imegen

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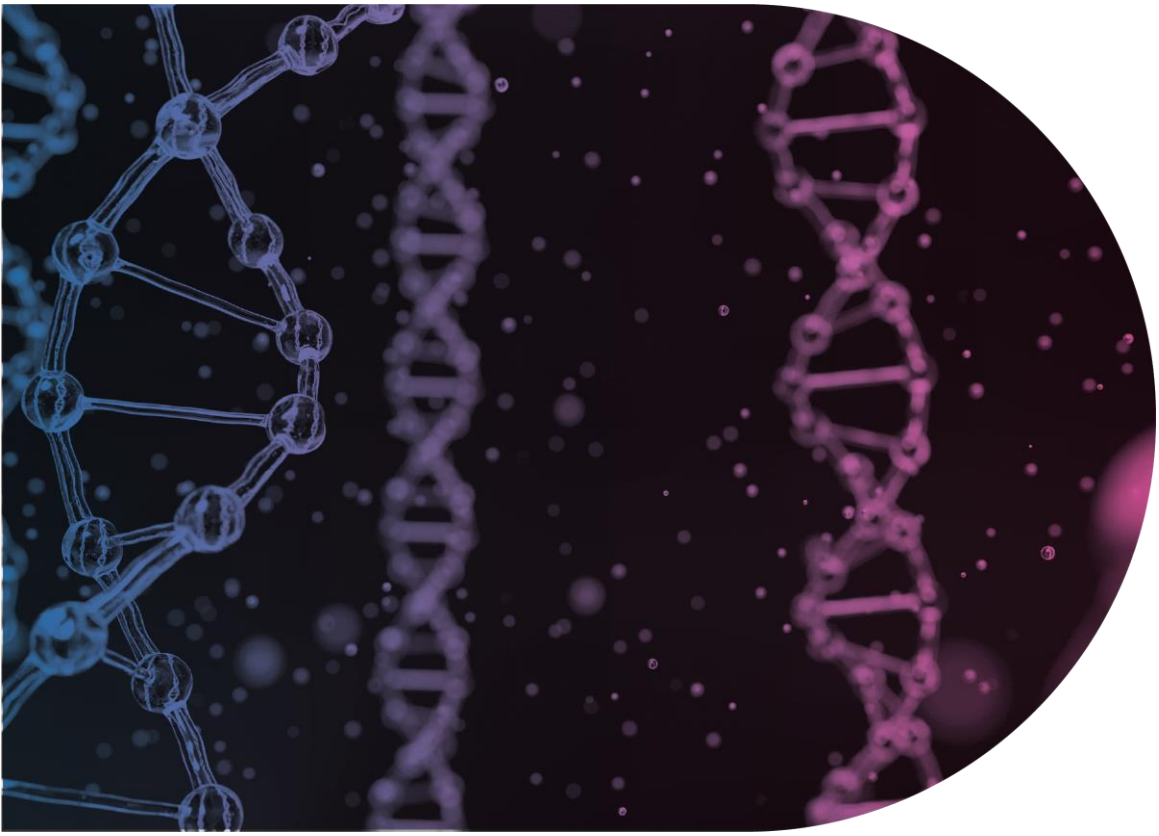

Instructions for use

HRR OncoKit

REF IMG-400

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Rev. 5 08/08/2022

Health in Code guarantees that its products are free of defects, both in materials and workmanship. This guarantee remains in force until the expiration date, as long as the conservation practices described in this manual are adhered to.

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

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

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

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

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Instructions for Use (IFU) modifications	
Rev.5	Section 3 updated and change of manufacturer's identification from Imegen to Health in Code
Rev.4	Sections 3, 7.7 and 7.9 updated.
Rev.3	Addition of pseudogenic panel region, section 10
Rev.2	Sections 1, 2, 3, and 8 added.
Rev.1	Document generated

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1. General information

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

One of these processes is homologous recombination DNA repair (HRR). Tumors showing deficiencies in this mechanism have a higher sensitivity to DNA-damaging agents. The detection of variants in genes involved in the homologous recombination DNA repair pathway allows administering personalized treatments with higher efficacy and lower toxicity.

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2. Intended use

HRR OncoKit has been designed to detect single-nucleotide variants and small insertions and deletions, as well as whole gene gains or losses, in a panel of genes directly or indirectly involved in the homologous recombination DNA repair pathway. The detection of alterations in these genes can allow administering PARP inhibitor drugs. Based on the mutations detected in any of the genes included in the panel, recommendations may include either the administration of drugs approved by regulatory agencies (FDA or EMA) for specific tumors and clinical scenarios or the possibility to enroll in clinical trials where PARP inhibitors are part of the assessed therapeutic regime.

To detect variants related to the homologous recombination DNA repair pathway, the whole exonic regions are sequenced for the following **38 genes**: *ATM, ATR, ATRX, BARD1, BLM, BRCA1, BRCA2, BRIP1, CDK12, CHEK1, CHEK2, ERCC3, FAM175A (ABRAXAS1), FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCL, FANCM, GEN1, HDAC2, MRE11A, NBN, PALB2, PPP2R2A, RAD50, RAD51, RAD51B, RAD51C, RAD51D, RAD52, RAD54L, RPA1,* and *WRN*.

In addition, **HRR OncoKit** integrates a 110-microsatellite panel that allows for microsatellite instability testing (MSI) based on NGS results. MSI testing with this panel corresponds with the Bethesda panel in >99% of cases.

Detection of copy number variations (**CNVs**) in the whole genome. Moreover, this analysis has been improved using a low-density SNP array through the capture of >996 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**).

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

HRR OncoKit is intended for research use only and is aimed at professionals in the sector of molecular biology.

3. Technical characteristics

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

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

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

Technical specifications:

- Type of sample: DNA from peripheral blood and paraffin-embedded tumor tissue.
- Necessary amount of DNA: 10-100 ng.
- Detection of microsatellite instability when at least 85 of the 110 markers included are analyzable.
- Limits of detection:
  - The detection limit of the analysis of point variants, small insertions, and deletions is 5%.
  - The limit of detection for CNV analysis is 3 copies for gains and 1 copy for deletions.
- Mean coverage: 4400X.
- Mean coverage after UMI analysis: 2700X.
- Coverage: 97.4% of bases covered at 200X depth.
- Uniformity: 96.6% of bases covered at >20% mean coverage.
- Specificity: > 99%
- Sensitivity: > 99%
- Repeatability: > 99.99%
- Reproducibility: <98%

HRR OncoKit is compatible with Illumina's massive sequencing platforms.

This product complies with the quality requirements established by ISO 9001, both in its validation and manufacturing process as well as in the materials used.

4. Safety warnings and precautions

1. It is recommended to strictly follow the instructions in this manual, especially regarding the handling and storage conditions of the reagents.
2. Do not mouth-pipette.
3. Do not smoke, eat, drink, or apply cosmetics in areas where kits and samples are handled.
4. Any cuts, abrasions, and other skin injuries must be properly protected.
5. 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.
6. In the case of an accidental spill of any of the reagents, avoid contact with the skin, eyes, and mucous membranes and rinse with a large amount of water.
7. Safety data-sheets (MSDS) of all dangerous substances contained in this kit are available on request.
8. This product requires the manipulation of samples and materials of human origin. It is recommended to consider all materials of human origin as potentially infectious and manipulate them according to level 2 of the OSHA norm on biosafety and bloodborne pathogens or other practices related to biosafety of materials that contain or are suspected to contain infectious agents.
9. The reagents included in this kit are not toxic, explosive, infectious, radioactive, magnetic, corrosive, or environmental biological pollutants.
10. This kit has been validated with specific equipment and under specific conditions that may vary widely among laboratories. Therefore, it is recommended that each laboratory verify compliance with the technical specifications of the manufacturer when the kit is to be used for the first time.
11. The manufacturer assumes no responsibility for any damage or failure of the assay caused by substituting reagents included in the kit for ones not provided by Health in Code.
12. The manufacturer does not guarantee the assay's reproducibility when the user uses reagents that have not been validated by Health in Code but are considered by the user equivalent to those provided in the kit.
13. The manufacturer is not liable for the obtained results when the bioinformatics analysis is carried out on an analysis platform different from DataGenomics.

5. Content and storage conditions of the kit

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

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

Here are the components of the kit listed:

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

Table 1. Reagents in box 1 of HRR OncoKit

Box 2 of 4			
Reagents	**Color indicator**	**Quantity**	**Conservation**
Fragmentation Buffer	Green cap	72 µL	-20 °C
Fragmentation Enzyme	White cap	36 µl	-20 °C
Reagent Plate	White/Black	3 plates	-20 °C
Index strip*	Black	3 strips	-20 °C

Table 2. Reagents in box 2 of HRR OncoKit

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

Box 3 of 4			
Reagents	**Color indicator**	**Quantity**	**Conservation**
HRR Probe Strip	White	3 strips	-80 °C

Table 3. Reagents in box 3 of HRR OncoKit

"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	Blue	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. "Magnis Empty Consumables" box reagents; box 4 of 4

NOTE: The kit contains two "Magnis Empty Consumables" boxes, one for each run of 3 samples on the Magnis instrument.

6. Equipment, reagents, and material not included in the kit

Equipment:

- 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, 0.2 mL strips, and 96-well plates; with adjustable sped of at least 1,000 rpm)
- Fluorometer (recommended: Qubit; ThermoFisher)
- Fragment analyzer (optional: TapeStation System by Agilent Technologies; LabChip GX Touch/GXII Touch by PerkinElmer)
- Thermal cycler with adjustable heated lid
- Automated library preparation system Magnis NGS Prep System by Agilent Technologies (cat. no. G9710AA)
- Illumina sequencer (recommended: MISeq)

Reagents:

- Extraction kit
- 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 1000µL)
- 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

HRR OncoKit is designed for use in combination with the **Imegen-Sample tracking components (REF: IMG-340)** kits, which allow tracking each sample from DNA dilution to bioinformatics analysis of the results via an integrated system that identifies samples. This guarantees that samples can be tracked during the whole protocol. These references are available upon request.

7. Assay protocol

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

The steps necessary to carry out the preparation of 8 libraries using **HRR OncoKit** are outlined below.

For correct coverage normalization, which is necessary for the CNV analysis, it is necessary to have the reference *fastq* libraries. Due to the intrinsic variability of the technique observed in different laboratories, it is necessary that the reference samples be processed in the same laboratory, with the same equipment, and following the same protocol as the test samples. To generate *fastq* files of germline reference samples of both sexes available, Health in Code provides the user with the "*Reference sample*2 reagent, which contains one sample to be processed with **HRR OncoKit**.

7.1 Preparation of the Magnis system for the execution of a protocol

1. Ensure that there are no materials from previous runs on the instrument deck, as they could interfere with the setup and launch processes.
2. Close the instrument door.
3. Turn on by pressing the power button on the front of the instrument (LED indicator lights will turn on). Wait while the system launches. This may take a few minutes.
4. UV decontamination is recommended prior to each run. In order to do so:
   - On the *Home* screen, press *Decontamination*.

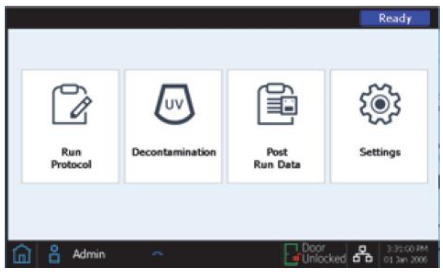


Figure 1. *Magnis NGS Prep* system *Home* screen

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

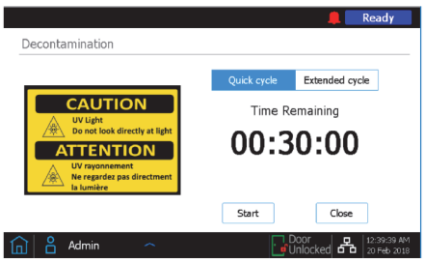


Figure 2. *Magnis NGS Prep Decontamination* screen

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

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

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

7.2 Preparation and fragmentation of the DNA template strand

The steps necessary to carry out the preparation of 8 libraries using **HRR OncoKit** are outlined below.

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

7.2.1 Quantification and dilution of DNA samples

1. Thaw DNA samples at room temperature.
2. Vortex and quantify DNA samples with a fluorometer, such as Qubit.
3. Dilute each DNA sample to 25 ng/µL with nuclease-free water to a final volume of 20 µL.

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

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

4. Mix on a vortex mixer and quantify each sample with a fluorometer, such as Qubit.

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

> If it is not possible to obtain a total of 100 ng in a volume of 7 µL:
> - Lower the concentration to a total of 50 or 10 ng. If this method is selected, it is advised to apply it to the 8 samples in the run. Otherwise, the Magnis equipment must be configured with the recommended program for the sample with the lowest input.
> - The final volume must be duplicated, also duplicating the volume of the rest of reagents used for fragmentation (see the end of this section).

6. **Optional:** To process the reference sample, for a concentration of 25 ng/µL, add to the corresponding 0.2 mL tube on the strip:
   - 5 µL of the reference sample.
   - 1 µL of the traceability reagent used (or nuclease-free water if the integrated traceability system is not used).
   - 1 µL of nuclease-free water

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

7.2.2 Quantification and dilution of DNA samples

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

1. Thaw the *Fragmentation Buffer* and keep cold. Keep the *Fragmentation Enzyme* at -20 °C until its use.
2. Prepare the required volume of the fragmentation mix at cold temperature, as described below, mixing each reagent before use. The *Fragmentation Buffer* should be vortexed vigorously, while the *Fragmentation Enzyme* is mixed by inversion several times. When processing several samples, we recommend preparing a 12% excess of all reagent mixes.

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

3. Vortex vigorously.
4. Add 3 µL of the fragmentation mix to each 0.2 mL tube with the fragmented sample. Mix by pipetting 20 times.
5. Seal the strip, spin the samples, and immediately place the tubes inside the thermal cycler and execute the fragmentation program.
   - Lid pre-heated 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 program requires that the lid be pre-heated to 100°C. When using thermal cyclers with high ramp rates, such as the one used during the validation of this protocol, GeneAmp PCR System 9700 (ThermoFisher), pre-heating the lid is not necessary. If this is not the case, pre-heat the lid for a few minutes before starting the protocol.

6. Once the fragmentation program is completed, remove samples from the equipment, spin, add 40 µL of nuclease-free water to each sample, transfer the whole volume to a *Sample Input Strip*, seal with the included foil seals, and keep cold until their use in the next step.

   Note: The samples 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 labels that could obscure the barcode of the *Sample Input Strip*.

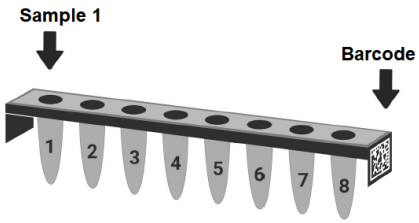


Figure 3. Required orientation of samples in the *Sample Input Strip*.

IMPORTANT: In the extraordinary case of needing to duplicate the final volume and the samples and reagents used for fragmentation, once said program has been completed, only 30 µL of nuclease-free water need to be added.

7.3 Preparation of the reagents and plasticware used by the Magnis system

Reagents to be used in this step:

Reagent	Color	Conservation
Reagent Plate	Blue plate	-20 °C
Beads/Buffers Plate	White plate	4 °C
Index Strip	Black strip	-20 °C
HRR Probe Strip	White strip	-80 °C
"Magnis Empty Consumables" box	Black strip	15-25 °C

1. Preparation of the *Reagents plate* reagent:
    - Thaw the plate at room temperature, keeping the plate in its white cardboard sleeve.
    - Once the contents of all wells are thawed, vortex the plate while keeping it in its white cardboard sleeve. Begin by pressing the long side of the plate on the vortex head for 10 seconds. Afterwards, rotate the plate 90° and press the short side of the plate on the vortex head for an additional 10 seconds. Repeat the rotation/mixing sequence until completed on all four sides of the plate.
    - Spin the plate (still in its white cardboard sleeve) in a centrifuge set at 250 x g for 1 minute.
    - Make sure that there are no bubbles at the bottom of the plate wells. If bubbles are present, repeat the spin step.
    - Keep plate in its packaging and at cold temperature to be used on the same day.
2. Preparation of the *Beads/Buffers plate*:
    - Keep the sleeved plate at room temperature for about 30 minutes.
    - Vortex the plate (still in its cardboard sleeve). Start by pressing the long side of the plate on the vortex head for 10 seconds. Afterwards, rotate the plate 90° and press the short side of the plate on the vortex head for an additional 10 seconds. Repeat the rotation/mixing sequence until completed on all four sides of the plate.
    - Spin the plate (still in its cardboard sleeve) in a centrifuge set at 150 x g for 10 seconds. Do not exceed recommended spin times to prevent beads from pelleting.

- Keep plate in its cardboard sleeve at room temperature to be used on the same day.

3. Preparation of the *Index strip:*

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

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

Table 6. Sequences of the indexes included in the kit.

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

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

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

Table 7. Correlation between the *Index* on the *Post-Run Data* screen and the inscription on the strip.

4. Immediately before use, thaw the *HRR 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.

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

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

7.4 Execution of the library preparation protocol

7.4.1 Start of the protocol

1. On the *Home* screen shown on the touchscreen, press *Run Protocol*. The system will lock the instrument door and carry out an *Instrument Health Check* (IHC), which may take several minutes.
2. Once this check is completed, the message *Enter Run Info* will automatically appear on the screen. In the *Protocol* menu, select *SSEL XTHS-RevB-ILM*.
3. Recommended: Mark the *Aliquot sample for QC* checkbox if you want the instrument to take an aliquot of each pre-capture library. This will enable quality control later on.
   Note: Quality control of the pre-capture libraries will only be available once the run has been fully completed.

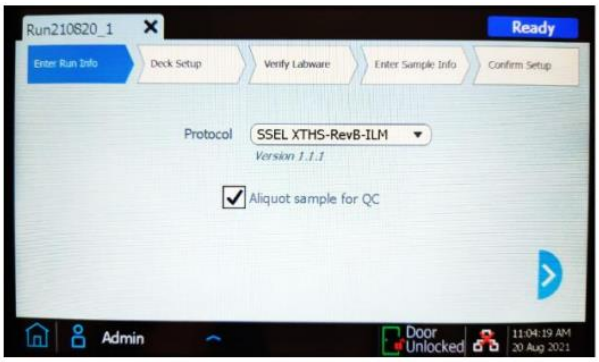


Figure 4. *Magnis NGS Prep* System *Enter Run Info* screen

4. Move on to the next screen.
5. Select appropriate sample type, *High Quality DNA*.
6. Select input DNA amount in the *Input Amount* menu. Even though the options for 10 ng, 50 ng, 100 ng, and 200 ng appear, an amount of 100 ng is recommended to prepare libraries using **HRR Plus OncoKit**. If any sample in the run is below 100 ng, select the amount of the sample with the smallest amount in the run.

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

7.4.2 Deck setup

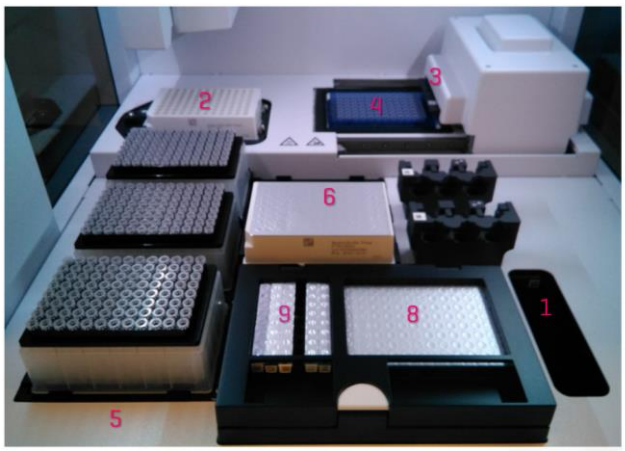
The deck can be very easily set up by following the steps on the Magnis touchscreen.

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: Carga del recipiente desechable en el cajón de desechos.
Step 2/10: Carga de Magnis Deep-Well HSM.
Step 3/10: Carga de Magnis Thermal Cycler Seal en la ranura del módulo termociclador.
Step 4/10: Carga de Magnis 96-Well PCR Plate en el bloque del módulo termociclador.
Step 5/10: Carga de 3 cajas de puntas completamente llenas.
Step 6/10: Carga de Beads and Buffers Plate.
Step 7/10: Comprobación de temperatura óptima en el módulo de enfriamiento.
Step 8/10: Carga de Reagent Plate en el módulo de enfriamiento.
Step 9/10: Carga de las tiras de tubos en el módulo de enfriamiento (QC Strip opcional).
Step 10/10: Cerrar la puerta del equipo Magnis.

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

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

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

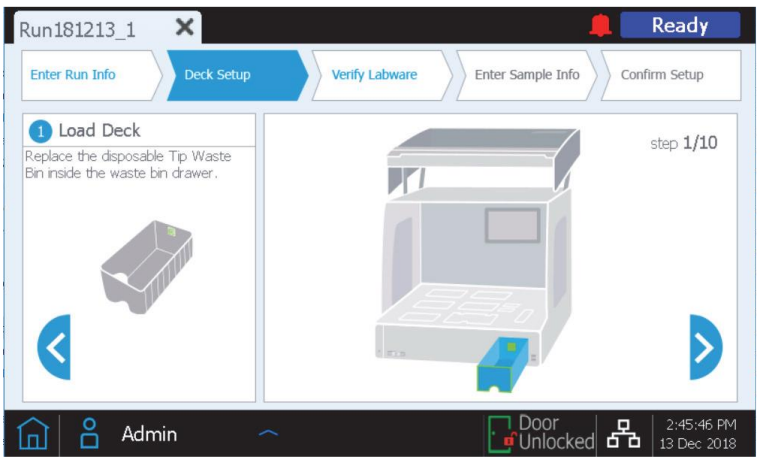


Figure 6. Step 1 of 10 on the *Deck Setup* screen of the Magnis NGS Prep System

2. 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.

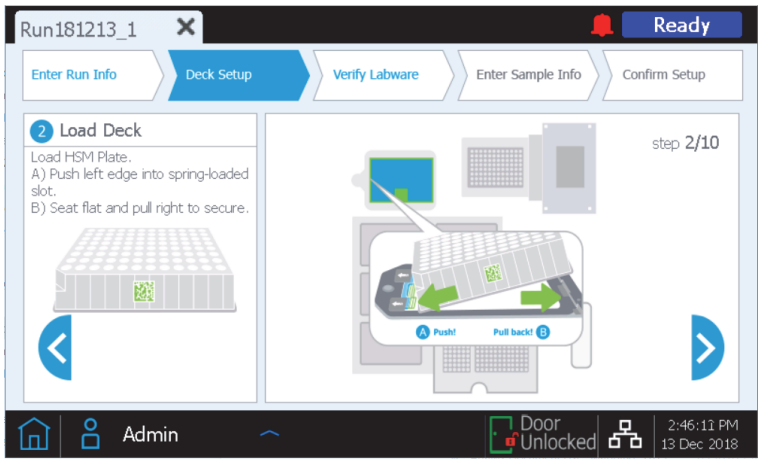


Figure 7. Step 2 of 10 of the *Deck Setup* screen of the Magnis NGS Prep System

3. 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.

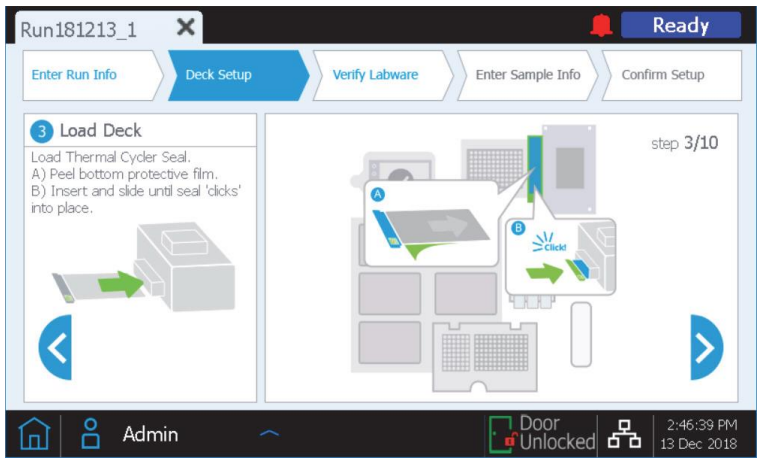


Figure 8. Step 3 of 10 of the *Deck Setup* screen of the Magnis NGS Prep instrument.

4. 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.

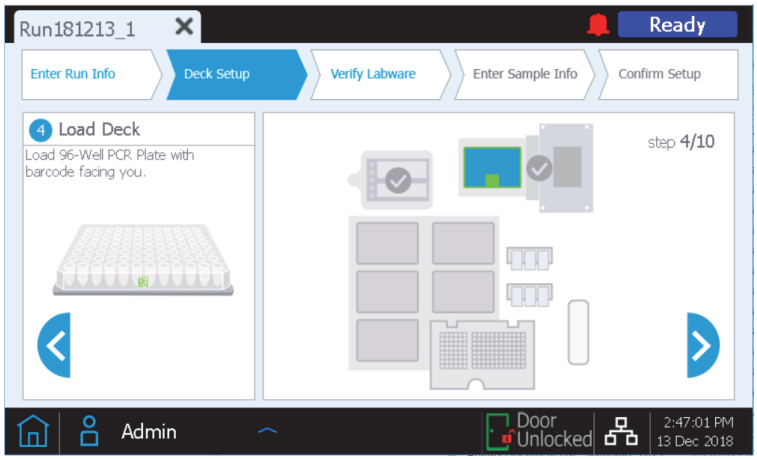


Figure 9. Step 4 of 10 of the *Deck Setup* screen of the Magnis NGS Prep instrument.

5. 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.

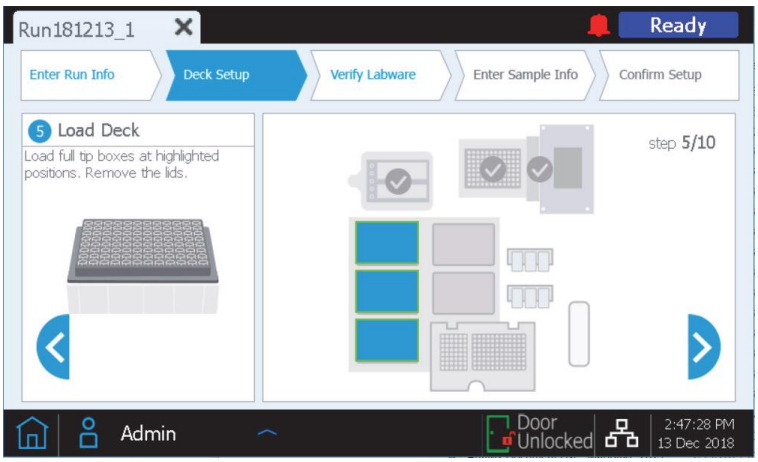


Figure 10. Step 5 of 10 of the *Deck Setup* screen of the Magnis NGS Prep instrument.

6. 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.

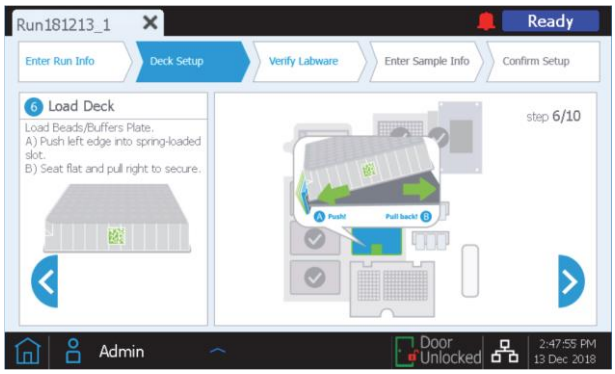


Figure 11. Step 6 of 10 of the *Deck Setup* screen of the Magnis NGS Prep instrument.

7. 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.

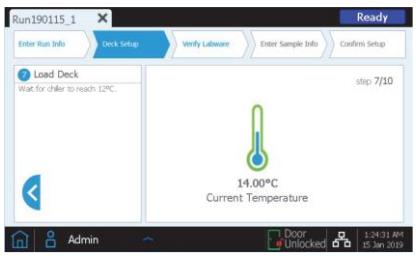


Figure 12. Step 7 of 10 of the *Deck Setup* screen of the Magnis NGS Prep instrument.

8. 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.

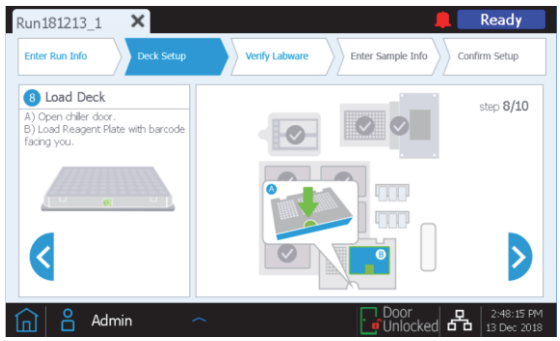


Figure 13. Step 8 of 10 of the *Deck Setup* screen of the Magnis NGS Prep instrument.

9. Load the strip tubes for the run in the indicated positions of the chiller, as shown on the instrument's touchscreen. Ensure that each strip is secured by pressuring firmly and evenly on the edges of the strip tubes. Avoid touching or damaging the foil covers. Make sure all the strip tubes have their *barcode* facing the user.

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

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

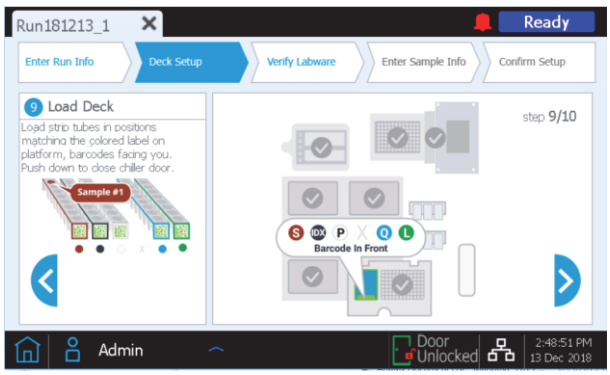


Figure 14. Step 9 of 10 of the *Deck Setup* screen of the Magnis NGS Prep instrument.

10. Close the instrument door.

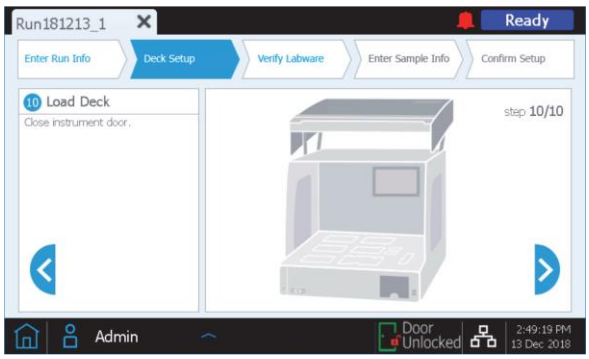


Figure 15. Step 10 of 10 of the *Deck Setup* screen of the Magnis NGS Prep instrument.

7.4.3 Labware verification

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

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

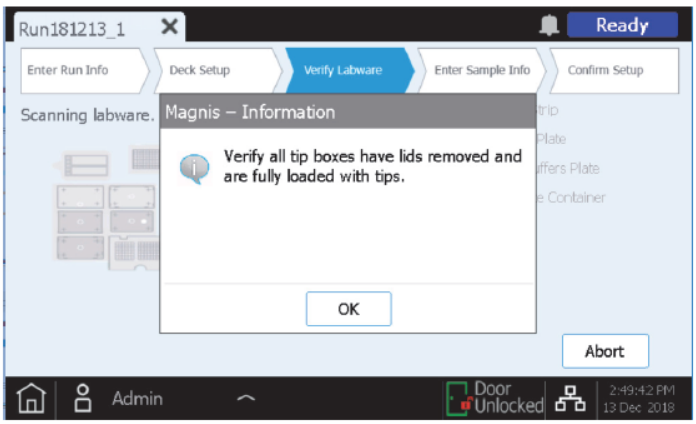


Figure 16. *Verify Labware* pop-up window of the Magnis NGS Prep System

During the verification of the material, 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. On the contrary, see section 9 of this document.

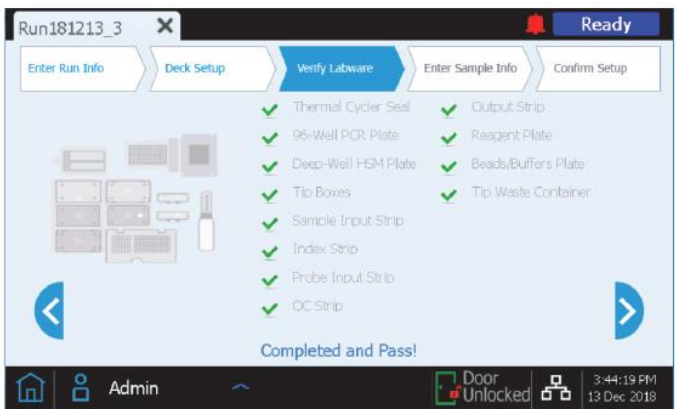


Figure 17. *Verify Labware* screen of the Magnis NGS Prep System after a correct verification of the material

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

7.4.4 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:

1. 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.

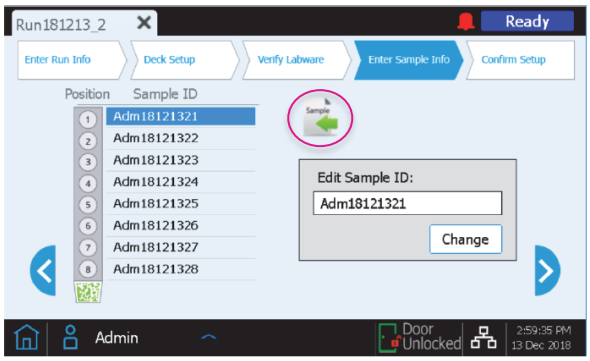


Figure 18. *Edit Sample Info* screen of the Magnis NGS Prep System, with a circle highlighting the load samples button.

2. Import of sample assignments using a .csv file:
    - Create a .csv file (comma-separated value) containing the sample names in the correct order. To enter the new sample names, you can use *Microsoft Excel* and save the file in .csv format.
    - Write '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 the .csv format.
    - Transfer the .csv file to an unencrypted USB disk and introduce the USB in the of the

Magnis ports.

- 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.

7.4.5 Confirm setup and start the run

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

2. 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 **HRR OncoKit.**

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

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

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

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

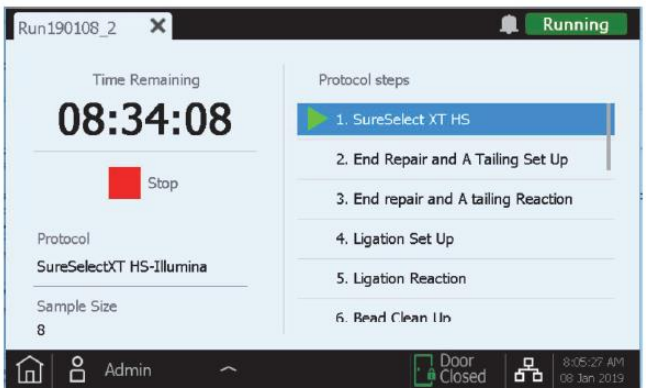


Figure 20. Running screen *during a run.*

7.4.6 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.

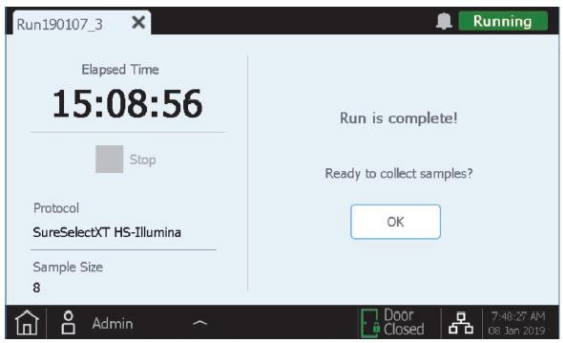


Figure 21. Running screen *after a run*.

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

The chiller module will be held at 12 °C for a 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.

It is possible to stop the protocol at this point, storing the libraries at 4 °C if they are to be used within the following 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.

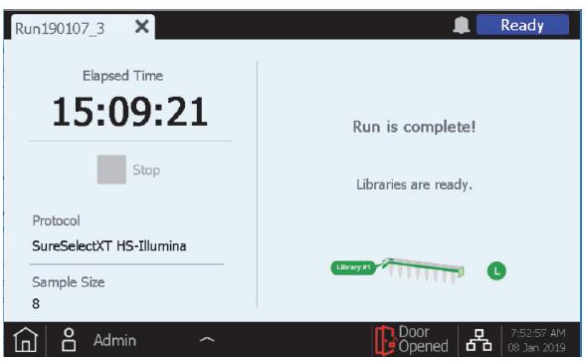


Figure 22. *Running* screen after a run and after removing the libraries

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

7.5 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 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.

7.6 Library validation and quantification

7.6.1 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 the 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, review the protocol or contact Health in Code's technical support team.

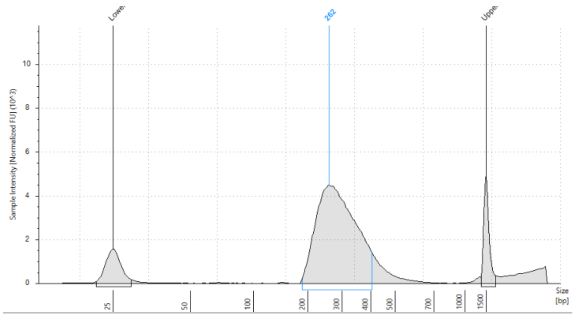


Figure 23. Expected result after analyzing pre-capture library size with TapeStation 2200

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 100 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).

7.6.2 Quality control of the post-capture library

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

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

The concentration of post-capture libraries will vary between 1 and 10 ng/µL.

For the quality analysis of the captured fragments, Health in Code recommends the use of *TapeStation 2200* 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 is between 260 and 340 bp. If unexpected sizes are obtained, review the protocol and pre-capture library quality control, carefully read the troubleshooting section, or contact Health in Code's technical support team.

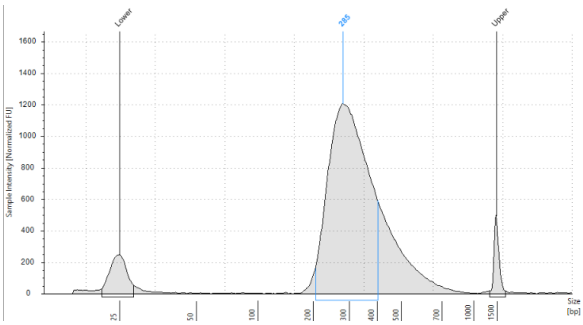


Figure 24. Expected result after the analysis of post-capture library size with TapeStation 2200

From DNA concentration and the mean captured library fragment size, we determine the concentration of each library through the following formula:

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

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

IMPORTANT: In processing samples with different inputs and qualities of the starting DNA, carry out the pool as specified below:

- **Libraries using fragmented DNA with a starting total amount equal to or greater than 50 ng:** These libraries within the pool will start from volume that will become the baseline. For example 2 µL.
- **Libraries with poor-quality DNA (old extractions, DIN less than 2.5, etc.) and/or with total starting amounts less than 50 ng:** These libraries must be represented in the pool four times more than the rest of the libraries. For example 8 µL.
- **Libraries with poor-quality DNA (old extractions, DIN less than 2.5, etc.) and total starting amounts equal to or greater than 50 ng:** These libraries must be represented in the pool twice as much as the rest of the libraries. For example 4 µL.

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

7.7 Library denaturation

A denaturation protocol must then be carried out prior to loading into an *Illumina NextSeq* sequencer, following the steps below:

1. Thaw the HT1 reagent (included in the Illumina reagent kit to be used during sequencing) and keep cold until its use.
2. 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 library denaturation protocol must be followed.
3. Add 5 µL of the library pool, previously diluted to 4 nM, and 5 µL of NaOH 0.2N to a 1.5 mL tube. Vortex and spin.
4. Incubate at room temperature for 5 minutes.
5. Add 5 µL of Tris-HCl 200 mM pH 7. Vortex and spin.
6. Add 985 µL of HT1 and vortex. The library should now be at 20 pM.
7. Transfer 97.5 µL of the 20 pM library to a new 1.5 mL tube.
8. Add 1202.5 µL of HT1.
9. Add 1.2 µL of denatured *PhiX* control diluted to 20 pM to the mix. At this point, the library should be diluted to 1.5 pM.
10. Load the entire volume contained in the 1.5 mL tube into the cartridge.

The following table specifies the maximum number of samples per run, according to the sequencing kit that is used, to guarantee a minimum number of PF clusters of approximately 9 millions per sample:

MiSeq Reagents Kit	Maximum no. of samples
NextSeq 500/550 Mid Output v2.5 kit (150 cycles). Ref:20024904	16
NextSeq 500/550 High Output v2.5 kit (150 cycles). Ref:20024907	32

Table 8. MiSeq Illumina kit and maximum number of samples to be analyzed with HRR OncoKit.

IMPORTANT: The necessary data on the number of samples per run and PF cluster are valid for runs in which all the sequenced libraries come from DNA with inputs equal to or greater than 50 ng. Libraries from poor-quality DNA (old extractions, DIN less than 2.5, etc.) and/or with total starting amounts less than 50 ng will require at least 25 millions of PF clusters, and libraries from poor-quality DNA (old extractions, DIN less than 2.5, etc.) and amounts of starting DNA equal to or greater than 50 ng total will require at least 12 millions of PF clusters.

7.8 Configuration of the NextSeq platform

1. Configure the platform by executing the independent mode ("Standalone"), since BaseSpace currently does not admit the sequencing of the "molecular barcode" as an index.
2. Follow the equipment's loading instructions.
3. 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

7.9 Generation of FastQ files necessary for bioinformatics analysis

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

For transformation of the bcl files to FastQ files, contact Health in Code's support team.

8. Analysis of results

Bioinformatic analysis of the results is done through an analysis *pipeline* designed specifically for **HRR OncoKit**, through the Data Genomics platform. To access this tool, visit: www.datagenomics.es.

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

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

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

8.1 Request for analysis

1. 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 two fastq files associated with each sample must be imported. Optionally, the *SampleSheet* file could be used as well; this file allows simultaneously importing all files from the same sequencing run.

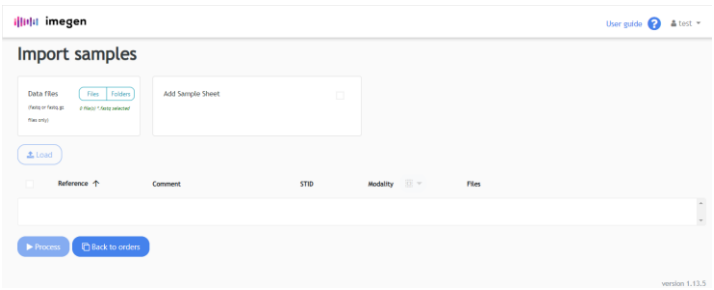


Figure 25. Screen for importing the fastq files and the sample sheet and commence the analysis request.

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

3. Before processing the sequencing files, it is necessary to fill in some required fields for each sample: tumor type and percentage of tumor cells. To access the pop-up screen with these fields, click the pencil icon. Apart from the required fields, there are additional fields that the user may find use (Figure 26). Once filled in, click "*Accept*".

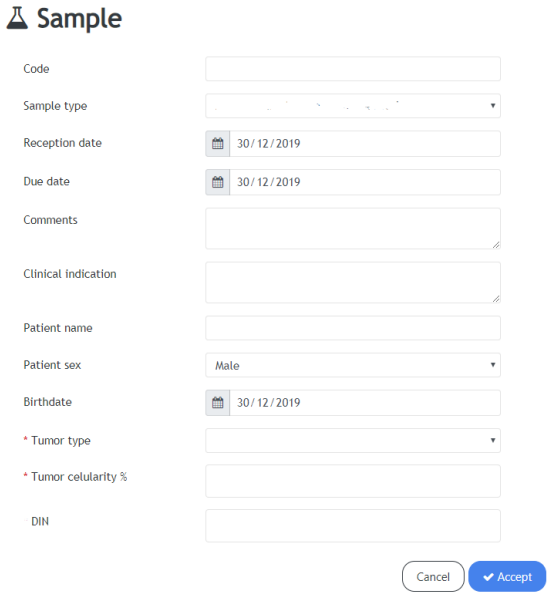


Figure 26. Pop-up screen with the required fields for each sample.

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

4. 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.

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

8.2 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 generated after the bioinformatics analysis: files from the microsatellite instability analysis

(.msi.txt), CNV testing (.CNVs.png and CNVs.txt), the alignment files (.bam and .bai), and the list of variants (.vcf), as well as other files with information on coverages and the quality report of the sequencing after the bioinformatics analysis.

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

- FASTQ: The established acceptance criteria are detailed in the *DataGenomics* instructions for use, available at: www.datagenomics.es.
- BAMs:
  - Mapped Reads (%):
    - Fail: < 98.1
    - Warn: 98.1 – 98.7
    - Pass: > 98.7
  - DP200 (%)
    - Fail: < 97.5
    - Warn: 97.5 – 98.4
    - Pass: > 98.4
  - On-target (%)
    - Fail: < 50.4
    - Warn: 50.4 – 57.6
    - Pass: > 57.6
- STIDs: Verification that the tracking reagent obtained matches the expected one (if it has been used), as shown in Figure 27.

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

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

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

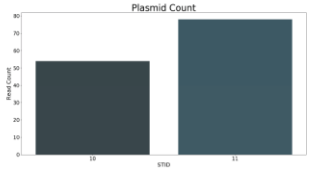


Figure 27. Quality control of the integrated tracking system.

To access the variants filter, in the "*Filtering*" request the *HRR OncoKit Default* filter will be applied, which is characterized by:

- Quality variants: **PASS; d200; pseudogenic homology** [*Fault summary*].
- Depth: ≥ **20X** [*Clean total count*].
- Reads with the balanced variant: ≥ **2 *forward* / 2 *reverse***. To filter artifacts.
- Allele frequency: ≥ **5%** *[Variant Freq]*. Limit of detection established for samples with DIN >3.
- 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.

When the first analysis is finished, it is recommended to apply a second filter "HRR OncoKit II" in which all the variants cataloged by Clinvar as pathogenic or possibly pathogenic are shown.

8.3 Analysis of large rearrangements (CNVs)

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

Since the number of 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:

1. 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.
2. 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 **HRR OncoKit**.

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

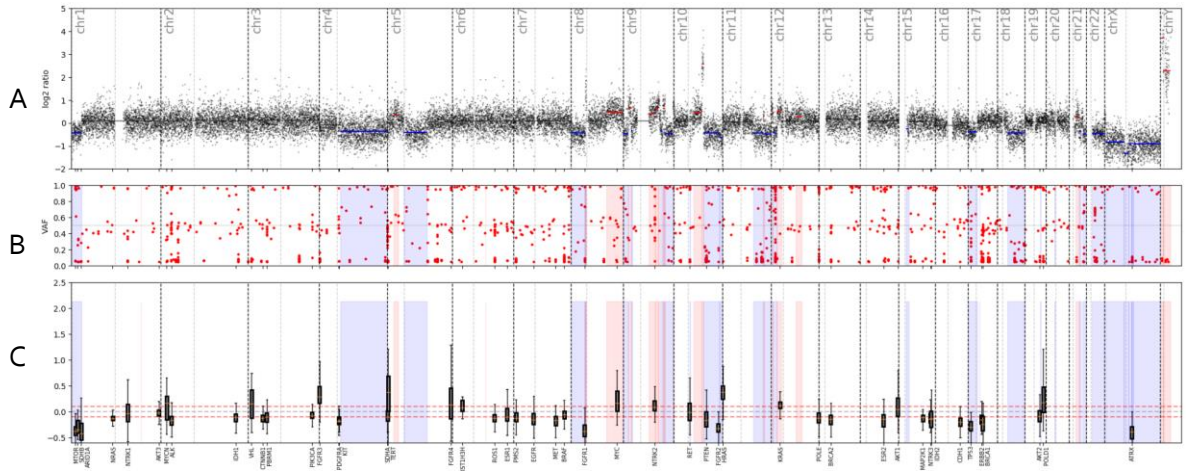


Figure 28. The log2_ratio value (A), allelic frequency of the variant (B), and deviation of the normalized coverage of a gene in the tumor with respect to the coverage of that same gene in germline DNA (C) are plotted on the ordinate axis; the abscissa axis represents the 24 chromosomes present in human cells and the genes included in the HRR OncoKit panel according to their location in the genome. A) Result of CNV testing of the entire genome by means of *off-target* analysis. Each point represents 100 Kb of sequence. Deleted regions appear in blue and amplified regions in red. B) Result of CNV testing of the entire genome by means of SNP array analysis. Each red dot is one of the >996 SNPs tested for. C) CNV analysis per gene in which the difference between gene coverage in tumor DNA and germline DNA is represented, as well as the location of the gene in the genome.

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

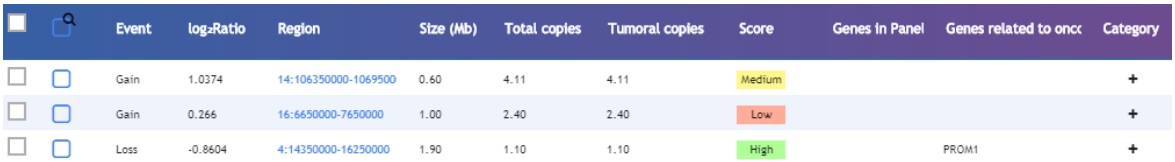
To analyze CNV results with *DataGenomics*, access the results of the "*Filtering*" request, specifically the CNVs tab. On this screen, you can apply different filters and select the variants that you wish to include in the results report.

For each CNV call, the *software* calculates a *Score*, by taking into account parameters such as CNV size, nearby calls (<3 Mb), the value of the *log_2 ratio*, and if the gene or genes affected by the CNV are included in this panel; this information is used to classify the variants as *High-Score, Medium-Score*, and *Low-Score*.

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

The calculation of the number of copies will be adjusted according to the degree of infiltration of the tumor with non-tumorous cells, a value that will be indicated to the user in the "*Tumor cellularity*" field.

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



Event	log2Ratio	Region	Size (Mb)	Total copies	Tumoral copies	Score	Genes in Panel	Genes related to oncc	Category
Gain	1.0374	14:106350000-106950000	0.60	4.11	4.11	Medium			+
Gain	0.266	16:6650000-7650000	1.00	2.40	2.40	Low			+
Loss	-0.8604	4:14350000-16250000	1.90	1.10	1.10	High		PROM1	+

Figure 29. CNV results

8.4 Microsatellite instability analysis (MSI)

Traditionally, microsatellite instability has been analyzed by immunohistochemistry or by fragment analysis using at least five microsatellites. However, a panel of 110 microsatellite markers that allows the determination of the instability condition in the same sequencing reaction has been developed for **HRR OncoKit**.

The results of the MSI analysis can be found in *DataGenomics* in the "*Filtering*" request in the "*Microsatellites*" tab.

As an acceptance criterion for the MSI analysis in a sample, it has been established that there must be more than 85 valid markers for study. In this case, instability can be assigned according to Table 9.

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

Table 9. Instability range using HRR OncoKit

In the opposite scenario (<85 valid markers for analysis), there will be an alert indicating that the MSI analysis could not be carried out.

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

Inestability fraction	Analysed markers	Results	Clinical interpretation	Unstable Markers
0.17	104/110	MSS	El tumor presenta inestabilidad d	18

Figure 30. MSI results

8.5 Variant filtering

By pressing the *Request: "Filtering"* button, a pop-up screen with the different tests on variants generated so far appears (see Figure 31).

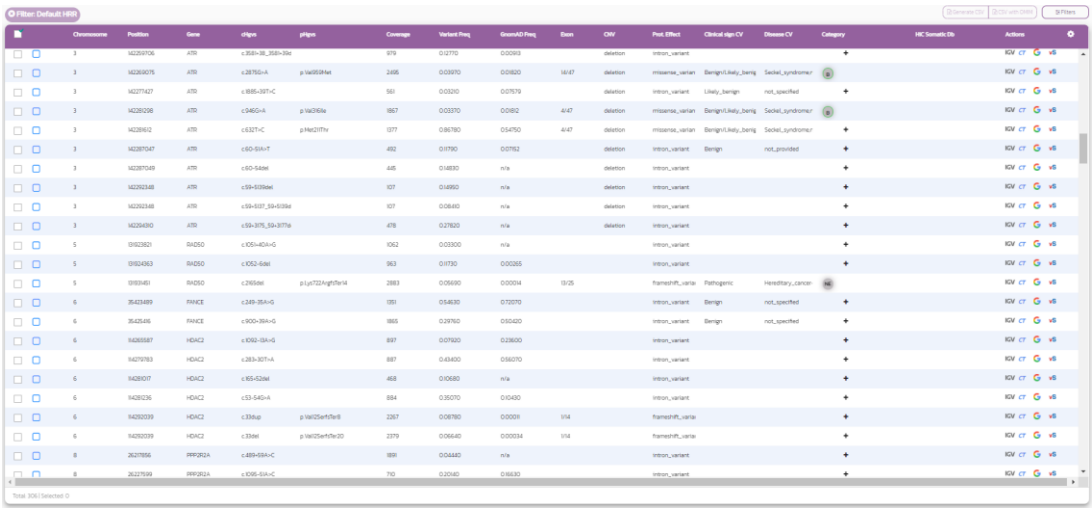


Figure 31. Variant filtering with *DataGenomics*

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

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

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

Below are shown the quality labels exclusive to **HRR OncoKit**:

- d200: Read depth less than 200.
- f0.03: Allele frequency lower than 0.03.

In samples with a DIN <3, it is recommended that variants with allele frequencies lower than 10% be not reported, since the degradation of the sample may have affected the detection limit of the technique.

Categorization of SNV, INDEL, CNV, and SV variants

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

By clicking on the "*Category*" column, a drop-down list appears for each variant (see Figure 32), with the different categories that can be assigned to the variant.

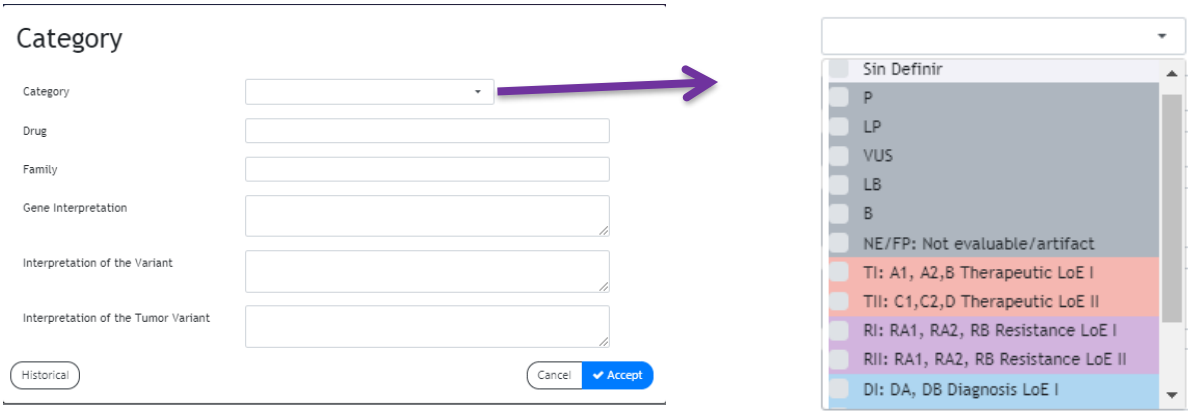


Figure 32. Drop-down list in the "*Category*" column.

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

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

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

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

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

(green).

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

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

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

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

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

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

Figure 33. Example: Category assigned by the user does not match the one recommended by Health in Code.

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

Should you have any doubt about the results analysis, please contact Health in Code's technical

support, and your issue will be responded to within 24 hours.

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

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

9. Troubleshooting

A list of possible unexpected results throughout the library preparation and sequencing protocol using **HRR OncoKit** 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 for the instrument.

- **Using the touchscreen for run setup presents usability issues:**

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

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

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

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

  1. 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.
  2. 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.
  3. Reboot the system. After logging in, the instrument will perform another IHC. If the 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 by following the steps below.

  1. 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.
  2. Once the Auto Teaching process is finished, start configuration of the assay by pressing Run Protocol on the Home screen.

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

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

  1. In the error dialog box, press Cancel to reject the start of the diagnostic test.
  2. Press the error icon at the bottom of the screen and record the error code for potential use in troubleshooting with Agilent Technical Support.
  3. Turn off the instrument by pressing the power button on the front of the instrument.
  4. 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.
  5. Turn on the instrument by pressing the power button.
  6. After logging in, the instrument will perform another IHC. If this check is successful, begin run setup. If the IHC fails again, contact Agilent Technical Support for assistance.

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

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

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

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

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

  - The scanned labware is past its expiration date:

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

  - The scanned labware is incorrectly positioned:

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

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

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

- **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.

- **Cluster density different than expected:**

  In this case, it is advised to review library quantification and the library pool generation protocol prior to sequencing.

- **Errors in STID:**

  Should sample tracking reagents provided by Health in Code be used, it is possible that the STID will not match the expected one. In this case, checking the STID specified in the samples sheet is recommended.

- **Coverage issues:**

  Coverage issues that affect other regions and not covered in the section about kit limitations may arise due to low DNA quality or issues during the library preparation and/or capture protocol. It is advised to check the quality of the input DNA; if the quality problem affects all samples, all steps of the protocol must be verified.

10. Limitations

10.1 Analytical

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.

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.

The employed technique cannot distinguish between regions with high sequence homology, such as homologous genes, pseudogenes, etc., which can lead to false positives or negatives. A list of pseudogenic regions is shown in Table 10. 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.

Cromosoma	Posición de inicio	Posición final	Gen	Secuencia de referencia
3	10105932	10106079	FANCD2	NM_033084

Tabla 10. Listado de regiones pseudogénicas

10.2 Equipment

HRR OncoKit has been validated using the following equipment for DNA fragmentation:

- GeneAmp PCR System 9700 (Applied Biosystems) for enzymatic fragmentation.

HRR OncoKit has been validated using the following automated library preparation system:

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

HRR OncoKit has been validated using the following massive sequencing platform:

- NextSeq System (Illumina)

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

10.3 Reagents

HRR OncoKit 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 Product stability

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