure 28). If an excessive number of CNVs are observed, the user can select the "*Extensive chromosomal abnormalities*" option; this way, the high number of copy number variants will be reflected in the report.

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

Event	log-Ratio	Region	Size (Mb)	Total copies	Score	Genes in Panel	Genes related to oncology	Lineage	Category
Gain	0.3981	2:240250000-240850000	0.60	2.64	High		HDAC4	UNITY	+
Gain	0.413	10:86950000-87550000	1.00	2.66	High		GRID1	UNITY	+
Loss	-0.3746	12:11750000-13750000	2.00	1.54	High		ETV6,GRIN2B,CDKN1B	UNITY	+
Gain	0.3891	13:19250000-19550000	0.30	2.62	Low			UNITY	+
Gain	1.1306	14:106250000-107250000	1.00	4.38	Medium			UNITY	+

Figure 30. Results of CNVs per genome

08.3.2 | CNVs per gene

Data Genomics integrates an alert system to warn the user about the reliability of the results based on sample quality parameters. According to these parameters, the

reliability of the results will be considered high (*High confidence*), intermediate (*Medium confidence*), or low (*Low confidence*). The parameters that have been taken into account are the following: similarity with reference samples, z-score and ratio, mean coverage, number of reference samples selected for the analysis, uniformity between samples from the same batch, and number of detected CNVs before variant filtering.

Regarding CNV results, the PASS variants will be shown by default. These are good-quality variants with a p-value ≤0.005 and a ratio ≤0.7 or ≥1.3. The filters allow the user to choose to display No Pass variants.

If the CNVs analysis could not be carried out, **Data Genomics** will display a warning indicating the reason.

In the CNVs per gene tab, you can access the dynamic representation graph (Figure 31) of the sample's coverage profiles vs. the reference samples or of the same batch for all the genes and NMs of each gene. This graph allows expanding the regions of interest and visualizing SNPs and INDELs, as well as the regions of conflict of homology with pseudogenes (orange area).

In case of finding a positive result or a sample with suboptimal quality, it is recommended to confirm said result using an alternative technique, such as MLPA or dPCR.

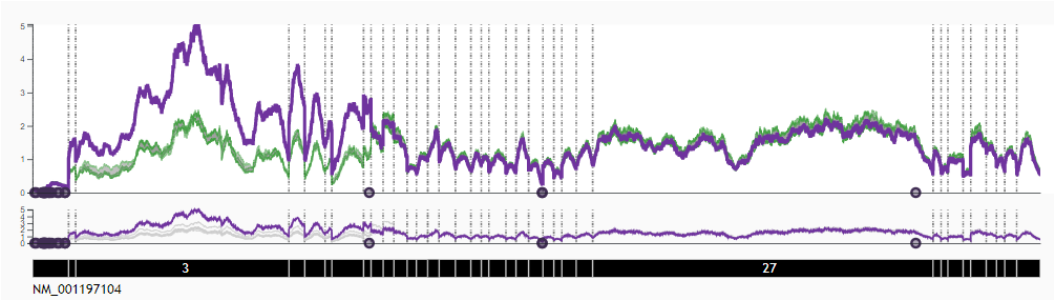


Figure 31. Dynamic graph offered in the results of the analysis of CNVs per gene

08.4 | Analysis of structural variants (SVs)

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

- **Split reads**: Reads whose sequence maps to different locations of the genome. These reads also provide information on where exactly the event takes place. (see Figure 32).

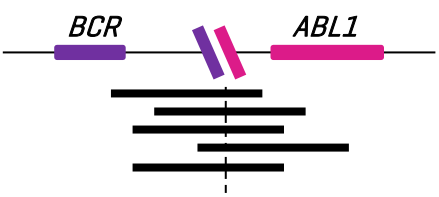


Figure 32. Representation of the readings that include the fusion point, represented as a dashed vertical line, and whose bases map to different regions of the genome (Split reads).

- ***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 33).

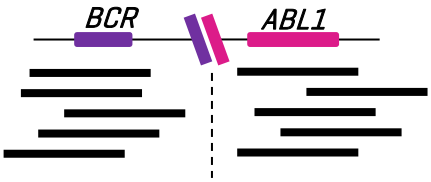


Figure 33. Representation of the readings that do not include the fusion point, represented as a dashed vertical line, and whose search for the sequencing pair (Discordant read pairs) allows identifying the translocation.

The combination of these two detection types enables the identification of structural variants with high reliability, as well as of other types of rearrangements, such as deletions or duplications, all while preventing the possible generation of artifacts during sequencing.

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

In the results screen (Figure 34), you can see all the detected events, classified within three quality ranges: *High*, *Mid*, and *Low*. The quality range assigned to each event will mostly depend on the readings obtained relative to mean coverage.

On this screen, you can select the variants that you want to include in the results report. It is recommended to filter out variants that do not have a high quality (of greater reliability), as well as to examine the sequences of each variant in the IGV viewer. It is also recommended to review variants with lower scores, since these could be real but show a lower score due to their frequency being close to the detection limit.

	Event type	Region 1	Gene 1	Region 2	Gene 2	Split	Mate	Total	Quality	Lineage	Category
☐	Deletion	12:12026415	ETV6	12:12027153	ETV6	393	72	465	High	EMPTY	+
✓	Translocation	9:133729876	ABL1	22:23631909	BCR	280	49	329	High	EMPTY	+

Figure 34. Example of results for structural variants

08.5 | Pharmacogenetic testing

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

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

- Verify that the version of the available IFUs contains information on the pharmacogenetics employed in the kit used (see page 2 of this document).
- Bear in mind that, among all the analyzed regions (see section 2 of this document), the genotypes that have some sort of clinical implication will be shown by default, among which it is possible to select those that will appear in the results report.
- Using the *PharmaGKB* database, verify the interpretation of the variants found.

08.6 | Variant filtering

By pressing the *Request: Filtering* button, a pop-up screen appears, showing the different tests on variants generated so far.

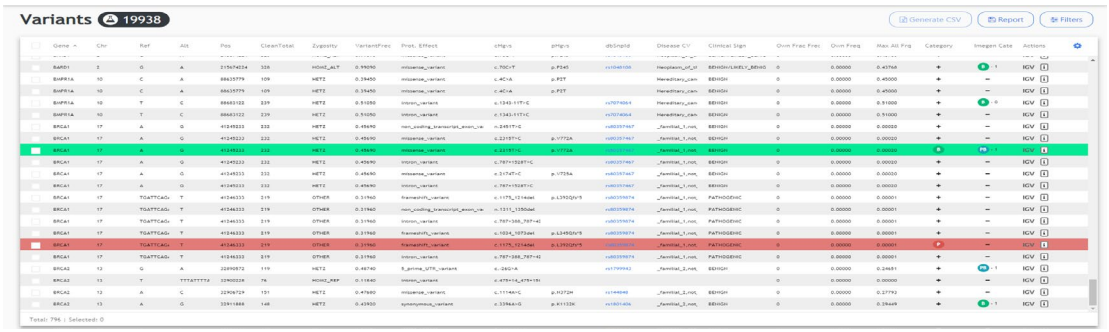


Figure 35. Variant filtering with Data Genomics

Once the variant analysis is opened, the variants that have met the *Haematology OncoKitDx Default* filter criteria appear.

Variant filtering is meant to show the best-quality variants identified within the target regions of Haematology OncoKitDx.

Once all variants shown after the first variant filtering step have been reviewed and categorized, it is recommended to apply a second filter, *Haematology 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 *Haematology 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 a new filter can be created appears. The user must adjust the different options as desired. Once the filter parameters have been selected, they can be saved (*Save*) and used on the current sample by clicking "*Apply*".

Each variant found 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 DataGenomics instructions for use, available at: www.datagenomics.es.

The quality labels exclusive to Haematology OncoKitDx are shown below:

- d200: Read depth covering that position lower than 200.
- f0.02: Allele frequency lower than 0.02.

NOTE: In case of large ITDs expansions in FLT3, the variant may not be detected with the normal procedure while still being detected by the algorithm designed to detect rearrangements; therefore, it is recommended to check both the *Variants* tab and the *Structural* variants tab. If it appears in *Variants*, it is recommended to select the variant if it is to be included in the report; if it only appears in *Structural* variants, it is recommended to not select the variant in this tab and to indicate the existence of an ITD in FLT3 in the interpretation tab.

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

Once the desired filters have been set up by the user, each variant found, be it a point variant, a small deletion/insertion, CNV, or SV, can be categorized.

By clicking on the "*Category*" column, a drop-down list appears for each variant (see Figure 36), showing the different categories that can be assigned to the variant.

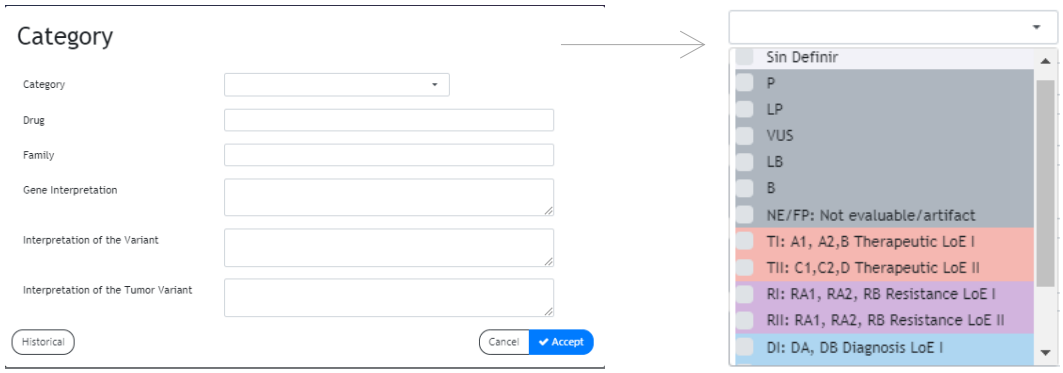


Figure 36. Drop-down menu for the "Category" column

There are different categorization groups, all color-coded by the tool, and each variant may only belong to one of the different groups.

First, it is recommended that each variant be assigned 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 a variant does not need to be assessed or is suspected to be a false positive, it is recommended that it be categorized as "*not evaluable/artifact*", which, if selected, would prevent any further selection.

If a variant is considered benign or probably benign at the functional level, categorizing it in any of the groups of clinical relevance is not recommended. 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 could have a deleterious effect on protein function; however, it is recommended to assess each case in particular, taking into account the alteration and the type of tumor studied.

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

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

- **Gene**: Information on the role of the gene in cancer development.
- **Variant**: Information on and relevance of the variant type in this gene.
- **Tumoral type**: Information on and clinical implications of the variant in the type of tumor 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*, column, Health in Code S.L. provides the categorization for somatic variants, considering both the functional impact that the variant is expected to 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 continuously updated.

By selecting the category recommended by Health in Code S.L. in this column (for example DI PII), a pop-up window can be accessed, which allows viewing 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 Health in Code category*". The accepted categorization will automatically become a 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 database, the user will be warned by the following alert icon: ⚠

Figure 37: An example a category assigned by the user not matching the one recommended by Health in Code S.L.

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

Should you have any questions about the results analysis, please contact Health in Code's technical support team, who will reply to your query within a period of 24 hours.

09 Troubleshooting

A list of possible unexpected results throughout the library preparation and sequencing protocol using Haematology OncoKitDx and the steps to follow for their solution can be found below. For all other general issues related to the Magnis instrument and not listed in this section, please see the instrument 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.

Library fragment size is larger than expected in electropherograms:

Verify the correct execution of the enzyme fragmentation protocol (section 7).

Consider repeating the experiment with control DNA to verify that the experimental samples do not contain fragmentation reaction inhibitors.

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

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

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

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

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

10 Limitations

10.1 | Analytical

- The employed technique cannot distinguish between regions with high sequence homology, such as homologous genes, pseudogenes, etc., which can lead to false positives or negatives. A list of specific pseudogenic regions can be found in Table 16. 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
5	170837600	170837649	NPM1	EX11	NM_002520.6
7	151919612	151919673	KMT2C	EX21	NM_170606.3
7	151921227	151921256	KMT2C	EX20	NM_170606.3
7	151921606	151921750	KMT2C	EX19	NM_170606.3
7	151926959	151926981	KMT2C	IN18*	NM_170606.3
7	151927001	151927097	KMT2C	EX18	NM_170606.3
7	151927115	151927159	KMT2C	IN17*	NM_170606.3
7	151927255	151927328	KMT2C	EX17	NM_170606.3
10	89623781	89623821	PTEN	EX1	NM_000314.8
10	89692789	89692888	PTEN	EX5	NM_000314.8
10	89692929	89693002	PTEN	EX5	NM_000314.8

*Table 10. List of pseudogenic regions *IN=Intron*

- The correctness of any obtained result with quality parameters below the established criteria cannot be ensured.
- NGS is not yet considered the *Gold Standard* for some types of mutation; therefore, it is recommended, whenever possible, to confirm positive results using a complementary standardized technique.
- Haematology OncoKitDx allows detecting 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 patient.

- All the obtained data and information must be clinically evaluated and interpreted by the clinician 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

Haematology OncoKitDx has been validated by using the following thermal cycler for DNA fragmentation:

- *GeneAmp PCR System 9700* (Applied Biosystems)

  If a different brand or model of thermal cycler is used, the amplification program may need to be adjusted. Should you need further information or advice, please contact our technical support team.

Haematology OncoKitDx has been validated using the following automated library preparation system:

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

Haematology OncoKitDx has been validated using the following massive sequencing platform:

- *NextSeq 500/550 System* (Illumina)

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

10.3 | Reagents

Haematology OncoKitDx 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 advised to use the reagents recommended by the sequencer manufacturer: **Illumina**.

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

10.4 | Bioinformatics analysis platform

Haematology OncoKitDx has been validated using **Data Genomics**, which is a platform for in vitro bioinformatics analysis. This platform includes an analysis pipeline tailored specifically for Haematology OncoKitDx, 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

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

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

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