Instructions for use

Hereditary Plus OncoKitDx

CE
IVD

Imegen 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.

Our products are intended for *in vitro* diagnostic use. Imegen provides no guarantee, whether explicit or implicit, that extends beyond the proper functioning of the components of this kit. Imegen'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.

Imegen 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 Imegen products undergo strict quality control. **HereditaryPlus 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, feel free to contact our Technical Department:

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Instructions for Use (IFU) modifications	
Version: 05	Section 7.7 updated
Version: 04	Adding the **Hotspot** flag in the default filter
Version: 03	Content modification
Version: 02	Change the protocol of the Magnis equipment and adjust the sequencer parameters

Table of contents

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.

Hereditary cancer occurs when a person is born with a genetic mutation leading to a higher predisposition to develop a specific type of cancer. Approximately 5-10% of all cancers are hereditary. Moreover, carriers of pathogenic variants in genes associated with a specific type of cancer are at higher risk of developing tumors in tissues other than the one affected by the original tumor. This risk can be higher or lower depending on the gene in which the mutation has been identified.

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2. Intended use

Hereditary Plus OncoKitDx uses high–throughput massive sequencing technology (NGS) to detect single nucleotide variants (SNVs), small insertions and deletions (INDELs), copy number variations (CNVs), and large insertions such as ALU insertions, associated with familial cancer.

Hereditary Plus OncoKitDx has been designed to analyze the sequences of coding regions of 100 genes selected to study predisposition to some of the most frequent types of hereditary cancer (breast, ovarian, colorectal, uterine, melanoma, renal, prostate, pancreatic, multiple endocrine neoplasia, pheochromocytoma, paraganglioma, and retinoblastoma).

The following genes are included in the panels:

ABRAXAS1 (FAM175A), ACD, AIP, AKT1, ALK, APC (incl. 5' UTR), *ATM, ATR, AXIN2, BAP1, BARD1, BLM, BMPR1A, BRCA1, BRCA2, BRIP1, CDC73, CDH1, CDK4, CDKN1B, CDKN1C, CDKN2A, CHEK2, CTNNA1, CYLD, DICER1, EPCAM* (incl. 3' UTR), *FANCC, FANCG, FANCM, FH, FLCN, GALNT12, GALNT14, GDNF, GEN1, GREM1, HABP2, HOXB13, KIF1B, KLLN, LZTR1, MAX, MC1R, MEN1, MET, MITF, MLH1, MLH3, MRE11A, MSH2, MSH3, MSH6, MUTYH, NBN, NF1, NF2, NTHL1, PALB2, PHOX2B, PIK3CA, PMS2, POLD1, POLE, POT1, PRKAR1A, PRSS1, PTCH1, PTEN, RAD50, RAD51, RAD51B, RAD51C, RAD51D, RB1, RECQL4, RET, RINT1, SDHA, SDHAF2, SDHB, SDHC, SDHD, SEC23B, SLX4, SMAD4, SMARCA4, SMARCB1, SMARCE1, SPRED1, STK11, SUFU, TERF2IP, TERT, TMEM127, TP53, TSC1, TSC2, VHL, WT1* y *XRCC2.*

Moreover, other intergenic regions of interest for the calculation of CNVs in genes *EPCAM* (3' UTR) and *MSH2* (5' UTR) have been included, as well as 50 intronic regions to cover hotspots described in genes*APC, ATM, ATR, BRCA1, BRCA2, CHEK2, FH, LZTR1, MET, MLH1, MSH2, NF1, PMS2, PTEN, RB1, RET, SDHB, STK11* and *TERT*.

The assay uses DNA extracted from peripheral blood or saliva, and libraries are built by hybridization and capture of the regions of interest; NGS sequencing is subsequently performed using Illumina technology, and bioinformatic analysis is carried out using the DataGenomics tool provided by Imegen.

The results yielded by this assay provide the clinician with the variants detected in the coding regions of the 101 genes included in the panel. This information can be used to determine the patient's predisposition to hereditary cancer.

Hereditary Plus OncoKitDx can be used for in vitro diagnosis and is aimed at professionals in molecular biology.

3. Technical characteristics

Hereditary Plus OncoKitDx has been validated in Illumina's MiSeq System and NextSeq System platforms via the analysis of DNA reference samples from the Coriell Institute and from clinically relevant samples that have been previously genotyped by 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 kit allows detecting SNVs, INDELs, ALUs, and CNVs.

Technical specifications:

- Sample type: DNA from peripheral blood and saliva.
- Initial amount of DNA: 50–100 ng.
- Mean coverage: >350 X.
- Coverage: 99% of bases covered at 20X depth.
- Uniformity: 98.8% of bases covered at >20% mean coverage.
- Specificity: > 99%
- Sensitivity: > 99%
- Repeatability: > 99.99%
- Reproducibility: <98%

Hereditary Plus OncoKitDx is compatible with Illumina's massive sequencing platforms.

Imegen is certified against the norm **UNE–EN ISO 13485:2018 Medical devices: Quality management systems – Requirements for regulatory purposes** by the SPANISH AGENCY OF MEDICINES AND MEDICAL DEVICES 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

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 Imegen.
12. The manufacturer does not guarantee the assay's reproducibility when the user uses reagents that have not been validated by Imegen 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 48 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.

▪ Reagent Plate: Plate containing all the necessary reagents for DNA end repair reactions and Illumina adaptor binding, as well as for the amplifications within the library preparation protocol.

▪ Beads/Buffers Plate: Plate containing the required magnetic particles and wash buffers to perform the necessary capture and purification within the library preparation protocol.

▪ Index Strip: Oligonucleotides with a unique 8-nucleotide sequence compatible with Illumina adaptors. They are necessary to label libraries for each sample, leading to a unique combination that will allow for their analysis after sequencing. The kit includes 48 indexes distributed in single-use strips.

▪ Hereditary 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 bonding property between biotin and streptavidin molecules.

▪ Sample Input Strips: Empty 8-well strips in which sample DNA is placed.

▪ Magnis Library Output Strips, QC Strips, and Foil Seals: 8-well strips for generated libraries, strips for 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.

- <u>Magnis Deep-Well HSM Plate:</u> Plate for the capture and purifications necessary for the library preparation protocol.

- <u>Magnis Thermal Cycler Seal:</u> Seal for the 96-well plate.

- <u>Magnis Tip Waste Bin:</u> Container for tip waste created during the protocol.

Here are the components of the kit listed:

Box 1 of 7			
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	3 x 1 mL	4°C

Table 1. Reagents in box 1 of Hereditary Plus OncoKitDx

Box 2 of 7			
Reagents	Color indicator	Quantity	Conservation
Sample input strips	Red	3 strips	15-25°C
Beads and buffer plates	White	3 plates	4°C

Table 2. Reagents in box 2 of Hereditary Plus OncoKitDx

Box 3 of 7			
Reagents	Color indicator	Quantity	Conservation
Fragmentation Buffer	Green cap	96 µL	-20°C
Fragmentation Enzyme	White cap	48 µL	-20°C
Reagents Plate	White/Black	3 plates	-20°C
Index strip*	Black	3 strips	-20°C

Table 3. Reagents in box 3 of Hereditary Plus OncoKitDx

Box 4 of 7			
Reagents	Color indicator	Quantity	Conservation
Reagents Plate	White/Black	3 plates	–20°C
Index strip*	Black	3 strips	–20°C

Table 4. Reagents in box 4 of Hereditary Plus OncoKitDx

Note: Each kit includes 6 strips with four possible index combinations: A1, A2, A3, and A4.

Box 5 of 7			
Reagents	Color indicator	Quantity	Conservation
Hereditary Probes strips	White	3 strips	–80°C

Table 5. Table 4. Reagents in box 5 of Hereditary Plus OncoKitDx

Box 6 of 7			
Reagents	Color indicator	Quantity	Conservation
Hereditary Probes strips	White	3 strips	–80°C

Table 6. Table 4. Reagents in box 7 of Hereditary Plus OncoKitDx

"Magnis Empty Consumables" box; box 7 of 7			
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 7. "Magnis Empty Consumables" box reagents; box 7 of 7

NOTE: The kit contains 6 "Magnis Empty Consumables" boxes containing the necessary consumables to perform 6 runs using the Magnis instrument.

6. Equipment, reagents, and material not included

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 least 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 Technologies (cat. no. G9710AA)
- Illumina sequencer (recommended: MiSeq and 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 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

Hereditary Plus OncoKitDx is designed to be used in combination with **Imegen-Sample tracking component (REF: IMG-340)** kits, 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 guarantees that samples can be tracked during the whole protocol. These references are available upon request.

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7. Assay protocol

The reagents included in **Hereditary PlusOncoKitDx** to be used by the Magnis NGS Prep System come pre-dosed for 48 libraries, used in 6 runs with 8 libraries each, thus optimizing the performance of the instrument.

The necessary steps to carry out the preparation of 8 libraries using **Hereditary Plus OncoKitDx** are outlined below.

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 door of the device.

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).
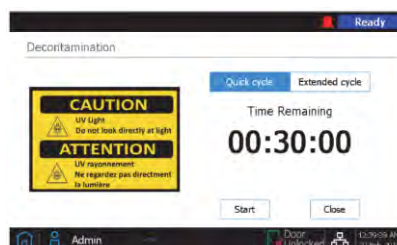

Figure 2. *Magnis NGS Prep Decontamination* screen

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

Note: During the 30-minutes 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 by using **Hereditary Plus 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.

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 30 ng/µL with nuclease-free water to a final volume of 25 µL.
    Optional: If Imegen's integrated tracking system is used (Sample tracking components; Ref. IMG-340), carry out this step by replacing 2.5 µL of nuclease-free water with the same quantity of one single tracing reagent per sample.

4. Vortex and quantify each sample again with a fluorometer, such as Qubit.

5. Dilute each DNA sample with nuclease-free water to obtain a input of 100 ng, to 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 ng. If this method is selected, it must be carried out for the 8 samples in the run.

6. Vortex all dilutions, spin, and keep cold until they are used in the next step.

7.2.2 DNA fragmentation

In this section, the DNA is enzymatically fragmented to obtain DNA fragments between 200 and 250 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. For processing several samples, we recommend preparing the reagent mixes with a 12% excess.

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	10 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 thermal cycler, 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*.

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
Hereditary Probe Strip	White strip	–80 °C
"Magnis Empty Consumables" box	N/A	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 cold temperature, vortex for 5 seconds 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 a run has not been recorded, it can be reviewed on the *Post-Run Data* screen of the *Magnis Prep System* device. 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 found on the right 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 *Hereditary 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 legible labels that show the specific identity of the probe design. It is recommended that special care be exercised to guarantee the traceability of this reagent in both storage and during the protocol.

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

7.4   Running the library preparation protocol

7.4.1   Start of the protocol

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

2. Once this check is completed, the message *Enter Run Info* will automatically appear on 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 allow for quality control later on.

   Note: Quality control of the pre–capture libraries will only be available once the run has been fully completed.
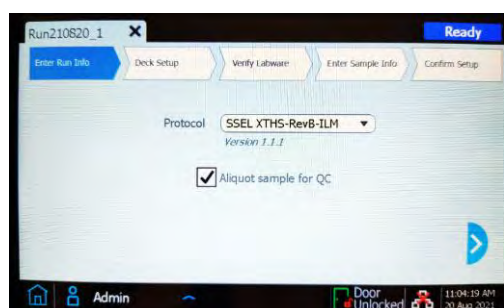

Figure 4. *Magnis NGS Prep* System *Enter Run Info* screen

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 **Hereditary Plus OncoKitDx**. Change the amount of DNA in case a different input amount is used (minimum 50 ng).

   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 will have the same amount of input DNA.

7.4.2   Deck setup

The deck can be very easily set up by following the steps in 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*.

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

The following figure shows a completely loaded 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.

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.
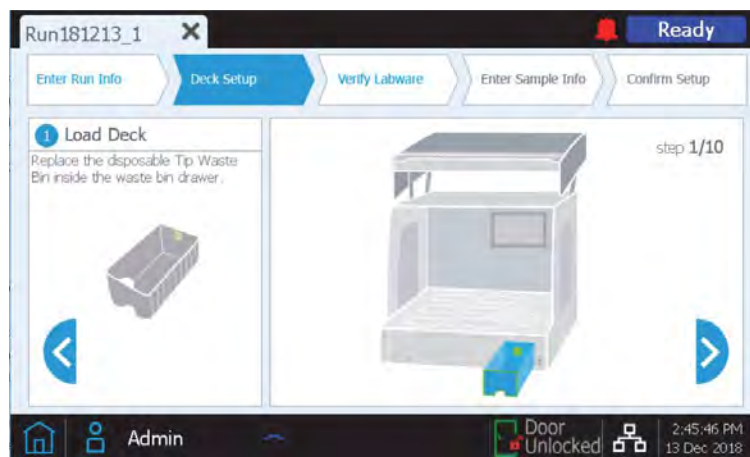

Figure 6. Step 1 of 10 on the *Deck Setup* screen of the Magnis NGS Prep System

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.
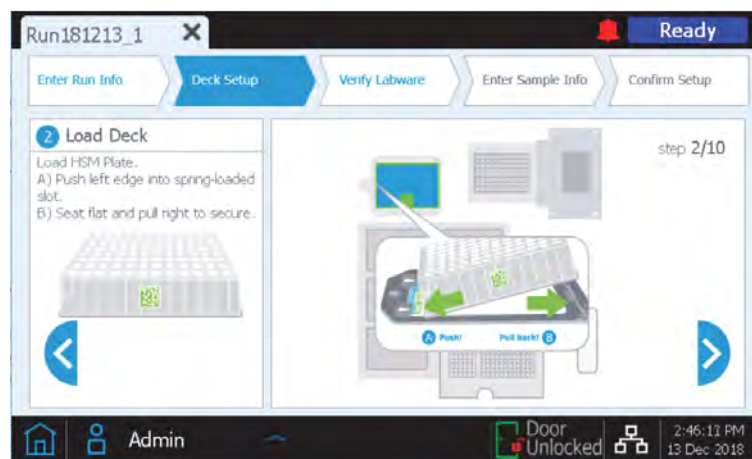

Figure 7. Step 2 of 10 of the *Deck Setup* screen of the Magnis NGS Prep System

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.
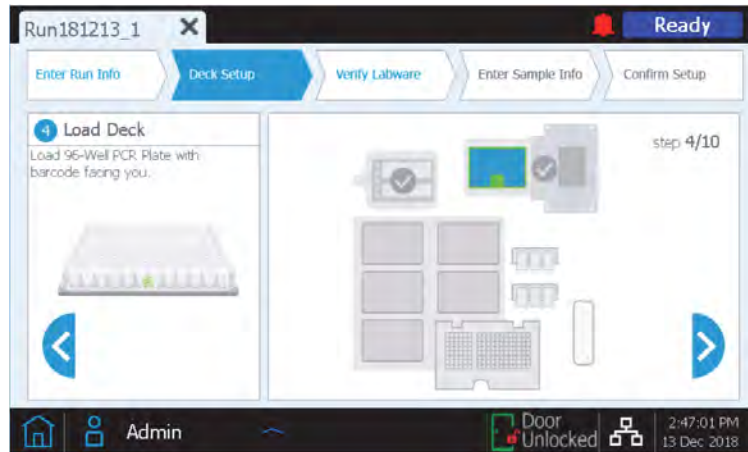

Figure 9. Step 4 of 10 of the *Deck Setup* screen of the Magnis NGS Prep instrument.

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.
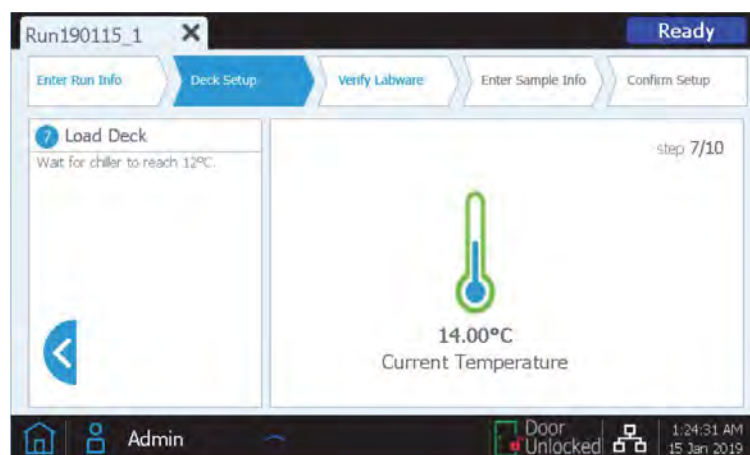

Figure 12. Step 7 of 10 of the *Deck Setup* screen of the Magnis NGS Prep instrument.

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 *Hereditary Probe Strip* (white strip), prepared in section 7.3 of this document, into the chiller holder position labeled with **P**.

   - Load the *Magnis Library Output Strip* (green strip), included in the "**Magnis Empty Consumables**" box, into the chiller holder position labeled with *P*.

   - Optional: If the run will include the collection of aliquots of the pre-capture libraries for quality control, as recommended by Imegen, 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 door of the device.

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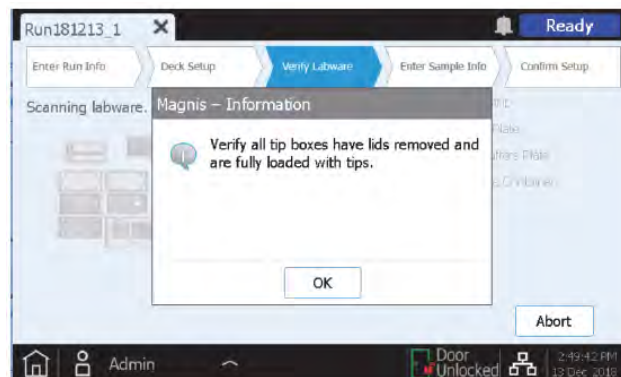
Figure 16. *Verify Labware* pop-up window of the Magnis NGS Prep System

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 have not exceeded 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.
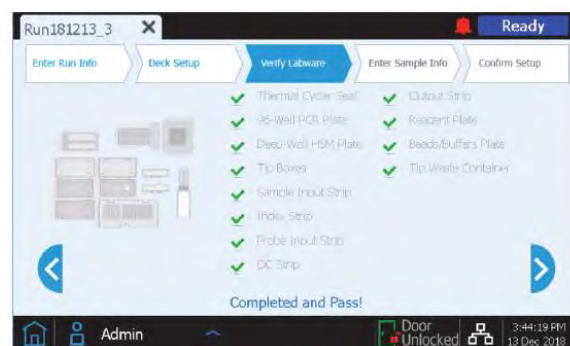

Figure 17. *Verify Labware* screen of the Magnis NGS Prep System after a correct verification of the material

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 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.
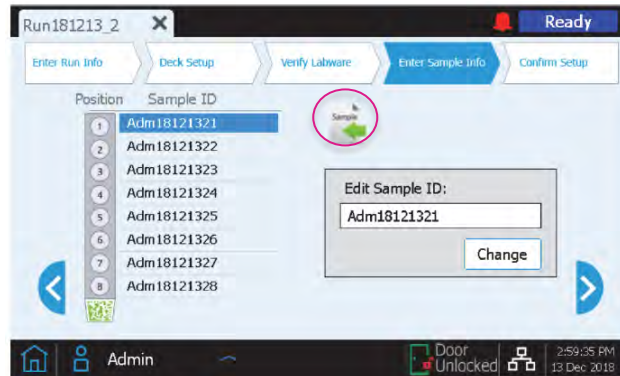

Figure 18. *Edit Sample Info* screen of the Magnis NGS Prep System, with a circle highlighting the load samples button.

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.

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

- Type the name of each sample from cell A2 to A9. The entry file of the sample shall 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.

- Dowload the .csv file in a non-encrypted USB stick and plug said USB stick in one of the 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.

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.
    <u>Important:</u> 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 **Hereditary Plus 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.
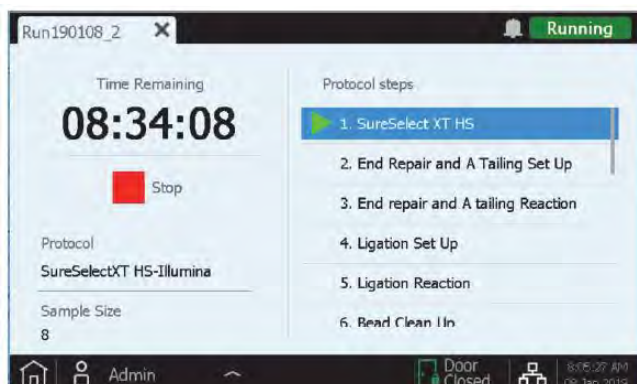
Figure 20. *Running* screen during a run.

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.
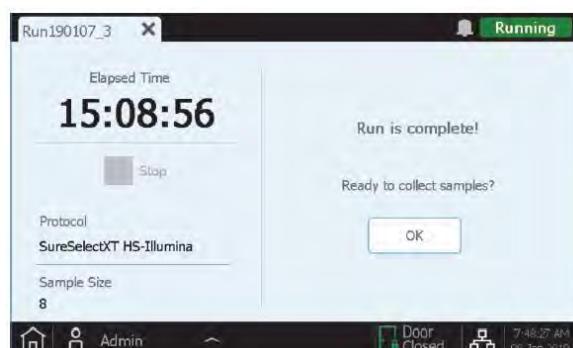

Figure 21. *Running* screen after a run.

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 12 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.
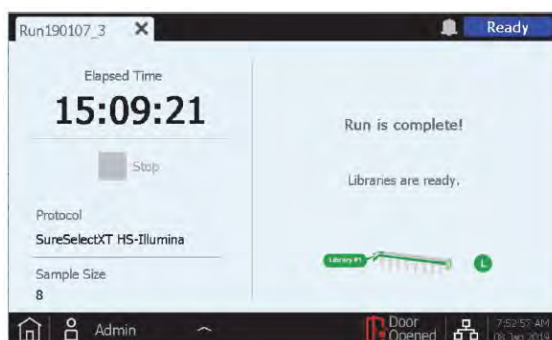

Figure 22. *Running* screen after a run and after removing the libraries

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.

- M*agnis Deep-Well HSM.*

- M*agnis Thermal Cycler Seal.*

- M*agnis 96-Well PCR Plate.*

- All tip boxes, including any partially filled ones.

- *Beads/Buffers Plate.*

- R*eagent 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 required, resuspend the dried library samples in 6 μL of nuclease-free water to achieve a concentration suitable for analysis, using the recommended Tapestation 2200 and commercial kits D1000 Reagents (cat. no. 5067-5583) and D1000 ScreenTape (cat. no. 5067-5582) by Agilent Technologies.

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

After analysing the samples using a TapeStation system, a library size of 300-400 bp should be obtained (Figure 23). In case unexpected sizes are obtained, review the protocol or contact Imegen's technical support team.
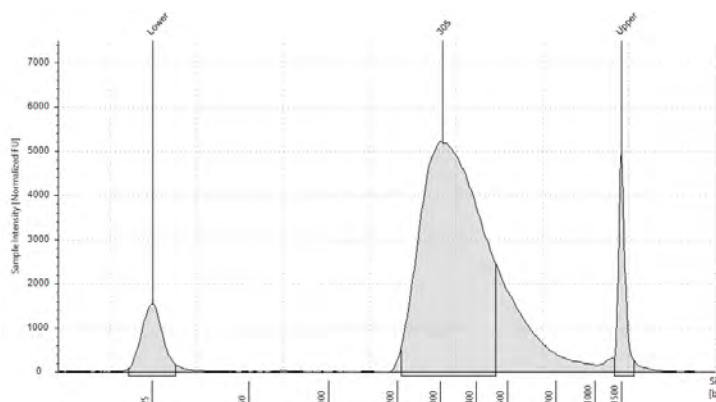

Figure 23. Expected result after analysing pre-capture library size with TapeStation 2200

To determine DNA concentration, the peak area corresponding to the expected library size must be integrated. The amount of obtained library DNA will vary depending on the concentration of the starting 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 Optional quality control of the post-capture library

Prior to sample pooling for multiplexed sequencing, it is necessary to analyze DNA 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 10 and 20 ng/μL.

For the quality analysis of the captured fragments, Imegen 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 300 and 450 bp. In case an unexpected size is obtained, review the protocol and pre-capture library quality control, carefully read section 9, or contact Imegen's technical support team.
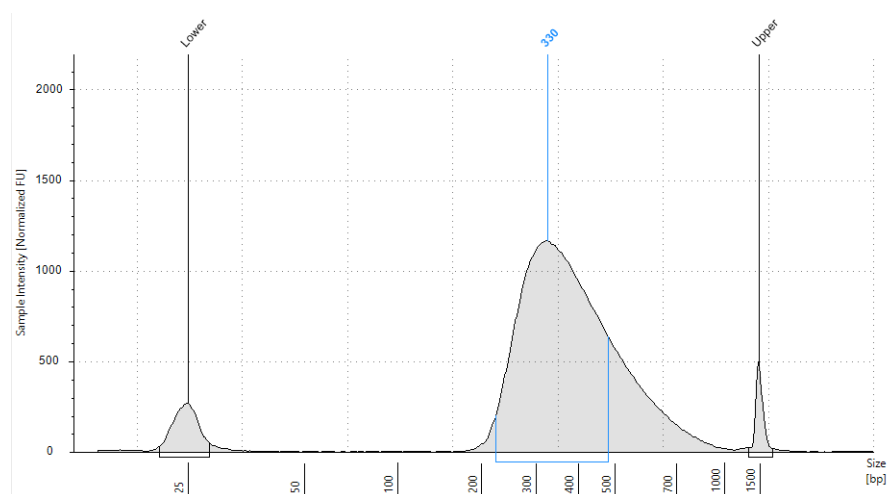

Figure 24. Expected result after the analysis of post-capture library size with TapeStation 2200

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

$$Concentración\ librerías\ (nM) = \left[Concentración\ (^{ng}/_{\mu L}) \cdot \frac{1500}{Tamaño\ (pb)} \right]$$

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

It is possible to stop the protocol at this point, storing the libraries at 4 °C if they are to be used within the following 12 hours or at -20°C for longer storage periods.

7.7 Library denaturation to load onto the Illumina MiSeq equipment

1. Thaw the HT1 reagent (included in the Illumina reagent kit to be used during sequencing, e.g., MiSeq Reagents Kit v2, 300 cycles; Ref: MS-103-1001) 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 12.5 pM (following the denaturation process of PhiX Control v3 provided along with Illumina reagents).

3. Dilute the library pool to 2 nM, adding to a new 1.5 mL tube, 10 µL of the library pool and 10 µL of Elution Buffer reagent.

4. 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.

5. Incubate at room temperature for 5 minutes.

6. Add 990 µL of HT1 and vortex.

7. Add 10 µL of denatured PhiX control diluted to 12.5 pM to the mix. The libraries should now be at 10 pM.

8. Load the entire volume contained in the 1.5 mL tube into the sample loading port on the cartridge.

The following table specifies the maximum number of samples per run to guarantee a minimum number of PF clusters of approximately 1.25 millions per sample:

MiSeq Reagents Kit	Maximum no. of samples
MiSeq V2 (300 cycles). Ref: MS-102-2002	16

Table 8. MiSeq Illumina Kit and maximum number of samples to be analyzed with Hereditary Plus OncoKitDx

7.8 Denaturation of libraries to be loaded onto the Illumina NextSeq system

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

8.   Add 1222 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 1.2 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 to guarantee a minimum number of PF clusters of approximately 1.25 millions per sample:

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

Table 9. NextSeq Illumina kit and maximum number of samples to be analyzed with Hereditary Plus OncoKitDx

7.9 Parameters for the sequencer

▪   *Read Type: Paired End.*

▪   *Cycles: Read 1: 150*
          Read 2: 150
          Index 1 (i7): 8

8   Analysis of results

Bioinformatics analysis of the results is done through the DataGenomics platform, using an analysis *pipeline* specifically designed for **Hereditary Plus OncoKitDx**. 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.

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.

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.
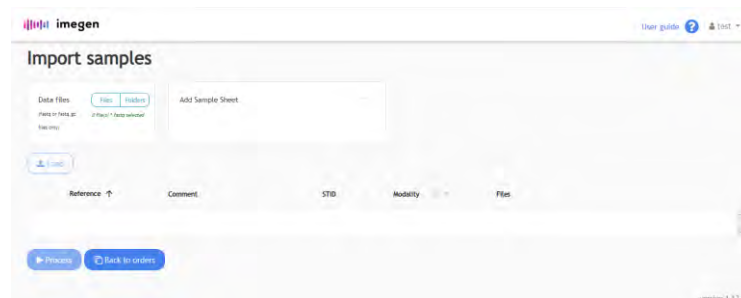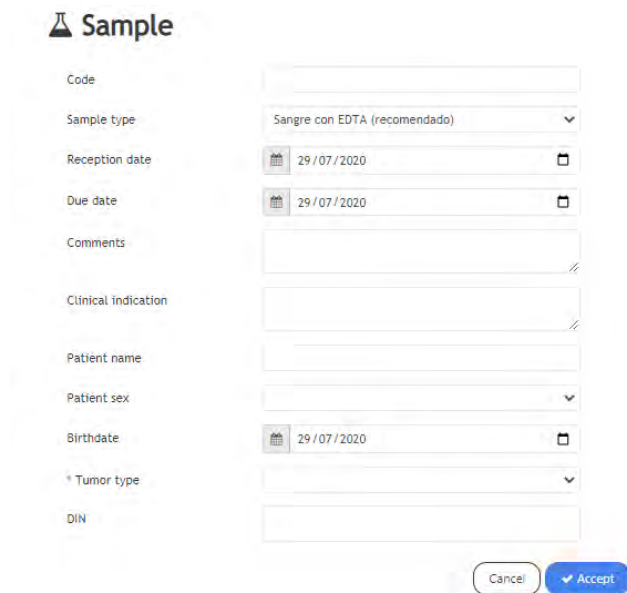

Figure 25. Screen to import the fastq files and the *SampleSheet* and start the analysis request.

2. Once the files are uploaded, the sequencing run name must be entered, and the study modality (*Hereditary OncoKitDx V2*Action OncoKitDx) 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 tumor type for each sample. 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.

4. 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 resulting files from the bioinformatics analysis: analysis of ALU regions and its report, alignment files (bam and bai), and list of variants (vcf), as well as other files containing

information on coverage and the sequencing quality report 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).

For a sample to pass the bioinformatics quality control established for the **Hereditary Plus OncoKitDx** assay, the parameters taken into account in the different files generated during sequencing are:

- FASTQ: The established acceptance criteria are detailed in the *DataGenomics* instructions for use, available at: www.datagenomics.es.

- BAMs:

  - On-target (%):
    - Fail: ≤ 53
    - Warn: 53–60
    - Pass: ≥ 60

  - DP20 (%)
    - Fail: ≤ 96
    - Warn: 96 – 98
    - Pass: ≥ 98

- 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 **Hereditary Plus OncoKitDx** assay, VCF files are not taken into account for quality control, since this panel is too small to be representative and consistent.

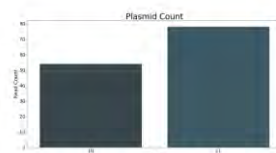| Feature	Obtained	Expected	Status
STID	1011	1011	PASS
Gender	Mujer	Mujer	PASS

Figure 27. Quality control of the integrated tracking system.

To access the variants filter, the *Filtering* request will optionally apply the *Hereditary Plus OncoKitDx d20 or Hereditary Plus OncoKitDx d50* filters, which only differ in the depth at which the GAPs are called: below 20 or 50 reads. The filter is characterized by:

- Panel genes

- Quality variants: **PASS; d20; pseudogenic homology; Hotspot*** (*Fault summary*).

- Depth: ≥ **20X** (*Clean total count*).

- Allele frequency: ≥ **30%** (*Variant Freq)*.

- Distance to the exon: **10** (*Exon distance*).

* Note: Hotspots intronic variants can be observed with this filter despite being more than 10 bp from the exon.

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 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:

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.

Hereditary Plus OncoKitDx offers an analysis of CNVs that may affect one or several exons of a gene or an entire gene that is included in the panel (CNVs per gene).

To analyze CNV results with *DataGenomics*, access the results of the "*Filtering*" request and, specifically, the CNVs tab.

DataGenomics integrates an alert system to warn the user about the reliability of the results based on sample quality parameters. According to these parameters, the credibility 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 lot, and number of detected CNVs before variant filtering.

Regarding CNV results, the PASS variants will be shown by default. These will be the good quality variants, that have a p–value ≤0.005 and a ratio ≤0.7 or ≥1.3. The filters allow the user to choose the option of showing the No Pass variants.

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

DataGenomics offers a dynamic graphic representation (Figure 28) of sample coverage profiles against reference samples or same–batch sample for all genes and all 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.
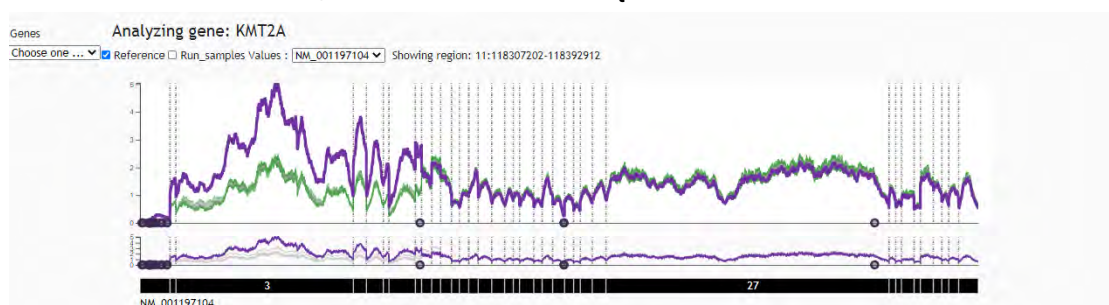

Figure 28. Dynamic graph displayed with CVS analysis results

8.4 Analysis of ALUs or large insertions

The methodology for detecting this type of variants is based on identifying sudden coverage increases or drops along the panel's coverage profiles. Therefore, events due to copy number variations (CNVs) may be reported, while these CNVs will in turn be reported in the CNV analysis.

To analyze the ALUs results, access the results of the "*Filtering*" request and, specifically, the ALUs tab. By default, good-quality PSS events will be displayed, although the filters allow choosing to display all the events.

Events classified as PASS are those with a frequency higher than 7% and more than two readings (both sense and antisense), with the sole exception of the known ALUs in *BRCA2*, c.5007insALU (13:32968896) and c.156_157insALU (13:3289302), which, due to their high diagnosis value and known prevalence in certain populations, have been granted an acceptance criteria that is more permissive, being classified as PASS whenever they have a frequency >3% and more than two readings (both sense and antisense).

Imegen provides a report on the analysis of large deletions, detailing PASS events. This report can be downloaded from the downloads section. An example of the information provided on the analysis of these variants is shown in the following table.

CHR	POSITION	GENE NAME	ALLELE DEPTH	TOTAL DEPTH	ALLELE FRACTION
13	3289302	BRCA2	19	174	0.11

Table 10. Results of the analysis of large insertions

Note: These regions interfere with the efficiency of the capture process; therefore, they present a low allelic frequency (>5%).

8.5 Variant filtering

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

Figure 29. Variant filtering with *DataGenomics*

Once variant analysis is opened, the variants that have met the selected filtering criteria appear. For the user to assess if a variant is validated as pathogenic, even if it does not meet some of the filtering criteria, it is recommended to clear any previous filters and apply a new filter to view pathogenic variants in the proprietary database and/or in 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.com

8.5.1 Categorization of SNV, INDEL, and CNV variants

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

By clicking on the "Category" column, a drop-down menu will appear, listing the different categories with which the variant can be associated. Among these categories are: pathogenic

(P), likely pathogenic(LP), variant of uncertain significance (VUS), likely benign (LB), and benign (B).

If assessing the variant is not necessary or if it is suspected to be a false positive, it is recommended to categorize it as "not evaluable/artifact", which, if selected, will prevent any further selection.

In the *HIC Germinal Db* column, Imegen will provide the categorization of the variants, considering the functional impact they would cause at the biological level.

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.

Should you have any doubt about the analysis of the results, please contact Imegen'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 **Hereditary Plus OncoKitDx** and the steps to solve them 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 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.

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 complete, 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 shouldbe restarted after a failed IHC, following the steps below:

  1. In the error dialog box, press *Cancel* to decline initiation of diagnostic testing.

  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 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.

- *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 *Imegen* 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

▪ The employed technique cannot distinguish between regions with high sequence homology, such as homologous genes, pseudogenes, etc., which can lead to false positives or negatives. A list of pseudogenic regions can be found in Table 11. In the analysis of results, the label "*Pseudogenic_homology*" will appear in the column "*Fault summary*" whenever a variant is detected in a region of homology with pseudogenes.

Chromosome	Initial position	Final position	Gene	Exon	Reference sequence
1	161332141	161332340	SDHC	EX5	NM_001035511.2
3	178937348	178937413	PIK3CA	EX12	NM_006218.4
3	178937747	178937850	PIK3CA	EX13	NM_006218.4
5	251442	251527	SDHA	EX13	NM_004168.4
7	6013019	6013183	PMS2	EX15	NM_000535.7
7	6017208	6017322	PMS2	EX14	NM_000535.7
7	6022444	6022632	PMS2	EX12	NM_000535.7
7	6027210	6027261	PMS2	EX11	NM_000535.7
10	89725087	89725232	PTEN	EX9	NM_000314.8

Table 11. List of pseudogenic regions

▪ Moreover, due to the considerable homology between the PMS2 gene and its pseudogenes in exons 11–15, the CNV analysis performed will not allow distinguishing between the number of copies from the gene and those from the pseudogene.

▪ Any results obtained with quality parameters below the established criteria cannot be guaranteed.

▪ 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.

10.2 Equipment

Hereditary Plus 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 service.

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

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

Hereditary Plus OncoKitDx has been validated using the following massive sequencing platform:

- MiSeq System (Illumina)

- 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 these platforms.

10.3 Reagents

Hereditary Plus OncoKitDx has been validated using the reagents included in the kit and those recommended in section 6 of this manual (Equipment, reagents, and material not included).

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

Should you have any questions, please contact Imegen's technical support team.

10.4 Bioinformatics analysis platform

Hereditary Plus OncoKitDx has been validated using DataGenomics, which is a platform for *in vitro* bioinformatics analysis. This platform includes a *pipeline* tailored specifically for **Hereditary Plus OncoKitDx**, which enables the detection of all the targets specified in section 2 of this document.

If a different analysis platform is used, Imegen cannot guarantee the correctness of the results.

10.5 Product stability

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