



# Instructions for use

## Inherited CardioKitDx

Ref. IMG-390



Manufactured by:

**HEALTH IN CODE, S.L.**

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**The product is intended for *in vitro* diagnostic use.** Health in Code, S.L. provides no other guarantee, whether explicit or implicit, that extends beyond the proper functioning of the components of this kit. Health in Code, S.L.'s sole obligation, in relation to the aforementioned guarantees, shall be to either replace the products or reimburse the price thereof, at the client's choice, provided that, however, materials or workmanship prove to be defective. Health in Code, S.L. shall not be liable for any loss or damage, whether direct or indirect, resulting in economic loss or harm incurred as a result of use of the product by the buyer or user.

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

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

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#### Instructions for Use (IFU) modifications

Version 10	JAN 2025	Modification of section 7.7: follow recommendations from Illumina. Section 10.2 Instruments (Limitations): added the load concentration for the libraries validated in each sequencing instrument.
Version 09	FEB 2024	Revision and update of the document's content. (Sections 5, 7.2., 7.3, 7.4.2, 7.6, 7.7, 8, 9, and 10.1).
Version 08	DEC 2023	Review and update of section "3. Technical characteristics".
Version 07	SEP 2023	Revision of contents of sections 7.2.1, 7.2.2, 7.7, 9 and 10. Name change of box previously called "Magnis Empty Consumables". Change of "Sample input strip" kit component from box 1 to 4 (section 5).
Version 06	NOV 2022	Change of the manufacturer's address: Health in Code, S.L., Calle de la Travesía s/n, 15E Base 5, Valencia 46024, Spain.
Version 05	OCT 2022	Update of sections 3, 5 and 10. Update of quality QC of pre-capture and post-capture libraries (sections 7.6.1 and 7.6.2)
Version 04	MAY 2022	Update by certified CE/IVD and change of the manufacturer's identification, going from Imegen to Health in Code, S.L.
Version 03	DEC 2021	Section 7.2.2 updated
Version 02	NOV 2021	Sections 2, 3 and 6 updated

# index

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<b>01</b>	<b>General information</b>	<b>4</b>
<b>02</b>	<b>Intended use</b>	<b>5</b>
<b>03</b>	<b>Technical characteristics</b>	<b>8</b>
<b>04</b>	<b>Safety warnings and precautions</b>	<b>9</b>
<b>05</b>	<b>Content and storage conditions of the kit</b>	<b>10</b>
<b>06</b>	<b>Equipment, reagents, and material not included in the kit</b>	<b>12</b>
<b>07</b>	<b>Assay protocol</b>	<b>14</b>
07.1	Preparation of the Magnis system for the execution of a protocol	14
07.2	Preparation and fragmentation of the DNA template strand	15
07.3	Preparation of the reagents and plastic materials used by the Magnis system	18
07.4	Execution of the library preparation protocol	20
07.5	Instrument cleaning after a run	29
07.6	Library validation and quantification	29
07.7	Denaturing libraries for loading into the Illumina <i>NextSeq 500/550</i>	31
07.8	<i>NextSeq</i> platform setup	32
<b>08</b>	<b>Analysis of results</b>	<b>33</b>
08.1	Sending sequencing files	33
08.2	Accessing the platform and managing orders	35
08.3	Coverage statistics	36
08.4	Variant filtering	36
08.5	Variant categorization	37
08.6	Analysis of large rearrangements (CNVs)	38
<b>09</b>	<b>Troubleshooting</b>	<b>42</b>
<b>10</b>	<b>Limitations</b>	<b>45</b>
10.1	Analytical	45
10.2	Equipment	46
10.3	Reagents	47
10.4	Bioinformatics analysis platform	47
10.5	Product stability	47

# 01 General information

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Hereditary cardiovascular diseases, including cardiomyopathies and channelopathies, are heterogeneous conditions with highly variable morphological and functional characteristics, clinical presentation, evolution, and prognosis. Hundreds of mutations in different genes have been associated with each of these diseases, and it is likely that this genetic heterogeneity is one of the main reasons for the variability in their clinical expression.

Information from genetic studies can help clinicians diagnose diseases in early stages, identify at-risk family members, those who do not require regular follow-up, and provide prognostic information. Proper and accurate interpretation of genetic tests is required to obtain the full potential benefits of these studies. This interpretation is not straightforward and requires information, expertise, and dedication.

**Inherited CardioKitDx** is a clinical guidance tool designed to assist healthcare professionals in the management of inherited cardiovascular diseases.

## 02 Intended use

**Inherited CardioKitDx** is aimed at the study of cardiomyopathies (those associated with a high risk of developing them, sudden death or supraventricular arrhythmias (atrial fibrillation), channelopathies, and cardiac conduction diseases. It has been designed mainly for the diagnosis of clinical scenarios in which it is not possible to establish a clearly defined phenotype but in which patients present cardiac arrhythmias as the main manifestation, and it is mainly intended for individuals with a family history of sudden death and a history of syncope of undetermined origin with ventricular fibrillation that meet the aforementioned characteristics. All genes associated with diseases that can produce an arrhythmic phenotype, linked or not with the presence of structural heart disease, are included in the kit.

**Inherited CardioKitDx** contains 261 cardiomyopathy- and channelopathy-related genes and has been designed to ensure the capture of all coding regions +/- 50 bp (for some genes the design also includes the capture of deeper intronic regions), as well as clinically relevant promoters and UTR regions.

The following genes are included in the panel:

*A2ML1, AARS2, ABCC9, ACAD9, ACADVL, ACTA1, ACTC1, ACTN2, AGK, AGL, AGPAT2, AKAP9, AKT1, ALMS1, ALPK3, ANK2, ANK3, ANKRD1, ANO5, ATP5F1E, ATPAF2, BAG3, BRAF, BSCL2, C10orf71, CACNA1C, CACNA1D, CACNA2D1, CACNB2, CALM1, CALM2, CALM3, CALR, CALR3, CAPN3, CASQ2, CASZ1, CAV3, CAVINI, CAVIN4, CBL, CDH2, COA5, COA6, COL7A1, COQ2, COX15, COX6B1, CRYAB, CSNK1A1, CSRP3, CTNNA1, CTNNA3, CTNNB1, CHRM2, DES, DLD, DMD, DNAJC19, DNML1, DOLK, DSC2, DSG2, DSP, DTNA, ELAC2, EMD, EYA4, FAH, FBXO32, FGF12, FHL1, FHL2, FHOD3, FKRP, FKTN, FLNC, FOXD4, FOXRED1, FXN, GAA, GATA4, GATA5, GATA6, GATAD1, GFMI, GJA1, GJA5, GLA, GLB1, GNB2, GNPTAB, GPD1L, GREM2, GSK3B, GUSB, GYG1, HCN4, HFE, HRAS, IDH2, ILK, IRX3, ISM2, JARID2, JPH2, JUP, KAT6B, KCNA5, KCND2, KCND3, KCNE1, KCNE2, KCNE3, KCNE5, KCNH2, KCNJ2, KCNJ5, KCNJ8, KCNK17, KCNK3, KCNQ1, KLF10, KLHL24, KRAS, LAMA2, LAMA4, LAMP2, LDB3, LDLR, LIAS, LMNA, LMOD2, LZTR1, MAP2K1, MAP2K2, MAP3K8, MEF2C, MIB1, MIR208A, MIR208B, MLYCD, MRPL3, MRPL44, MRPS22, MTO1, MYBPC3, MYBPHL, MYH11, MYH6, MYH7, MYL2, MYL3, MYLK2, MYOM1, MYOT, MYOZ2, MYPN, NEBL, NEXN, NF1, NKX2-5, NKX2-6, NNT, NONO, NOS1AP, NOTCH1, NPPA, NRAP, NRAS, OBSCN, OBSL1, OPA3, PDHA1, PDLIM3, PERP, PHKA1, PITX2, PKD2, PKP2, PKP4, PLN, PMM2, PPA2, PPCS, PPP1CB, PPP1R13L, PRDM16, PRKAG2, PSEN1, PSEN2, PTPN11, QRSL1, RAF1, RANGRF, RASA1, RASA2, RBM20, RBM24, RIT1, RRAS, RYR2, SCN10A, SCN1B, SCN2B, SCN3B, SCN4B, SCN5A, SCO2, SDHA, SGCA, SGCB, SGCD, SGCG, SHOC2, SLC22A5, SLC25A3, SLC25A4, SLMAP, SNTA1, SOS1, SOS2, SPEG, SPRED1, SPRY1, SRY, SURF1, SYNE1, SYNE2, SYNGAP1, TAZ, TBX20, TBX5, TCAP, TECRL, TGFB3, TMEM175, TMEM43, TMEM70, TMOD1, TNNC1, TNNI3, TNNI3K, TNNT2, TOR1AIP1, TPM1, TRDN, TRIM54, TRIM63, TRPM4, TSFM, TTN, TTR, TXNRD2, VCL, WISP1, WT1, XK, ZBTB17, ZFH3 y ZFPM2*

In combination with bioinformatics analysis and prediction tools designed to this end, this kit can detect SNPs, small indels, and copy number variants, coupled with optional variant annotation and clinical interpretation of results.

Below is a description of the different gene panels included in **Inherited CardioKitDx** based upon the disease under study:

#### ➤ Hypertrophic cardiomyopathy [118 genes]

*AARS2, ACAD9, ACADVL, ACTA1, ACTC1, ACTN2, AGK, AGL, AGPAT2, AKT1, ALPK3, ANK2, ANKRD1, ATP5F1E, ATPAF2, BAG3, BRAF, BSCL2, C10orf71, CACNA1C, CALR, CALR3, CASQ2, CAV3, CAVIN4, CBL, CDH2, COA5, COA6, COQ2, COX15, COX6B1, CRYAB, CSRP3, DES, DLD, DSP, ELAC2, FAH, FHL1, FHL2, FHOD3, FLNC, FOXRED1, FXN, GAA, GATA6, GFM1, GLA, GLB1, GNPTAB, GUSB, GYG1, HRAS, JPH2, KCNJ8, KLF10, KLHL24, KRAS, LAMP2, LDB3, LIAS, LMNA, LZTR1, MAP2K1, MAP2K2, MEF2C, MLYCD, MRPL3, MRPL44, MRPS22, MTO1, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYLK2, MYOM1, MYOZ2, NEXN, NF1, NRAS, PDHA1, PDLIM3, PHKA1, PLN, PMM2, PPA2, PPP1CB, PRKAG2, PTPN11, QRSL1, RAF1, RIT1, SCO2, SHOC2, SLC22A5, SLC25A3, SLC25A4, SOS1, SOS2, SURF1, TAZ, TCAP, TMEM70, TMOD1, TNNC1, TNNI3, TNNT2, TPM1, TRIM54, TRIM63, TSFM, TTN, TTR, VCL, WISPI*

#### ➤ Dilated cardiomyopathy [121 genes]

*ABCC9, ACTA1, ACTC1, ACTN2, AKT1, ALMS1, ALPK3, ANKRD1, ANO5, BAG3, BRAF, CALR3, CASZ1, CAV3, CAVIN4, CHRM2, COL7A1, CRYAB, CSRP3, DES, DMD, DNAJC19, DNML, DOLK, DSC2, DSG2, DSP, DTNA, EMD, EYA4, FBXO32, FHL1, FHL2, FHOD3, FKRP, FKTN, FLNC, GAA, GATA4, GATA5, GATA6, GATAD1, GLA, GLB1, GSK3B, GYG1, HFE, IDH2, ILK, JARID2, JUP, KCNJ2, LAMA2, LAMA4, LAMP2, LDB3, LMNA, LMOD2, MEF2C, MIB1, MYBPC3, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYOT, MYPN, NEBL, NEXN, NKX2-5, NONO, NRAP, OBSCN, OPA3, PDLIM3, PKD2, PKP2, PLN, PPA2, PPCS, PPP1R13L, PRDM16, PRKAG2, PSEN1, PSEN2, PTPN11, QRSL1, RAF1, RBM20, RBM24, RYR2, SCN5A, SDHA, SGCA, SGCB, SGCD, SGCG, SLC22A5, SPEG, SYNE1, SYNE2, TAZ, TBX20, TCAP, TMEM43, TMOD1, TNNC1, TNNI3, TNNI3K, TNNT2, TOR1AIP1, TPM1, TRIM63, TTN, TTR, TXNRD2, VCL, WISPI, XK, ZBTB17*

#### ➤ Arrhythmogenic cardiomyopathy [26 genes]

*CASQ2, CDH2, CTNNA1, CTNNA3, CTNNB1, DES, DSC2, DSG2, DSP, EMD, FLNC, ILK, ISM2, JUP, LMNA, PERP, PKP2, PKP4, PLN, PPP1R13L, RBM20, RYR2, SCN5A, TGFB3, TMEM43, TTN*

#### ➤ Non-compaction cardiomyopathy [48 genes]

*ACTC1, ACTN2, AKT1, ANKRD1, BAG3, CDH2, CSRP3, DMD, DNAJC19, DSP, DTNA, FHL1, FHOD3, FLNC, HCN4, JARID2, KCNH2, KCNQ1, KRAS, LAMP2, LDB3, LMNA, MIB1, MLYCD, MYBPC3, MYH6, MYH7, MYL2, MYL3, NKX2-5, NNT, NONO, NOTCH1, PLN, PRDM16, PTPN11, RBM20, RYR2, SPEG, TAZ, TBX20, TNNC1, TNNI3, TNNT2, TPM1, TRPM4, TTN, WT1*

➤ Restrictive cardiomyopathy [23 genes]

*ACTC1, ACTN2, ALMS1, BAG3, CRYAB, DES, FHL1, FHOD3, FLNC, GLA, HFE, LMNA, MYBPC3, MYH7, MYL2, MYL3, MYPN, TNNC1, TNNI3, TNNT2, TPM1, TTN, TTR*

➤ RASopathy syndromes [26 genes]

*A2ML1, ALPK3, BRAF, CBL, HRAS, KAT6B, KRAS, LZTR1, MAP2K1, MAP2K2, MAP3K8, NF1, NRAS, PPPICB, PTPN11, RAF1, RASA1, RASA2, RIT1, RRAS, SHOC2, SOS1, SOS2, SPRED1, SPRY1, SYNGAP1*

➤ Long QT syndrome [32 genes]

*AKAP9, ANK2, CACNA1C, CALM1, CALM2, CALM3, CAV3, CAVIN1, FHL2, HCN4, KCNA5, KCND2, KCND3, KCNE1, KCNE2, KCNE3, KCNE5, KCNH2, KCNJ2, KCNJ5, KCNQ1, NOS1AP, RYR2, SCN1B, SCN3B, SCN4B, SCN5A, SLC22A5, SNTA1, TECRL, TRDN, TRPM4*

➤ J-wave syndrome [27 genes]

*ABCC9, ANK2, ANK3, CACNA1C, CACNA1D, CACNA2D1, CACNB2, FGF12, GPD1L, HCN4, IRX3, KCND2, KCND3, KCNE3, KCNE5, KCNH2, KCNJ8, PKP2, RANGRF, SCN10A, SCN1B, SCN2B, SCN3B, SCN4B, SCN5A, SLMAP, TRPM4*

➤ Short QT syndrome [9 genes]

*CACNA1C, CACNA1D, CACNA2D1, CACNB2, KCNH2, KCNJ2, KCNQ1, SLC22A5, TMEM175*

➤ Catecholaminergic polymorphic ventricular tachycardia [10 genes]

*ANK2, CALM1, CALM2, CALM3, CASQ2, KCNJ2, RYR2, SCN5A, TECRL, TRDN*

➤ Cardiac conduction disorders [44 genes]

*ACTC1, CACNA1D, CALR, CAVIN4, DES, DSC2, DSG2, DSP, EMD, GAA, GATA4, GJA5, GLA, GNB2, HCN4, HFE, IRX3, JUP, KCNE2, KCNH2, KCNJ2, KCNK17, KCNQ1, LAMP2, LDB3, LMNA, MYBPHL, MYH6, MYH7, NKX2-5, NPPA, PITX2, PKP2, PRKAG2, RYR2, SCN1B, SCN4B, SCN5A, SLC22A5, SLMAP, TBX5, TNNI3K, TRPM4, TTR*

➤ Atrial fibrillation [46 genes]

*ABCC9, ACTC1, CACNB2, EMD, GATA4, GATA5, GATA6, GJA1, GJA5, GNB2, GREM2, HCN4, IRX3, JPH2, KCNA5, KCND2, KCND3, KCNE1, KCNE2, KCNE3, KCNE5, KCNH2, KCNJ2, KCNJ5, KCNJ8, KCNK3, KCNQ1, LMNA, MYBPHL, NKX2-5, NKX2-6, NPPA, PITX2, RYR2, SCN1B, SCN2B, SCN3B, SCN4B, SCN5A, TBX5, TNNI3, TNNI3K, TNNT2, TPM1, TTR, ZFH3*

**Inherited CardioKitDx** is solely intended for *in vitro* diagnosis and is aimed at professionals in the molecular biology sector.

## 03 Technical characteristics

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**Inherited CardioKitDx** has been validated on the Illumina *NextSeq 500/550 System* platform via the analysis of reference DNA samples from the *Coriell Institute* and DNA samples that have been genotyped from the Health in Code, S.L. collection. This validation has confirmed the specific detection of variants present in the selected genes (see above), as well as the repeatability, reproducibility, and limit of detection of the technique.

The kit also employs molecular barcoding to detect cross-contamination and sample swaps during the protocol.

### Technical specifications:

- ◇ Sample type: DNA from peripheral blood
- ◇ Necessary amount of DNA: 200 ng
- ◇ Mean coverage: >450X
- ◇ Coverage: >99% of regions covered at >30X
- ◇ Uniformity: >98% in the 20th percentile of average coverage
- ◇ Specificity and sensitivity: >99%
- ◇ Repeatability and reproducibility: >99%

## 04 Safety warnings and precautions

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

## 05

# Content and storage conditions of the kit

This kit contains sufficient reagents for the preparation of 16 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 16 different indexes distributed in single-use strips.
- **Cardiovascular Probes Strips:** 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.
- **Sample Input Strips:** 8-well strips for DNA of the samples.
- **Magnis Library Output Strips, QC Strips, and Foil Seals:** 8-well strips to collect the generated libraries, strips to collect the pre-capture libraries, which can be used for an optional quality check, and seals for the well strips included in the kit.
- **Magnis 96-Well PCR Plate:** Plate for amplification reactions.
- **Magnis Deep-Well HSM Plate:** Plate for the capture and purifications necessary for the library preparation protocol.
- **Magnis Thermal Cycler Seal:** Seal for the 96-well plate.
- **Magnis Tip Waste Bin:** Container for tip waste created during the protocol.

The components of the kit are listed below:

Table 1. Reagents of box 1 of Inherited CardioKitDx

Box 1 of 4			
Reagents	Color indicator	Quantity	Conservation
Beads and buffer plates	White	2 plates	4°C
Elution Buffer	Green disc	2 x 1 mL	4°C

Table 2. Reagents of box 2 of Inherited CardioKitDx

Box 2 of 4			
Reagents	Color indicator	Quantity	Conservation
Fragmentation Buffer	Green cap	32 µL	-20°C
Fragmentation Enzyme	White cap	16 µL	-20°C
Reagents Plate	Black/White	2 plates	-20°C
Index strip*	Black	2 strips	-20°C

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

Table 3. Reagents of box 3 of Inherited CardioKitDx

Box 3 of 4			
Reagents	Color indicator	Quantity	Conservation
Cardiovascular Probes strips	White	2 strips	-80°C

Table 4. Reagents of box 4 of Inherited CardioKitDx

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

**NOTE:** The kit will include 2 units of Box 4, one for each run of 8 samples on the Magnis instrument.

## 06

# Equipment, reagents and material not included in the kit

## Equipment:

- 10 µL, 20 µL, 200 µL, and 1000 µL micropipettes
- Vortex (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 96 well plates; with adjustable speed of at least 1,000 rpm)
- Fluorometer (recommended: *Qubit*; Thermo Fisher Scientific)
- 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)
- Thermal cycler or sonicator (recommended: *ME220 Focused-ultrasonicator™*, Covaris) or thermal cycler with adjustable temperature lid
- Illumina sequencer (recommended: *NextSeq*)

## Reagents:

- Extraction kit (recommended: *QIAamp DNA Investigator Kit*; cat. no. 56504; Qiagen)
- Nuclease-free water
- Fluorometer reagents. Recommended: *Qubit dsDNA BR Assay kit* (cat. no. Q32853; Invitrogen), *Qubit dsDNA HS Assay kit* (cat. no. Q32854; Invitrogen).
- *NaOH 0.2N* (cat.no. 1091401000; Fluka)
- *TRIS-HCl 200 mM pH 7*
- *PhiX Control v3* (cat. no. FC-110-3001; Illumina)
- Fragment analyzer reagents. Optional:
  - ◇ *TapeStation D1000 Reagents* (cat. no. 5067-5583; Agilent), *High Sensitivity D1000 Reagents* (cat. no. 5067-5585; Agilent)
  - ◇ *DNA High Sensitivity Reagent Kit* (cat. no. CLS760672; PerkinElmer)

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

## Materials:

- Filter pipette tips (10 µL, 20 µL, 200 µL, and 1000 µL)
- Sterile filter tips compatible with *Magnis NGS Prep System* (Ref: 19477-022; Agilent)
- Sterile 1.5 mL tubes
- Sterile tubes or strips of 0.2 mL.
- 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

*Inherited CardioKitDx* is prepared to be used in combination with the *Imegen-Sample Tracking Components* (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. This reference is available upon request.

# 07 Assay protocol

The reagents included in **Inherited CardioKitDx** that are to be used by the *Magnis NGS Prep System* come pre-dosed for 16 libraries, used in 2 assays of 8 libraries each, thus optimizing the performance of the instrument.

The steps necessary to carry out the preparation of eight libraries using **Inherited CardioKitDx** are outlined below.

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

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

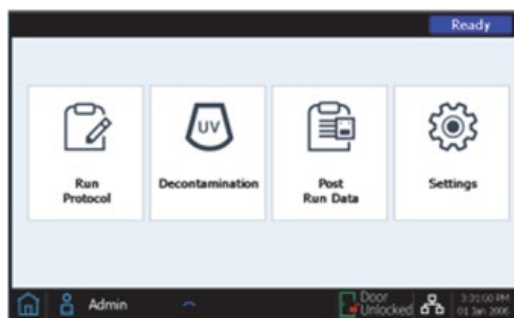


Figure 1. Magnis NGS Prep System Home screen

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

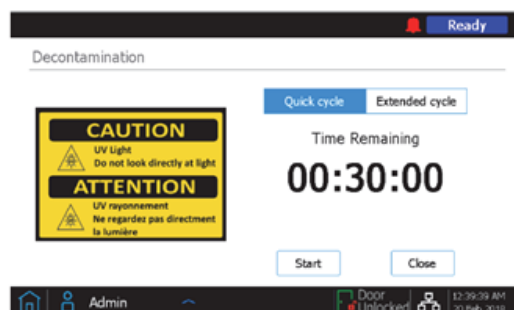


Figure 2. Magnis NGS Prep system Decontamination screen

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

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

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

## 07.2 | Preparation and fragmentation of the DNA template strand

The steps necessary for the preparation of eight libraries by **Inherited CardioKitDx** are outlined below.

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

### 07.2.1 | Quantification and dilution of DNA samples

- 01 Thaw DNA samples at room temperature.
- 02 Mix and quantify the DNA samples with a fluorometer, such as *Qubit*.
- 03 Perform DNA quality control using one of the following methods:
  - ◇ Quality analysis using Agilent's *Genomic DNA ScreenTape*, together with its associated reagents, and following the instructions provided by the instrument and analysis kit suppliers.

This analysis makes it possible to determine the sample's DNA integrity and provides a DNA integrity number (DIN) for each sample
  - ◇ Quality analysis using PerkinElmer's *LabChip GX Touch/GXII Touch*, together with its associated reagents, and following the instructions provided by the instrument and analysis kit suppliers.

This analysis makes it possible to determine the sample's DNA integrity and provides a quality value (DQS) for each sample.

**NOTE:** DNA samples that are too degraded can lead to a decrease in the specificity and sensitivity of the analysis. Therefore, Health in Code, S.L. recommends using DNA with a quality higher than DIN 3 if using *TapeStation System* and DQS 15 if using *LabChip*.

- 04 Dilute each DNA sample to 50 ng/μL with nuclease-free water to a final volume of 25 μL.

**Optional:** If Health in Code, S.L.'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.
- 05 Mix on a vortex mixer and quantify each sample with a fluorometer, such as *Qubit*.

## 07.2.2 | ADN fragmentation

Two DNA fragmentation protocols are described below (enzymatic fragmentation and mechanical fragmentation), of which only one is to be selected, depending on the equipment and reagents available to the user.

➤ **OPTION A. Mechanical fragmentation:** It requires a sonicator (recommended: *ME220 Focused-ultrasonicator™*; Covaris).

Covaris employs their adaptive focused acoustics (AFA) technology for the sonication of DNA by isothermal cavitation. Temperature is a crucial factor, as small changes in temperature affect the size of the generated DNA.

- 01 Dilute each DNA sample with *Elution Buffer* to obtain a concentration of a total of 200 ng, to a final volume of 50 µL.
- 02 Vortex all dilutions, spin, and keep cold until they are used in the next step.
- 03 Place the 130 µL *microTUBE* in the sonicator.
- 04 In order to obtain DNA fragments of 150 to 250 bp in size, follow configurations in Table 5, at a temperature between 2–8 °C.

Table 5. Optimal fragmentation program for Covaris ME220

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

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

- 05 Once the program has ended, spin the tube.
- 06 Immediately transfer the whole volume from each 130 µL *microTUBE* to a corresponding *Sample Input Strip* well, seal with the provided aluminum seals, and keep cold until use in the next step.

**NOTE:** The 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*.

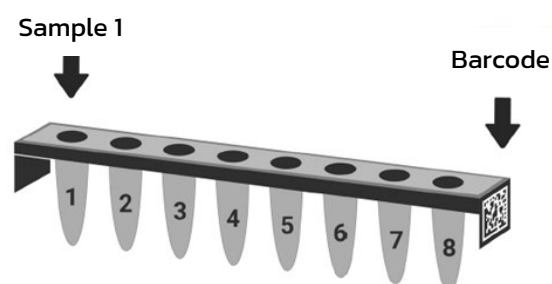


Figure 3. Required orientation of samples in the *Sample Input Strip*

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

➤ **OPTION B. Enzymatic fragmentation:** Thermal cycler with adjustable temperature lid.

In this section, the DNA is enzymatically fragmented to obtain DNA fragments between 100 to 150 bp in size.

Reagents to be used in this step:

Reagent	Color	Conservation
<b>Fragmentation Buffer</b>	Green cap	-20°C
<b>Fragmentation Enzyme</b>	White cap	-20°C

- 01 Thaw the *Fragmentation Buffer* and keep cold. Keep the *Fragmentation Enzyme* at -20 °C until its use.
- 02 Dilute each DNA sample with nuclease-free water to obtain a total concentration of 200 ng, to a final volume of 7 µL, on a 0.2 mL well strip.

If you do not have a total of 200 ng at a volume of 7 µL, choose one of the following options:

- ↘ Increase the sample volume by preparing the last dilution at a final volume of 17 µL. Once this last dilution has been carried out, fragmentation should be performed by maintaining the reagent volume and PCR program described in Section 7.2.2 of this document.
- ↘ Lower the total concentration to a total of 100 ng. If this option is chosen, it is advisable to perform it only for samples below a total of 200 ng, and all the samples with these characteristics should be combined into one run. Otherwise, set up the Magnis instrument with the recommended program for the sample with the lowest input.

**! IMPORTANT**

There are certain disadvantages to the second option, which should be assessed beforehand. Lowering the starting concentration will involve an increase in the sequencing duplicates and a decrease in the coverage metrics, which could affect the sensitivity and specificity of the technique.

- 03 Mix all dilutions on the vortex mixer, spin, and keep cold until their use.
- 04 Prepare the required volume of the fragmentation mix at cold temperatures, as described below, mixing each reagent before use. The *Fragmentation Buffer* should be vortexed vigorously, while the *Fragmentation Enzyme* should be 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)
<i>Fragmentation Buffer</i>	2 $\mu$ L	18 $\mu$ L
<i>Fragmentation Enzyme</i>	1 $\mu$ L	9 $\mu$ L

- 05 Vortex vigorously.
- 06 Add 3  $\mu$ L of the fragmentation mix to each 0.2 mL tube with the fragmented sample. Mix by pipetting 20 times.
- 07 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

Table 6. Optimal fragmentation program

Temperature	Time	Cycles
37°C	15 minutes	1
65°C	5 minutes	1
4°C	$\infty$	

**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 (Thermo Fisher Scientific), 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.

- 08 Once the fragmentation program is completed, remove samples from the thermal cycler, spin, add nuclease-free water to each sample to a total final volume of 50  $\mu$ L, 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:** If the fragmentation volume has been modified, less nuclease-free water will be needed to obtain the final volume of 50  $\mu$ L.

**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*.

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

Reagents to be used in this step:

Reagent	Color	Conservation
<b>Reagents Plate</b>	Blue plate	-20°C
<b>Beads/Buffer Plate</b>	White plate	4°C
<b>Index Strip</b>	Black strip	-20°C
<b>Cardiovascular Probe Strip</b>	White strip	-80°C
<b>Box 4</b>	N/A	15-25°C

**01** Preparation of the *Reagents plate*:

- ◇ Thaw the plate at room temperature, keeping the plate in its white cardboard sleeve.
- ◇ Once the contents of all wells are thawed, vortex the plate while keeping it in its white cardboard sleeve. Begin by pressing the long side of the plate on the vortex head for 10 seconds. Afterwards, rotate the plate 90° and press the short side of the plate on the vortex head for 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 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.

**02** Preparation of the *Beads/Buffer plate*:

- ◇ Keep the white cardboard 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.

**03** Preparation of the *Index strip*:

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

Table 7. Sequences of the indexes included in the kit

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

- ◇ 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*. 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.

Table 8. Correlation between the Index on the Post-Run Data screen and the inscription on the strip

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

- 04 Immediately before use, thaw the Cardiovascular probe strip at cold temperature. Vortex for 5 seconds and spin. It is important to check that bubbles have not formed at the bottom of the well.

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

- 05 Finally, prepare a unit of the Box 4 to be used during deck setup.

## 07.4 | Running the library preparation protocol

### 07.4.1 | Start of the protocol

- 01 On the Home screen shown on the touchscreen, press *Run Protocol*. The system will lock the instrument door and carry out an *Instrument Health Check (IHC)*, which may take several minutes.
- 02 Once this check is completed, the message *Enter Run Info* will automatically appear on the screen. In the *Protocol menu*, select SSEL XTHS-RevB-ILM.
- 03 **Recommended:** Tick 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.

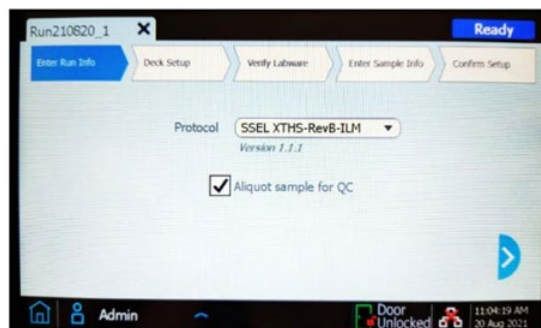


Figure 4. Magnis NGS Prep System Enter Run Info screen

- 04 Move on to the next screen.
- 05 Select appropriate sample type, *High Quality DNA*.

- 06 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 200 ng is recommended to prepare libraries using *Inherited CardioKitDx*.

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

## 07.4.2 | Deck setup

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

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

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

After removing the lid of the boxes of new and completely full tips, you should 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.

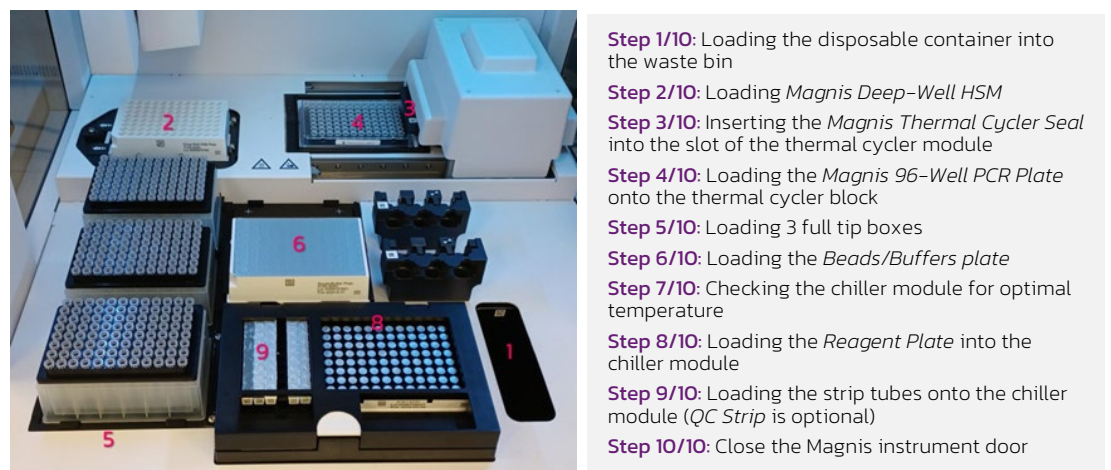


Figure 5. Magnis NGS Prep instrument deck loaded for the run and quick loading guide.

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

- 01 Place the disposable container *Magnis Tip Waste Bin* (included in the Box 4) in the waste bin drawer located in the lower left corner. The barcode must be facing the user, as shown on the touchscreen. Close the waste bin drawer.

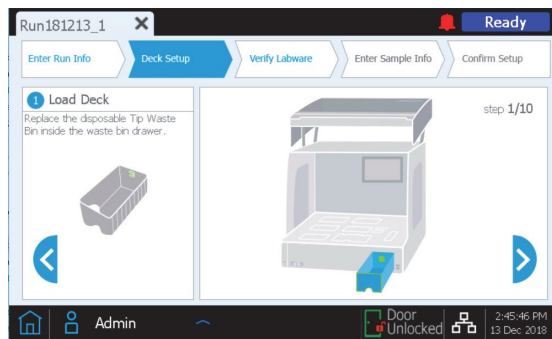


Figure 6. Step 1 of 10 on the Deck Setup screen of the Magnis NGS Prep System

- 02 Place the Magnis Deep-Well HSM Plate (included in the Box 4) as shown on the instrument's touchscreen. To do this, first insert the left edge of the plate into the spring-loaded slot and then lower the right edge of the plate down until it sits flat on the platform. Once flat, shift the plate slightly to the right and ensure that it is secured inside the holder.

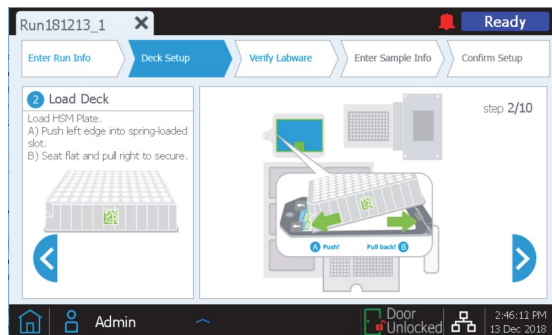


Figure 7. Step 2 of 10 of the Deck Setup screen of the Magnis NGS Prep System

- 03 Place the *Magnis Thermal Cycler Seal* (included in the Box 4) as shown on the instrument's touchscreen. To do this, peel the protective film from the white foam pad located below the metal plate. After the full sheet of film has been removed, insert the *Thermal Cycler Seal* into the slot of the thermal cycler, with the barcode facing up, and slide until it clicks into place.

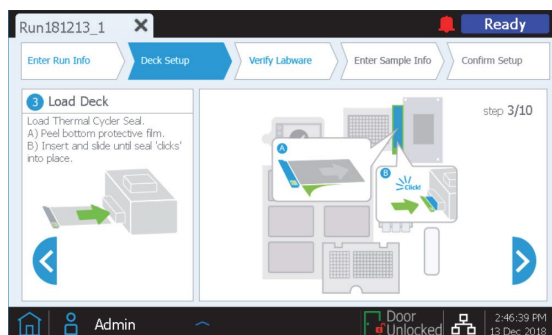


Figure 8. Step 3 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

- 04 Place the *Magnis 96-Well PCR Plate* (included in the Box 4) as shown on the instrument's touchscreen. To do this, insert the plate wells into the thermal cycler block wells, with the plate barcode facing the user. Make sure that the plate is fully seated by pressing evenly, first on the center of the plate and then on the corners.

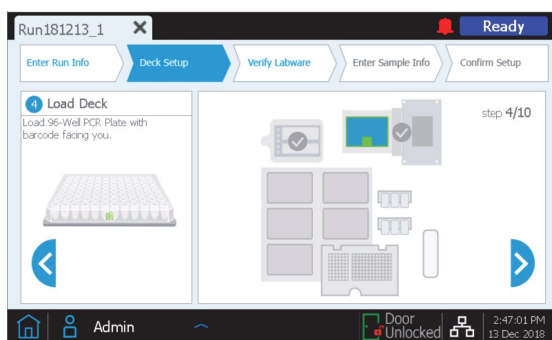


Figure 9. Step 4 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

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



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

- 06 Place the *Beads/Buffers Plate* (prepared in section 7.3 of this document). Remove the white cardboard sleeve and then load the plate as shown on the instrument's touchscreen, with the barcode facing the user. To do this, first insert the left edge of the plate in the spring-loaded slot and then lower the right edge of the plate down until it sits flat on the platform. Once flat, shift the plate slightly to the right and ensure that it is secured inside the holder.



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

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

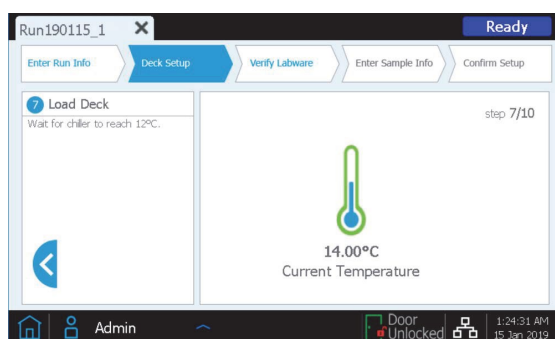


Figure 12. Step 7 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

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

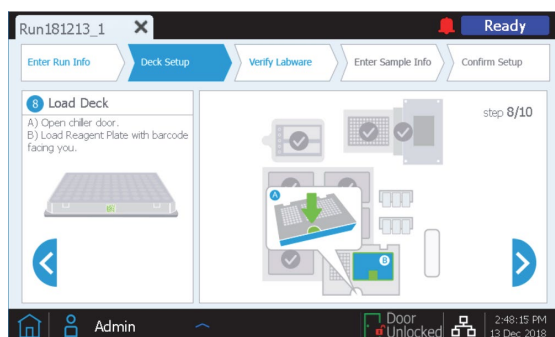


Figure 13. Step 8 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

- 09 Load the strip tubes for the run in the indicated positions of the chiller, as shown on the instrument's touchscreen. Ensure that each strip is secured by pressing firmly and evenly on the edges of the strip tubes. Avoid touching or damaging the foil covers. All the tube strips must have a barcode facing the user.
- ◇ Load the *Sample Input Strip* (red strip) containing the DNA samples, prepared in section 7.2 of this document, into the chiller holder position labeled with S.
  - ◇ Load the *Index Strip* (black strip), prepared in section 7.3 of this document, into the chiller holder position labeled with IDX.
  - ◇ Load the *Cardiovascular 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 Box 4, into the chiller holder position labeled with L.
  - ◇ **Optional:** If the run will include the collection of aliquots of the pre-capture libraries for quality control, as recommended by Health in Code, S.L., load the QC Strip (blue strip), included in the Box 4, into the chiller holder position labeled with Q.

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



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

10 Close the instrument door.

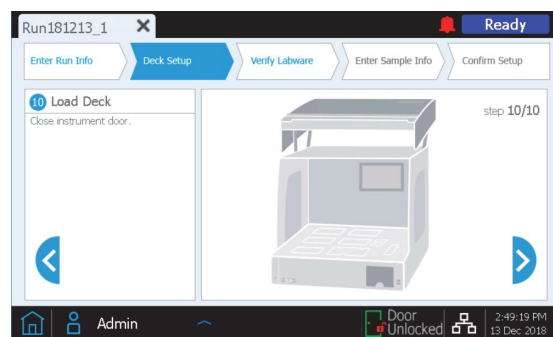


Figure 15. Step 10 of 10 of the Deck Setup screen of the Magnis NGS Prep instrument

### 07.4.3 | Labware verification

Once loading has been completed, proceed to the *Verify Labware* phase, in which the instrument scans the barcode of each of the components present in the unit.

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

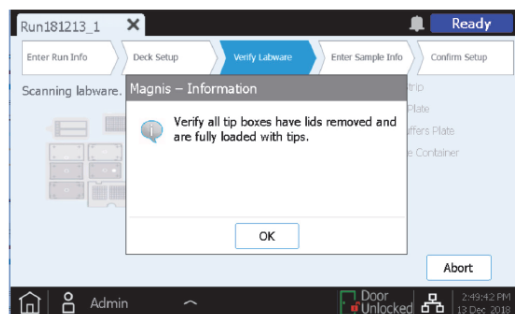


Figure 16. Verify Labware pop-up window of the Magnis NGS Prep System

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

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

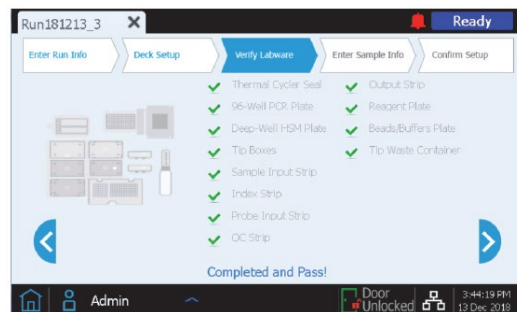


Figure 17. Verify Labwarescreen of the Magnis NGS Prep System after a correct verification of the material.

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

## 07.4.4 | Entering sample information

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

### 01 Manual sample assignment:

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

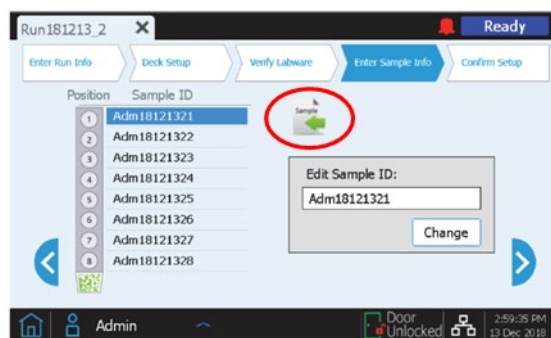


Figure 18. Edit Sample Info screen of the Magnis NGS Prep System, with a circle highlighting the load samples button.

### 02 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 *empty2*).
- ◇ Save file in the .csv format.
- ◇ Download the .csv file into an unencrypted USB disk and connect the disk to one of the USB ports of the Magnis instrument.
- ◇ When configuring the assay, on the *Enter Sample Info* screen, press the load samples button (highlighted with a circle in Figure 18).
- ◇ Follow the instructions of the protocol setup assistant to transfer sample IDs from the USB disk.

## 07.4.5 | Confirm setup and start the run

- 01 Confirm the run setup details. Once entries are confirmed to be correct, press the forward arrow to move on to the final setup screen.
- 02 Verifying run setup details related to characteristics of the DNA sample. After confirming that the run setup details are correct, press the  Start button to start the run.

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

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 completed, the prepared libraries are automatically held at 12 °C. Collect the libraries from the instrument within a maximum of 24 hours.

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

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

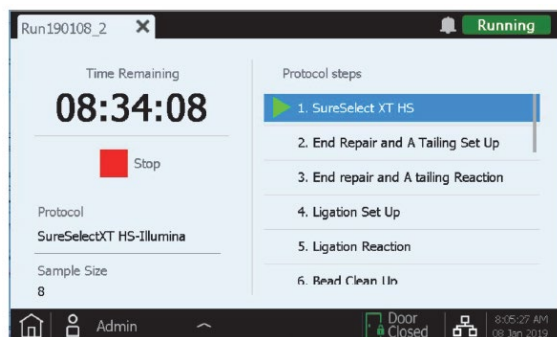


Figure 20. Running screen during a run.

## 07.4.6 | Collecting libraries from the instrument

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

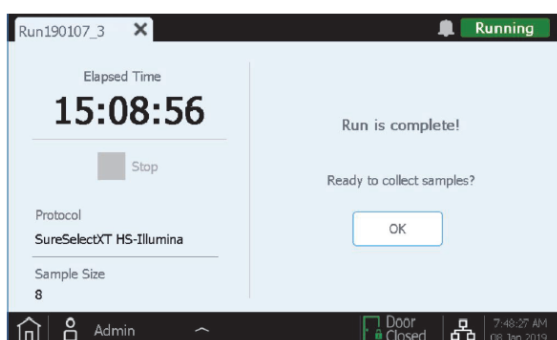


Figure 21. Running screen after a run

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

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

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

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

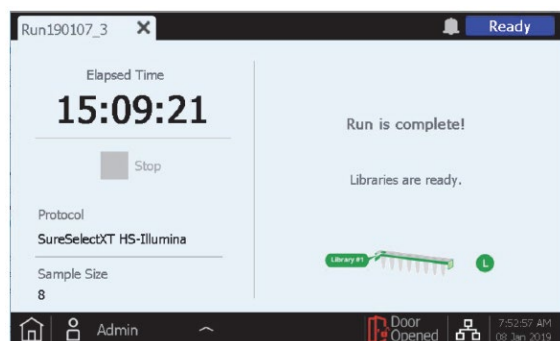


Figure 22. Running screen after a run and after removing the libraries

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

## 07.5 | Instrument cleaning after a run

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

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

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

## 07.6 | Library validation and quantification

### 07.6.1 | Optional quality control of the pre-capture library

If analysis of pre-capture libraries is required, resuspend the dried library samples in 6  $\mu\text{L}$  of nuclease free water to achieve a concentration suitable for analysis, using the recommended *TapeStation System* and commercial kits *D1000 Reagents* (cat. no. 5067-5583) and *D1000 ScreenTape* (cat. no. 5067-5582) by Agilent Technologies.

After adding 6  $\mu\text{L}$  of nuclease-free water, incubate at room temperature for 10 minutes. Last, vortex vigorously to ensure complete resuspension.

After analyzing the samples using a *TapeStation System*, a library size of 260–320 bp should be obtained (Figure 23). If unexpected sizes are obtained, review the protocol or contact Health in Code, S.L.'s technical support team.

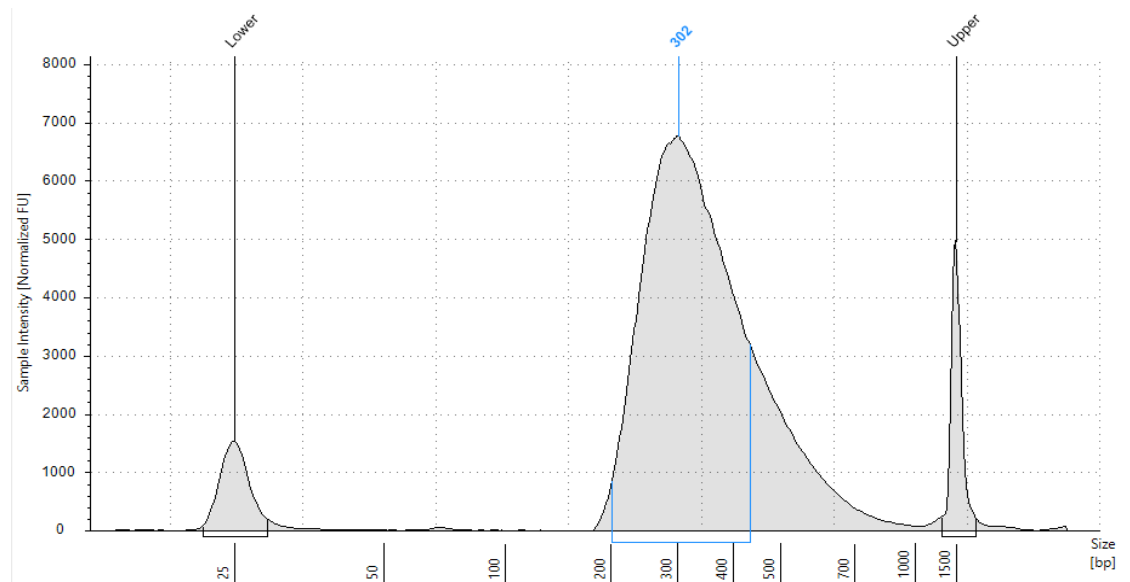


Figure 23. Expected result after analyzing pre-capture library size with TapeStation System

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

## 07.6.2 | Quality control of the post-capture library

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

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

The concentration of post-capture libraries will vary between 2 and 10 ng/ $\mu$ L.

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

The expected mean fragment size is between 250 – 350 bp. If an unexpected size is obtained, review the protocol and pre-capture library quality control, carefully read section 9 or contact Health in Code, S.L.'s technical support team.

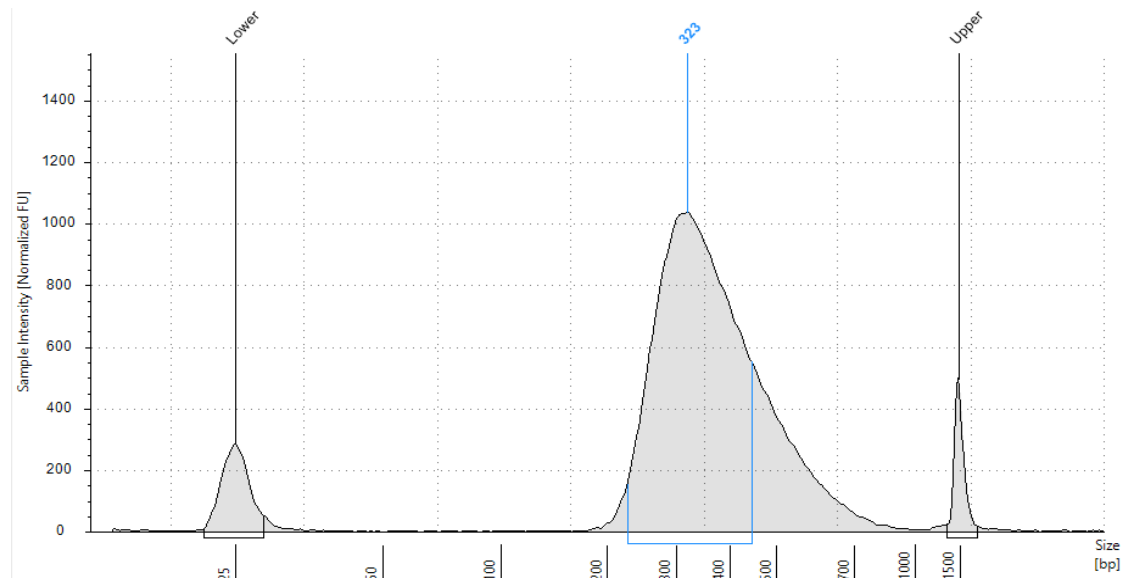


Figure 24. Expected result after the analysis of post-capture library size with TapeStation System

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

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

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

↘ The protocol can be stopped at this point, storing the libraries at 4 °C if they are to be used within the following 12 hours or at -20°C for longer storage periods.

## 07.7 | Denaturing libraries for loading into the Illumina *NextSeq 500/550* instrument

Prior to loading the libraries in the Illumina *NextSeq 500/550 System* sequencer, they must be denatured. To denature and load the libraries, Illumina’s instructions should be followed for each sequencer.

Similarly, the *PhiX Control* must also be denatured by following the denaturation protocol provided by Illumina for each instrument. *PhiX Control* should be present in the reaction at 1%.

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 8.2 million per sample:

Table 9. NextSeq Illumina kit and maximum number of samples to be analyzed with Inherited CardioKitDx

NextSeq 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

## 07.8 | NextSeq platform setup

- ◇ Read Type: Paired End.
- ◇ Cycles:
  - ↳ Read 1: 75
  - ↳ Read 2: 75
  - ↳ Index 1 (i7): 8

## 08 Analysis of results

Bioinformatics analysis of the results is done through the **Health in Code Client Site** platform, using an analysis pipeline specifically designed for **Inherited CardioKitDx**. To access this tool, visit: [www.clientsite.healthincode.com](http://www.clientsite.healthincode.com).

This pipeline uses five callers that significantly increase specificity and sensitivity and generates consensus coverage and quality parameters per variant, which can be individually accessed.

### 08.1 | Sending sequencing files

**01** Before accessing **Client Site** at Health in Code, S.L., it is necessary to upload the *FASTQ* files of each sample to the platform using the [transfer.healthincode.com](http://transfer.healthincode.com) tool.

For access, the "User" and "Password" data received must be entered in the pop-up window as shown in the Figure 25:

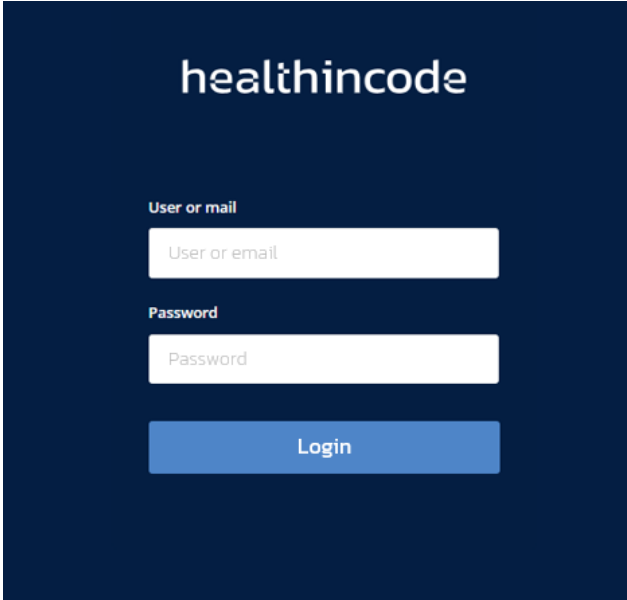


Figure 25. Login screen on to the [transfer.healthincode.com](http://transfer.healthincode.com) tool

⚠ **NOTE:** At the beginning of the project, Health in Code, S.L. will provide the user with the necessary template and configuration. Similarly, it will be necessary to specify which equipment has been used for the sequencing.

**02** After starting the session, the user will have different folders available in their user profile (Figure 26):

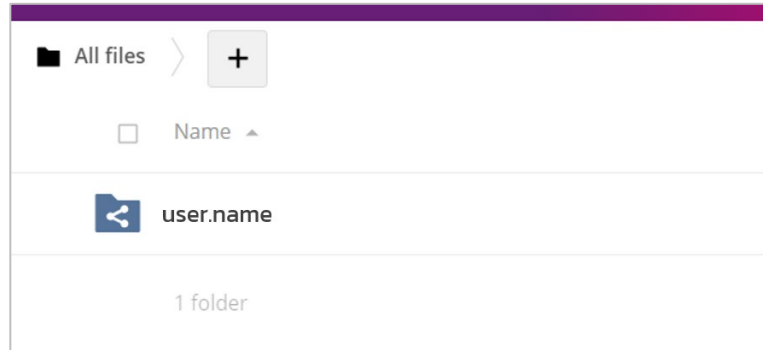


Figure 26. Access to user folder

**03** To load the files, enter the folder called Data (“*Datos*”) within the user folder and upload the necessary files indicated below to the “*Upload*” folder (Figure 27):

- + FASTQ files generated by the sequencer.
- + The *SampleSheet* for the assay.
- + A template containing the sample data and the FASTQ association with the following:
  - ◇ Center/Hospital
  - ◇ Requesting party
  - ◇ Phenotype or suspected phenotype
  - ◇ Patient initials
  - ◇ Family ID
  - ◇ Patient ID
  - ◇ DOB
  - ◇ Gender (Male/Female)
  - ◇ The name of the FASTQ file corresponding to the sample

**IMPORTANT:** You will need to specify the full name of the kit being used: **Inherited CardioKitDx**.

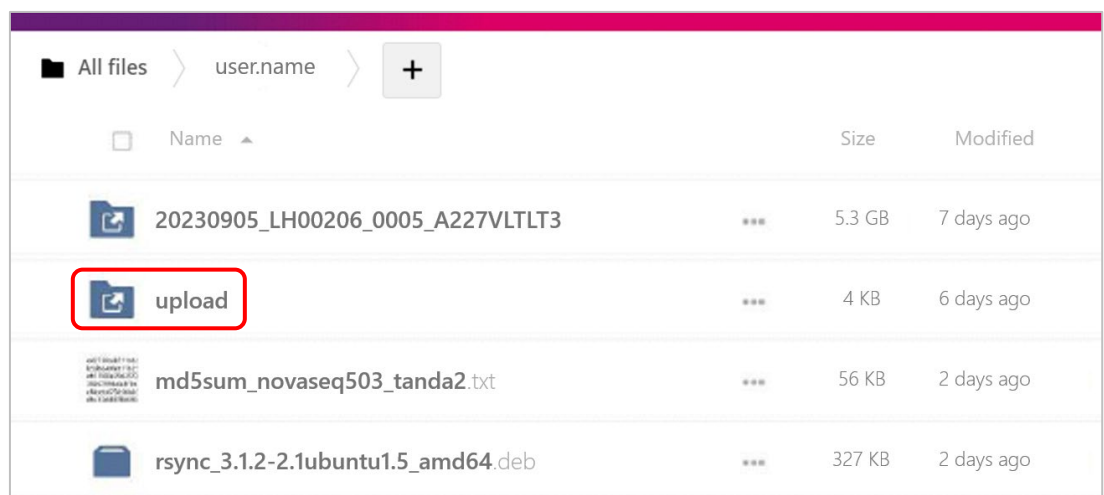


Figure 27. User folder structure.



Once the FASTQ files have been uploaded and the necessary information has been entered in the template, the Health in Code, S.L. team will generate the study corresponding to the project and the user will be able to start the analysis with the information available on the **Client Site**.

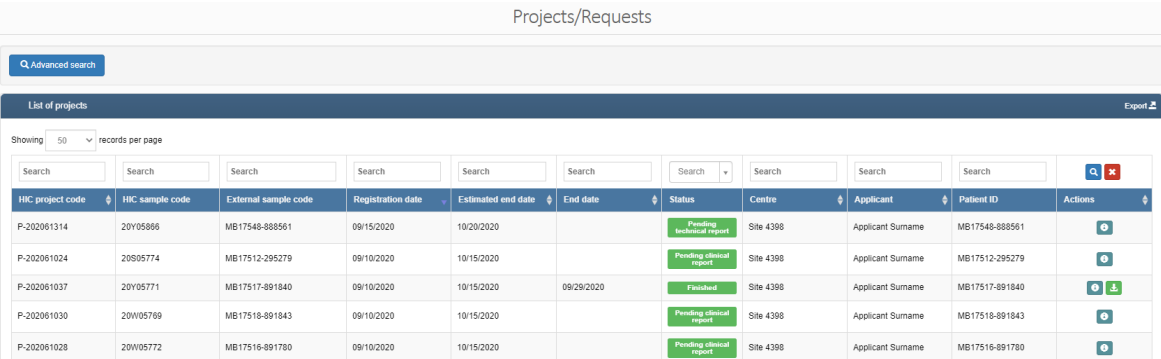
## 08.2 | Accessing the platform and managing orders

Our IT team at Health in Code, S.L. will email you the necessary username and password to access **Client Site**. If this is the first time that you log in, you will be asked to change your password for security reasons.

Once logged in, the home screen shows a series of plots and stat summaries on the requested studies.

The *"Projects/Orders"* menu shows a detailed list of the projects (Figure 28). The table contains the ID codes for projects/orders and associated samples, client/center, date of registration, estimated finishing date, finishing date (for finished projects), patient ID, and current status.

The  button gives access to project details. The user can find more specific information using the filters on the table header or perform a detailed search using the *"Advanced search"* option. The data displayed can be exported to XML or Excel format by clicking the  button at the top right corner of the screen.

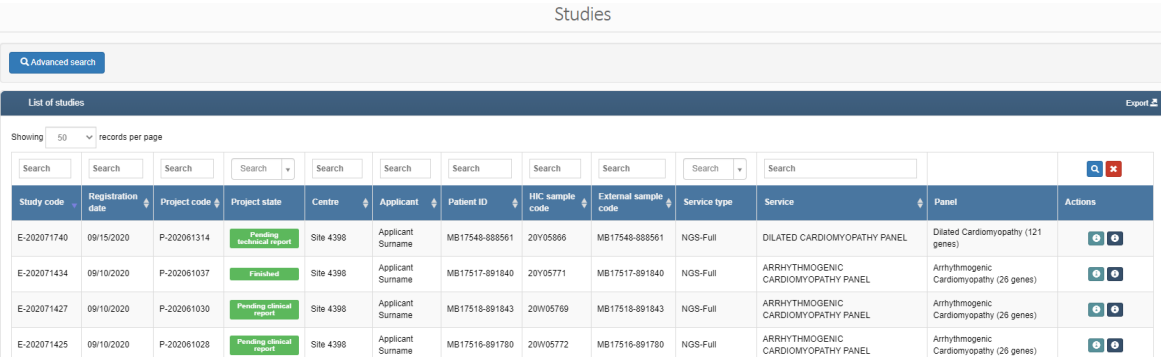


HIC project code	HIC sample code	External sample code	Registration date	Estimated end date	End date	Status	Centre	Applicant	Patient ID	Actions
P-202061314	20Y05866	MB17548-888561	09/15/2020	10/20/2020		Pending technical report	Site 4398	Applicant Surname	MB17548-888561	
P-202061024	20R05774	MB17512-295279	09/10/2020	10/15/2020		Pending clinical report	Site 4398	Applicant Surname	MB17512-295279	
P-202061037	20Y05771	MB17517-891840	09/10/2020	10/15/2020	09/29/2020	Finished	Site 4398	Applicant Surname	MB17517-891840	
P-202061030	20W05769	MB17518-891843	09/10/2020	10/15/2020		Pending clinical report	Site 4398	Applicant Surname	MB17518-891843	
P-202061028	20W05772	MB17516-891780	09/10/2020	10/15/2020		Pending clinical report	Site 4398	Applicant Surname	MB17516-891780	

Figure 28. Project list

The *"Project details"* screen displays information on the date of registration, associated sample, client/hospital, etc. Further information on each project can be found in the *"Studies"*, *"Documents"*, *"Status"*, *"Reports"*, and *"Access"* tabs.

The *"Studies"* menu within *"Projects/Orders"* shows a detailed list of the available projects (Figure 29). The table contains information about the services and panels requested for the samples, the client, the date of registration, the current status of the sample and patient and sample identification details.



Study code	Registration date	Project code	Project state	Centre	Applicant	Patient ID	HIC sample code	External sample code	Service type	Service	Panel	Actions
E-202071740	09/15/2020	P-202061314	Pending technical report	Site 4398	Applicant Surname	MB17548-888561	20Y05866	MB17548-888561	NGS-Full	DILATED CARDIOMYOPATHY PANEL	Dilated Cardiomyopathy (121 genes)	
E-202071434	09/10/2020	P-202061037	Finished	Site 4398	Applicant Surname	MB17517-891840	20Y05771	MB17517-891840	NGS-Full	ARRHYTHMOGENIC CARDIOMYOPATHY PANEL	Arrhythmogenic Cardiomyopathy (26 genes)	
E-202071427	09/10/2020	P-202061030	Pending clinical report	Site 4398	Applicant Surname	MB17518-891843	20W05769	MB17518-891843	NGS-Full	ARRHYTHMOGENIC CARDIOMYOPATHY PANEL	Arrhythmogenic Cardiomyopathy (26 genes)	
E-202071425	09/10/2020	P-202061028	Pending clinical report	Site 4398	Applicant Surname	MB17516-891780	20W05772	MB17516-891780	NGS-Full	ARRHYTHMOGENIC CARDIOMYOPATHY PANEL	Arrhythmogenic Cardiomyopathy (26 genes)	

Figure 29. Studies list

As in the case of the "Projects/Orders" panel, more specific information can be found using filters or performing an Advanced Search. The data displayed can be exported to XML or Excel format.

The ⓘ button gives access to study details. This page displays information about services and panels requested, selected library, genetic variants detected, coverage statistics, CNVs, IGV viewer to verify alignment, downloadable files (including fastq and alignment files), quality parameters (including STID), etc.

### 08.3 | Coverage statistics

Coverage statistics can be accessed on the tab with the same name. The user can first see a summary table with the coverage data of the libraries, followed by several interactive graphs representing the coverage obtained.

A list of coverage by region is included (Figure 30) below. It includes an advanced search filter which can be used to filter the data shown in the table, which can be later exported to an Excel file.

Gene	Coding region	Cod. Reg. Category	Chromosome	Initial position	Final position	Size (pb)	No. pb cov. > 15X	No. pb < 15X	No. pb cov. > 15X	Average coverage	No. pb uncovered	No. pb cov. [1-5]	No. pb cov. [6-14]	No. pb cov. [15-29]	No. pb cov. >= 30X
DES (***)	4588	exon-cdna	2	220283185	220283762	578	578	0	100%	225.61	0	0	0	0	578
DES (***)	4589	intron-spl	2	220283763	220283772	10	10	0	100%	42.80	0	0	0	0	10
DES (***)	4592	intron-spl	2	220284807	220284816	10	10	0	100%	328.80	0	0	0	0	10
DES (***)	4593	exon-cdna	2	220284817	220284877	61	61	0	100%	376.61	0	0	0	0	61
DES (***)	4594	intron-spl	2	220284878	220284887	10	10	0	100%	394.80	0	0	0	0	10
DES (***)	4596	intron-spl	2	220284963	220284972	10	10	0	100%	350.80	0	0	0	0	10
DES (***)	4597	exon-cdna	2	220284973	220285068	96	96	0	100%	299.89	0	0	0	0	96
DES (***)	4598	intron-spl	2	220285069	220285078	10	10	0	100%	250.40	0	0	0	0	10
DES (***)	4600	intron-spl	2	220285207	220285216	10	10	0	100%	241.10	0	0	0	0	10

Figure 30. Coverage statistics by region

### 08.4 | Variant filtering

On the "Variants" tab, you can access the pre-set variant filter, designed to show only the most relevant variants, with a quality value greater than 100 (the maximum quality value is 250).

The filter can be modified or eliminated by clicking the "Advanced Search" button, where you can filter by gene, priority (priority, secondary, or candidate), chromosome, variant type, etc. To remove a filter, the user must click on "Clear filter" and then "Search". If you want to modify the filter, once the parameters have been changed, click "Search".

The three available priority categories are:

- **Priority genes:** Genes with sufficient clinical and functional evidence to be considered associated with the disease. These genes are included in clinical guidelines.
- **Secondary genes:** Genes related to the disease, but with a lower level of evidence.
- **Candidate genes:** Genes potentially associated with the disease, but with insufficient evidence in humans to be placed in either of the previous categories.

The list of variants shows relevant information on each variant identified in the study:

- ↳ Different variant names: chromosomal, genomic, and cDNA.
- ↳ The count (number of times detected/number of times the gene has been studied) of the variant in the general HIC dataset, as well as the count only in patients associated with the orderer's account.
- ↳ Variant frequency in *gnomAD*.
- ↳ Variant pathogenicity from the HIC database and pathogenicity manually determined by the user.
- ↳ Various quality scores.
- ↳ Summary of population data related to the variant.
- ↳ Pathogenicity indicators reported in the *ClinVar* database and preselection of the variant as relevant by the HIC clinical team or by the user.

For each detected variant, there is a page that details information at the protein, cDNA, and DNA level, as well as information on the associated gene, population statistics, predictors, and articles related to the variant and their link to PubMed. This information can be accessed by pressing the ⓘ button next to each variant.

In *"Populations and predictors"*, in addition to population statistics, including one from the HIC database, it shows links to different external databases, such as OMIM, NCBI, HGNC, HPO, etc.

## 08.5 | Variant categorization

The users can select themselves the pathogenicity of variants identified in their studies. This can be modified in the details section of each variant in the *"Pathogenicity"* field (Figure 31), in a drop-down menu showing different options. Also, it is possible to review the history of the values that have been set over time. This value will appear in the results table under the acronym *"Pat."* Users can filter these values using the advanced search feature.

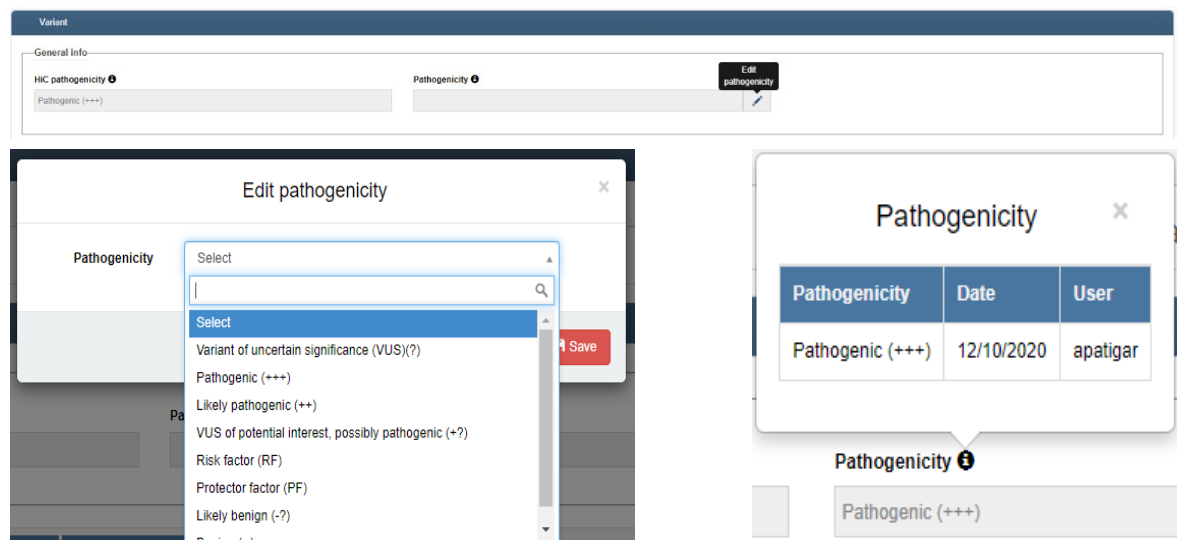




Figure 31. Select pathogenicity

All variants of interest can be marked and unmarked as a pre-selected variant, by clicking the  button located in the list of results. These variants will be indicated in the "info" column with a blue star symbol, while variants pre-selected by the HIC clinical team will appear with a red star. Pre-selected variants can be filtered using the advanced search feature.

In the advanced search, it is possible to filter by phenotypes that are included in the HPO database and that are associated with each of the variants. The info button shows the genes associated with the phenotype included in the filter. In addition, the page with details on each variant contains a table of the HPO phenotypes associated with that variant, together with a direct link to the HPO database. Advanced search can use these terms to filter the related variants.

The data shown in the table of variants can be exported to an Excel file by clicking the  button in the upper right part of the table. The exported data contain the details of each variant included in the initial table and relevant population information, and predictors, as well as pathogenicity values selected by the user and pre-selected variants.

By clicking on the "Generate report" button, located above the variant table, a report in .docx format will be generated, available in both English and Spanish. The report will include all the variants pre-selected by the user, as well as the comments associated with each variant and a space for the user to include comments about the study, which can be filled in the study details panel, by selecting the "Add result" button. In addition to variant data, the report will include information from databases such as *gnomAD* and *EVS*.

## 08.6 | Analysis of large rearrangements (CNVs)

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

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

- 01** 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.
- 02** Input DNA is another source of variability. Therefore, it is recommended that all analyzed DNA be extracted following the same extraction protocols.

When samples from the same assay are processed bioinformatically with the pipeline, a CNV analysis is started. A link through which the analysis can be accessed can be found in the "CNVs" tab within a study.

The analysis is based on the comparison of coverage patterns across all samples. First, a coverage model that uses all the samples a run contains is generated for each run. For a good coverage model, the software needs a minimum of eight samples processed with the same protocol and sequenced together, since the samples of a run will be grouped by similarity, so not all of them will be used to make the reference of all run libraries.

The analysis process will report all the candidate regions in which a potential CNV has been detected and will assign them a score and some measurements used for evaluating the confidence of each candidate CNV. Figure 30 shows an example of CNV detection.


In the table of candidate CNVs (Figure 32, A), each CNV is shown with some associated information and hovering the cursor over the header will show a brief explanation of the function of each column. Some of them are:

- + Quality score:** Ranging from 0 to 10, it shows, with increasing values, the confidence of the detected alteration call to be caused by a real CNV. This quality score is calculated on the basis of the size of the event, the breakpoints detected, the ratio, the variation of the ratio throughout the affected region, deviation from the model, and coverage.
- + Dev (deviation):** Represents the deviation of the signal of the sample studied with respect to the reference signal. The second value (highlighted in gray) represents the variation associated with this measurement.
- + Ratio:** Represents the ratio between the altered signal and the reference signal. The second value (highlighted in gray) represents the variation associated with this measurement.



Figure 32. CNV plot.

- A.** List of candidate loci affected by a CNV ordered from highest to lowest Quality score.
- B.** For the selected CNV, normalized coverage for all samples included in the analysis (in black), for the selected sample (in red), and for a reference model generated during validation (in green).
- C.** For the selected CNV, zygosity ratios of the selected sample (in red) and the rest of the samples included in the analysis (in black).

The CNVs shown can be filtered by clicking on the filter button , located in the upper left part of the table of candidate genes. Five recommended filters will appear. They are as follows:

- **Filter candidates with not compatible split reads information:** Filters out all partial CNVs in which there are no reads that cover both the zone with the CNV and the adjacent one, known as split reads, and therefore are likely to be a FP.
- **Filter not germinal characteristics ratios:** Filters out all CNVs with ratios outside the range established by the filter.
- **Filter not stable ratios:** Filters out all CNVs with unstable ratios according to the established values.
- **Filter not clear deviation:** Filters out all CNVs with low deviation of coverage with respect to the reference.
- **Filter low scored candidates:** Filters out all CNVs with a quality score lower than 7, due to their high probability of being a FP.

In addition, genes within the priority, secondary and candidate groups can be filtered in or out in the filter screen.

Due to their high predictive value, the use of the following filters is recommended: *Events without Split Reads, Low Scored Candidates, and Low Coverage Deviation.*

**IMPORTANT:** Review the sex assigned to the sample. If the assigned sex is not the expected one, it could lead to FPs on the sex chromosomes. Sex appears at the top of the screen, centered next to the sample's HIC code.

Finally, quality control is performed for each execution, which provides general quality values for each CNV analysis. It can be accessed through the icon 🏠, located at the top of the CNVs analysis screen.

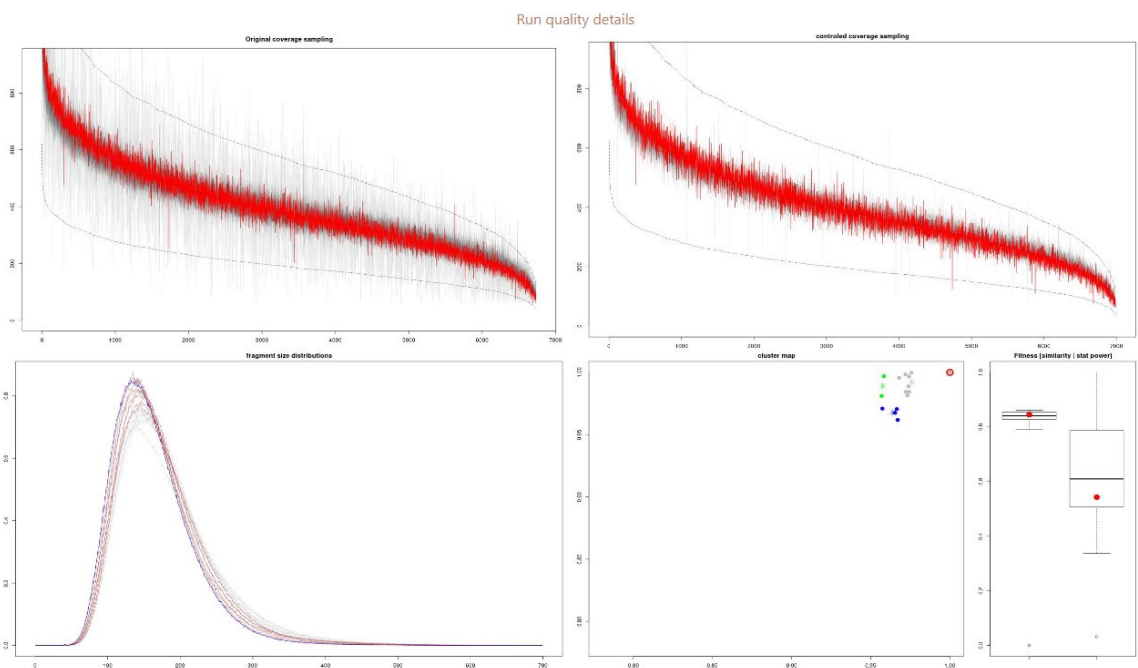


Figure 33. CNV analysis quality graphs

Figure 33 shows an example of this analysis. The upper graphs show coverage distribution of the sample with respect to the rest of the samples in the analysis—samples that have not been used in the CNV analysis of this sample are marked in gray on the left and a chart

without these samples is displayed on the right. The lower graphs show, from left to right: fragment size distribution, marking the sample in question in blue and the rest in different colors depending on size populations; group map, in which you can see each group of a color and compare the differences between the analyzed library (circled in red) with the rest of the libraries of the same group (marked with the same color) and with libraries of other groups (marked in other colors); box plot of similarity of the libraries and the predictive value of the analysis.

## 09 Troubleshooting

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

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

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

To reset touchscreen functionality, the system must be rebooted.

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

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

- 01 Verify that all deck positions are cleared of kit consumables and other debris. The presence of any materials on the instrument can prevent the successful detection of all verified teachpoint markers.
- 02 Clean the barcode scanner window according to the cleaning instructions in the Magnis Instrument User Guide. Debris or fingerprints on the scanner can obscure the teachpoints, consequently causing verification failure.
- 03 Reboot the system. After logging in, the instrument will perform another IHC. If this health check is successful, you can resume the setup process without performing the *Auto Teaching* routine.

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

- 01 On the *Home* screen, open *Settings* and press *Auto Teaching*. Follow the instructions displayed on the touchscreen. The *Auto Teaching* process takes approximately 30 minutes and requires the presence of the user to place the labware on the instrument.
- 02 Once the *Auto Teaching* process is finished, start configuration of the assay by pressing *Run Protocol* on the *Home* screen.

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

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

- 01 In the error dialog box, press *Cancel* to reject the start of the diagnostic test.
- 02 Press the error icon at the bottom of the screen and record the error code for potential use in troubleshooting with Agilent technical support.
- 03 Turn off the instrument by pressing the power button on the front of the instrument.
- 04 Verify that all deck positions are cleared of kit consumables and other debris. The presence of any materials on the instrument may interfere with IHC after restarting.
- 05 Turn on the instrument by pressing the power button.
- 06 After logging in, the instrument will perform another IHC. If this check is successful, begin run setup. If the IHC fails again, contact Agilent Technical Support for assistance.

- + The *Verify Labware* screen reports a problem with one or more laboratory material components after automated material verification:

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

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

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

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

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

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

- ◇ *The scanned labware is incorrectly positioned:*

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

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

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

**+ Larger sizes than expected after DNA mechanic fragmentation:**

Any bubbles present on the strand of the sonicator *microTUBEs* may interfere with fragmentation. Ensure that there are no bubbles before proceeding with fragmentation by centrifuging the *microTUBEs* briefly, if necessary.

**+ Larger sizes than expected after DNA enzymatic fragmentation:**

- ◇ The fragmentation protocol includes the thawing, temperature control, pipetting and mixing steps required to achieve optimal performance of the process.
- ◇ Using any thermal cycler other than the one indicated may require an adjustment to incubation times.
- ◇ Make sure all the instructions have been followed before beginning the protocol.
- ◇ Ensure that there are no bubbles before placing the fragmentation reactions in the thermal cycler. Bubbles may reduce the efficiency of the process.
- ◇ Make sure you are using consumables that are compatible with the thermal cycler being used. If not, you will not achieve optimum performance in the process.

**+ 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, S.L. 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

- ◇ **Inherited CardioKitDx** is designed for identifying SNV and INDEL variants in the coding regions of the genes specified in section 2 of this document. Health in Code, S.L. only guarantees correct identification of these types of variants in non-coding regions that fall into the following categories:
  - ↳ Distance to the coding exon of less than 10 bp.
  - ↳ They meet the established quality criteria.
  - ↳ Intronic variants of clinical interest, classified as hotspots and specified in section 2 of this document.
  - ↳ Variants in untranslated regions (UTRs) and in promoters that have been explicitly included in section 2 as target regions.
- ◇ The employed technology cannot sequence well GC-rich regions, which present high sequence homology, such as homologous genes, pseudogenes, etc. This may lead to false positives or negatives. The specific pseudogenic regions are listed in Table 10. The **Inherited CardioKitDx** regions with 100% homology to pseudogenic regions are listed below.

Table 10. List of pseudogenic regions.

Gene	Chromosome	Initial position	End position	Size (pb)	Category
CASZ1	chr1	10698999	10700116	1118	exon-cDNA
OBSCN	chr1	228522781	228522999	219	exon-cDNA
OBSCN	chr1	228523000	228523009	10	intron-spl
OBSCN	chr1	228566122	228566131	10	intron-spl
SPEG	chr2	220299700	220300087	388	exon-cDNA
SPEG	chr2	220312696	220313993	1298	exon-cDNA
SPEG	chr2	220347820	220349794	1975	exon-cDNA
OBSL1	chr2	220422341	220422350	10	intron-spl
PKD2	chr4	88928886	88929480	595	exon-cDNA
PKD2	chr4	88929481	88929490	10	intron-spl
SYNGAPI	chr6	33388042	33388108	67	exon-cDNA
SYNGAPI	chr6	33388109	33388118	10	intron-spl
KCNK17	chr6	39278659	39278668	10	intron-spl
GATAD1	chr7	92077293	92077302	10	intron-spl
GATA4	chr8	11565822	11566437	616	exon-cDNA

SURF1	chr9	136223176	136223185	10	intron-spl
SURF1	chr9	136223266	136223275	10	intron-spl
SURF1	chr9	136223276	136223329	54	exon-cDNA
NOTCH1	chr9	139440178	139440238	61	exon-cDNA
MYH6	chr14	23852530	23852539	10	intron-spl
SCN1B	chr19	35521725	35521764	40	exon-cDNA
SCN1B	chr19	35521765	35521774	10	intron-spl
RRAS	chr19	50139110	50139119	10	intron-spl
SNTA1	chr20	32031117	32031426	310	exon-cDNA
TXNRD2	chr22	19929214	19929223	10	intron-spl
TXNRD2	chr22	19929224	19929326	103	exon-cDNA

- ◇ The correctness of any obtained result with quality parameters below the established criteria cannot be ensured.
- ◇ NGS is not yet considered the Gold Standard for some types of mutation; therefore, it is recommended, whenever possible, to confirm positive results using a complementary standardized technique.
- ◇ 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.
- ◇ All the obtained data and information must be clinically evaluated and interpreted by the clinician, in an integrated way, together with the rest of the patient's clinical information and other results of complementary analytical or imaging tests.

## 10.2 | Equipment

**Inherited CardioKitDx** has been validated using the following equipment for DNA fragmentation:

- + GeneAmp PCR System 9700 (Applied Biosystems) for enzymatic fragmentation
- + ME220 Focused-ultrasonicator (Covaris) for mechanical fragmentation

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.

**Inherited CardioKitDx** has been validated using the following automated library preparation system:

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

**Inherited CardioKitDx** has been validated using the following massive sequencing platform with the load concentration specified:

- + **NextSeq 500/550 System (Illumina):**  
Validated load concentration → 1.2 pM

This kit is compatible with Illumina's massive sequencing platforms only. If massive sequencing equipment other than the *NextSeq 500/550 System* is used, the final

concentration of the libraries must be adjusted according to the instructions of the specific protocols of these platforms.

## 10.3 | Reagents

**Inherited CardioKitDx** has been validated using the reagents included in the kit and those recommended in section 6 of this manual (Equipment, reagents and materials required but 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.L.'s technical support team.

## 10.4 | Bioinformatics analysis platform

**Inherited CardioKitDx** has been validated using the **Client Site** platform, which is a platform for *in vitro* bioinformatics analysis. This platform includes a pipeline tailored specifically for **Inherited CardioKitDx** that is used for the detection of all targets outlined in section 2 of this document.

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

## 10.5 | Product stability

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

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

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