Instructions for use

Inherited NephroKitDx

CE IVD

REF IMG-370

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

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All Health in Code S.L. products undergo strict quality control. **Inherited NephroKitDx** 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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Modifications to the Instructions for Use (IFU)	
Version 6	Update of variant filtering (section 8.2).
Version 5	Update of sections 8.3.
Version 4	Update of sections 5 and 7.2.1.
Version 3	Modification of the fragmentation program
Version 2	CE-IVD marking update
Version 1	Document created

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1 General information

Kidney diseases are a wide spectrum of pathologies with different origins, diagnoses, and prognoses that affect millions of people worldwide, and they currently constitute one of the causes of mortality with the highest impact. In Spain, the prevalence of chronic kidney disease is estimated to affect one in seven individuals; therefore, these diseases are a subject of great interest for the public healthcare system in our country. Among kidney diseases, hereditary nephropathies account for more than 5% of all known genetic diseases. On the other hand, inherited nephropathies account for around 10% of nephropathies requiring transplantation in adults and virtually all of those that do in children. Their prevalence is highly variable and can range from diseases with a high prevalence in the general population, such as autosomal dominant polycystic kidney disease, to syndromes with a very low incidence. Due to the heterogeneity of these diseases and the nature of pathogenic variants, genetic diagnosis is clinically useful, enables genetic counseling, and often contributes to the prognosis of disease progression.

Thanks to the latest advances in the field of genetics and to the use of high-throughput sequencing techniques, traditionally known as next-generation sequencing (NGS), clinicians and researchers in the field of nephrology can rely on a test that is necessary in some contexts and that allows for accurate disease diagnosis, patient management optimization, prognosis of disease progression, and improved genetic counseling.

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- Medicines (Basel). 2020 Aug; 7(8): 44. Published online 2020 Jul 29. doi: 10.3390/medicines7080044 PMCID: PMC7459851 PMID: 32751108 "Genetic Alterations in Renal Cancers: Identification of The Mechanisms Underlying Cancer Initiation and Progression and of Therapeutic Targets" Ugo Testa,* Elvira Pelosi, and Germana Castelli.

2 Intended use

Inherited NephroKitDx has been designed to analyze the sequences of the coding regions of 529 genes selected for the study of the majority of kidney diseases. The pathologies addressed by this sequencing panel are the following:

- Glomerular diseases: nephrotic syndrome, FSGS, Alport syndrome, monogenic glomerulonephritis, Fabry disease, amyloidosis.
- Tubulointerstitial diseases: metabolic acidosis, metabolic alkalosis (Bartter syndrome, Liddle syndrome, Gitelman syndrome), nephrogenic diabetes, hypophosphatemic rickets, hyperoxaluria, cystinuria, nephrolithiasis and other metabolic kidney diseases.
- Cystic kidney diseases: polycystosis, nephronophthisis, syndromes with development of renal and/or liver cysts.
- CAKUT and other syndromes with kidney malformations, mainly corresponding to pediatric pathologies.
- Hereditary renal cancer.

This kit constitutes an approach to help healthcare professionals address hereditary kidney diseases, including genes associated with the development of glomerular diseases, tubulointerstitial diseases, cystic diseases, and polymalformative syndromes with kidney involvement, based on the current guidelines for the diagnosis and

management of hereditary kidney diseases, as well as on recommendations from scientific societies and selected scientific literature.

Below is the list of 529 genes selected for the study of kidney diseases covered by this sequencing panel:

ACE, ACTB, ACTG1, ACTN4, ADAMTS13, ADAMTS9, ADCY10, AGT, AGTR1, AGXT, AHI1, ALG1, ALG8, ALG9, ALMS1, ALPL, AMER1, ANKFY1, ANKS6, ANLN, ANOS1, AP2S1, APOA1, APOA4, APOE, APOL1, APRT, AQP2, ARHGAP24, ARHGDIA, ARL13B, ARL3, ARL6, ARMC9, ATN1, ATP6V0A4, ATP6V1B1, ATP6V1C2, AVIL, AVP, AVPR2, B2M, B3GLCT, B9D1, B9D2, BBIP1, BBS1, BBS10, BBS12, BBS2, BBS4, BBS5, BBS7, BBS9, BICC1, BMP2, BMP4, BMPER, BNC2, BSCL2, BSND, C1QA, C1QB, C1QC, C3, C4A, C4B, C8ORF37, CA1, CA2, CASP10, CASR, CC2D2A, CCBE1, CCDC28B, CCL2, CCNQ, CD151, CD2AP, CD46, CD81, CD96, CDC42, CDC5L, CDC73, CDK10, CDK20, CDKN1C, CENPF, CEP104, CEP120, CEP164, CEP290, CEP41, CEP55, CEP83, CFB, CFH, CFHR1, CFHR2, CFHR3, CFHR4, CFHR5, CFI, CFP, CHD1L, CHD7, CHRM3, CISD2, CIT, CLCN5, CLCN7, CLCNKA, CLCNKB, CLDN10, CLDN16, CLDN19, CNNM2, COL4A1, COL4A3, COL4A4, COL4A5, COL4A6, COPA, COQ2, COQ4, COQ6, COQ7, COQ8A, COQ8B, COQ9, CPLANE1, CPT1A, CRB2, CRKL, CSPP1, CTH, CTLA4, CTNS, CTU2, CUBN, CUL3, CYP24A1, DACH1, DACT1, DCDC2, DCHS1, DDX59, DGKE, DHCR7, DLC1, DMP1, DNAJB11, DNASE1, DSTYK, DVL1, DVL3, DYNC2H1, DYNC2LI1, DZIP1L, EGF, EHHADH, EMP2, ENPP1, EP300, ESCO2, ETFA, ETFB, ETFDH, ETV4, EXOC8, EYA1, FAH, FAM20A, FAN1, FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCL, FAS, FASLG, FAT1, FAT4, FCGR2A, FCGR3A, FGA, FGF10, FGF20, FGF23, FGFR2, FGFR3, FLCN, FLNA, FMN1, FN1, FOXI1, FOXP1, FRAS1, FREM1, FREM2, FUZ, FXYD2, G6PD, GANAB, GAPVD1, GATA3, GCM2, GDF11, GEMIN4, GLA, GLI3, GLIS2, GLIS3, GNA11, GPC3, GPHN, GREB1L, GRHPR, GRIP1, H19, HAAO, HAS2, HES7, HGD, HNF1A, HNF1B, HNF4A, HOGA1, HPRT1, HPSE2, HSD11B2, HSD17B4, HSPA9, IFT122, IFT140, IFT172, IFT27, IFT43, IFT46, IFT52, IFT74, IFT80, IFT81, INF2, INPP5E, INTU, INVS, IQCB1, IRF5, ITGA3, ITGA8, ITGAM, ITGB4, ITSN1, ITSN2, JAG1, JAM3, KANK1, KANK2, KANK4, KAT6B, KCNA1, KCNJ1, KCNJ10, KCNJ15, KCNJ16, KCTD1, KIAA0556, KIAA0586, KIAA0753, KIF14, KIF7, KLHL3, KMT2D, KYNU, LAGE3, LAMA5, LAMB2, LCAT, LMNA, LMX1B, LRIG2, LRP2, LRP4, LRP5, LYZ, LZTFL1, MAD2L2, MAFB, MAGED2, MAGI2, MAPKBP1, MBTPS2, MCM5, MKKS, MKS1, MMACHC, MNX1, MOCOS, MOCS1, MUC1, MYCN, MYH9, MYO1E, NAA10, NCAPG2, NDUFAF3, NDUFB8, NDUFS2, NEK1, NEK8, NEU1, NFIA, NIPBL, NOS1AP, NOTCH2, NPHP1, NPHP3, NPHP4, NPHS1, NPHS2, NR3C2, NRIP1, NSDHL, NUP107, NUP133, NUP160, NUP205, NUP85, NUP93, NXF5, NXN, OCRL, OFD1, OPLAH, OSGEP, PAX2, PAX8, PBX1, PCBD1, PCSK5, PDE6D, PDSS1, PDSS2, PEX1, PEX10, PEX11B, PEX12, PEX13, PEX14, PEX16, PEX19, PEX2, PEX26, PEX3, PEX5, PEX6, PGM3, PHEX, PHGDH, PIBF1, PIEZO2, PIGN, PIGT, PKD1, PKD2, PKHD1,

PLA2R1, PLCE1, PMM2, PODXL, PORCN, PRKCD, PRKCSH, PRPS1, PTEN, PTPN22, PTPRO, PUF60, RAD21, RAI1, RARRES1, REN, RERE, RET, RFWD3, RMND1, RNU4ATAC, ROBO2, ROR2, RPGRIP1L, RPL26, SALL1, SALL4, SARS2, SCARB2, SCNN1A, SCNN1B, SCNN1G, SDCCAG8, SEC61A1, SEC61B, SEC63, SEMA3E, SF3B4, SGPL1, SI, SIX1, SIX2, SIX5, SLC12A1, SLC12A2, SLC12A3, SLC12A7, SLC22A12, SLC26A1, SLC26A7, SLC2A2, SLC2A9, SLC34A1, SLC34A3, SLC36A2, SLC3A1, SLC41A1, SLC4A1, SLC4A2, SLC4A4, SLC5A1, SLC5A2, SLC6A19, SLC6A20, SLC7A7, SLC7A9, SLC9A3R1, SLIT2, SMARCAL1, SNRPB, SON, SOX11, SOX17, SPRY2, SRGAP1, STAT1, STAT4, STK11, STRA6, STS, SUFU, SYNPO, TBC1D24, TBC1D8B, TBX18, TBX4, TBXT, TCTN1, TCTN2, TCTN3, TFAP2A, THBD, THOC6, TMEM107, TMEM138, TMEM216, TMEM231, TMEM237, TMEM260, TMEM67, TNFSF4, TNIP1, TNS2, TNXB, TP53RK, TP63, TPRKB, TRAF3IP1, TRAP1, TREX1, TRIM32, TRIP11, TRPC6, TRPM6, TRPS1, TRRAP, TSC1, TSC2, TTC21B, TTC37, TTC8, TXNDC15, TXNL4A, UMOD, UPK3A, USP8, VANGL1, VANGL2, VDR, VHL, VIPAS39, VPS33B, VTN, WDPCP, WDR19, WDR34, WDR35, WDR60, WDR72, WDR73, WFS1, WNK1, WNK4, WNT3, WNT4, WNT5A, WNT7A, WT1, XDH, XPNPEP3, XPO5, XRCC2, XRCC4, ZAP70, ZIC3, ZMPSTE24, ZNF148, ZNF365 and *ZNF423.*

Furthermore, **Inherited NephroKitDx** includes a total of 98 intronic variants of interest, in the following 32 genes.

BBS1, BBS4, CD46, CEP290, CFH, CLCN7, COL4A5, CPLANE1, CPT1A, CUBN, FLCN, FLNA, GLA, HNF1A, IRF5, JAM3, OCRL, PHEX, PKHD1, PMM2, SDCCAG8, SLC12A3, SLC2A9, SNRPB, TMEM107, TSC1, TSC2, TXNL4A, UBA5, UMOD, VHL and *ZAP70.*

Inherited NephroKitDx is based on probe capture technology to capture the target regions of these 529 genes and includes all the necessary reagents for full library preparation. The results yielded by this assay identify variants in coding regions and splice-site variants detected in the genes included in the panel. This information can be used to determine the patient's predisposition to kidney disease.

Inherited NephroKitDx can detect SNVs, INDELs, and CNVs across the 529 study genes. CNV detection is outside the scope of the CE/IVD mark.

Inherited NephroKitDx is intended for *in vitro* diagnosis and is aimed at professionals in molecular biology.

3 Technical characteristics

Inherited NephroKitDx has been validated for Illumina's NextSeq System platform via the analysis of DNA reference samples from the *Coriell Institute* and of clinically relevant samples that have been previously genotyped with other technologies. In said validation, both the specific detection of the variants present in the selected genes and the repeatability and reproducibility have been verified.

Technical specifications:

- Sample type: DNA from peripheral blood.
- Necessary amount of DNA: 50-100 ng.
- Mean coverage: 1200X.
- Coverage: 99.7% of bases covered at read depth of 50X.
- Uniformity: 97.5% of bases covered at >20% of the mean coverage.
- Specificity: >99%.
- Sensitivity: >99%.
- Repeatability: >99%.
- Reproducibility: >99%.

Inherited NephroKitDx is compatible with Illumina's massive sequencing platforms.

Health in Code 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 event 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. 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.
10. The manufacturer assumes no responsibility for any damage or failure of the assay caused by substituting reagents included in the kit for ones not provided by Health in Code.
11. The manufacturer does not guarantee the assay's reproducibility when the user uses reagents that have not been validated by Health in Code but are considered by the user equivalent to those provided in the kit.
12. The manufacturer is not liable for the obtained results when the bioinformatics analysis is carried out on an analysis platform different from Data Genomics.

5 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 24 different indexes distributed in single-use strips.
- Nephropathies Probes Strips: Synthetic biotinylated oligonucleotides complementary to the kit's target regions which allow for the hybridization with said regions and which are later captured via streptavidin magnetic particles due to the biological property of bonding between biotin and streptavidin molecules.
- 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:

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

Table 1. Reagents of box 1 of Inherited NephroKitDx.

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
Reagent Plate	Blue	2 plates	-20 °C
Index strip*	Black	2 strips	-20 °C

Table 2. Reagents of box 2 of Inherited NephroKitDx.

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

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

Table 3. Reagents of box 3 of Inherited NephroKitDx.

"Magnis Empty Consumables" box; box 4 of 4			
Reagents	Color indicator	Quantity	Conservation
Magnis Library Output Strips	Green	1 strip	15–25 °C
QC Strips	Blue	1 strip	15–25 °C
Foil Seals	–	5	15–25 °C
Magnis 96-Well PCR Plate	Blue/Transparent	1 plate	15–25 °C
Magnis Deep-Well HSM Plate	White	1 plate	15–25 °C
Magnis Thermal Cycler Seal	–	1	15–25 °C

Table 4. Reagents in "Magnis Empty Consumables" box; box 4 of 4.

Note: Each kit contains two "Magnis Empty Consumables" boxes, one for each run of 8 samples on 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 mixer (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 Technolgies (cat. no. G9710AA).
- Illumina sequencer (recommended: NextSeq).

Reagents:

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

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

Materials:

- Filter pipette tips (10 µL, 20 µL, 200 µL, and 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

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

7 Assay protocol

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

The steps necessary to carry out the preparation of 8 libraries using **Inherited NephroKitDx** 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 instrument door.
3. Turn on by pressing the power button on the front of the instrument (LED indicator lights will turn on). Wait while the system launches. This may take a few minutes.
4. UV decontamination is recommended prior to each run. In order to do so:

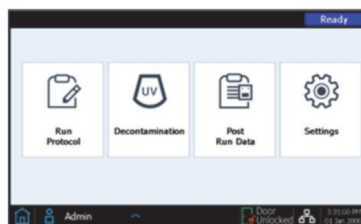


Figure 1. *Magnis NGS Prep* system *Home* screen

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

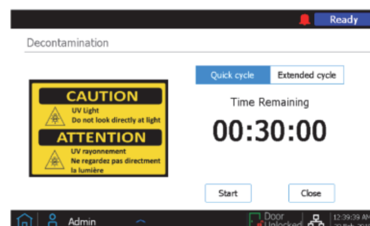


Figure 2. *Magnis NGS Prep Decontamination* screen

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

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

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

7.2 Preparation and fragmentation of the DNA template strand

The steps necessary for the preparation of eight libraries by **Inherited NephroKitDx** 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 using 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 Health in Code's integrated tracking system is to be used (Sample tracking components; Ref. IMG-340), this step must be carried out replacing 1.5 µL of nuclease-free water by the same amount of one tracing reagent per sample.
4. Vortex and quantify again each sample with a fluorometer, such as Qubit.
5. Dilute each DNA sample with nuclease-free water to obtain a total concentration of 100 ng, 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:

- Increase the total volume to 12 µL.
- It is also possible to lower the concentration of total DNA to 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

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* must 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)
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 nuclease-free water to each sample to reach 40 µL of total volume, 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.

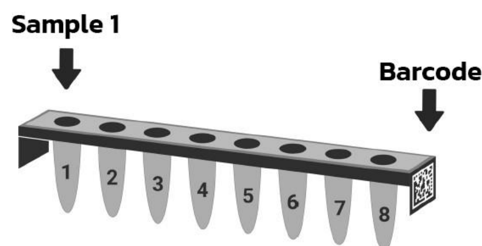


Figure 3. Required orientation of samples in the *Sample Input Strip*.

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
Nephopathies Probe Strip	White strip	-80 °C
"Magnis Empty Consumables" box	N/A	15-25 °C

1. 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 10 more 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 no bubbles have formed 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 10 more seconds. Repeat the rotation/mixing sequence until completed on all four sides of the plate.
   - Spin the plate (still in its cardboard sleeve) in a centrifuge set at 150 x g for 10 seconds. Do not exceed recommended spin times to prevent beads from pelleting.
   - Keep plate in its cardboard sleeve at room temperature to be used on the same day.
3. Preparation of the *Index strip:*
   - Determine and record the set of *indexes* that will be used for the run. The provided strips are inscribed with A1, A2, A3, or A4 on the strip tube end opposite to the barcode. The following table shows the order of the indexes of each strip and their sequence.

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

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

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

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

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

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

4. Immediately before it is used, thaw the *Nephropathies probe strip* in cold. 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. It is recommended that special care be exercised to guarantee the traceability of this reagent both during 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 on the touchscreen, press "*Run Protocol*". The system will lock the instrument door and carry out an *Instrument Health Check* (IHC), which may take several minutes.
2. Once this check is completed, the message *Enter Run Info* will automatically appear on the screen. In the *Protocol* menu, select *SSEL XTHS-RevB-ILM.*
3. Recommended: Mark the *Aliquot sample for QC* checkbox if you want the instrument to take an aliquot of each pre-capture library. This will enable quality control later

on.

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

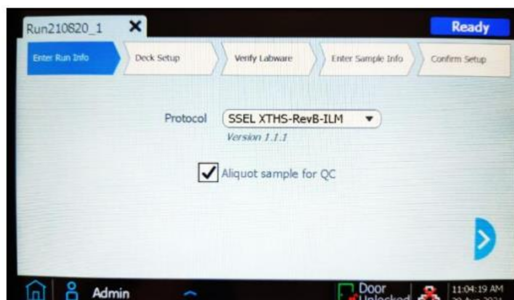


Figure 4. *Magnis NGS Prep* System *Enter Run Info* screen

4. Move on to the next screen.

5. Select appropriate sample type, *High Quality DNA*.

6. Select input DNA amount in the *Input Amount* menu. Even though the options for 10 ng, 50 ng, 100 ng, and 200 ng appear, an amount of 100 ng is recommended to prepare libraries using **Inherited NephroKitDx**. 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 have the same amount of input DNA.

7.4.2 Deck setup

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

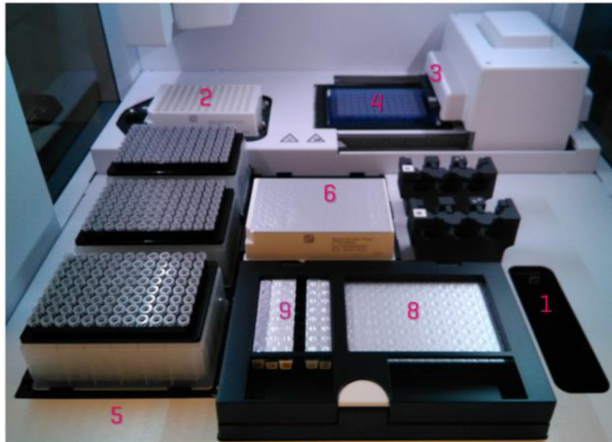
For each deck loading step, the deck position to be loaded will be shaded in blue on the touchscreen. Once each step is completed, move on to the next screen.

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

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

The following figure shows a completely loaded deck with each material numbered from

1 to 10, following the steps displayed by the Magnis instrument. As can be observed, both reagent plates, as well as the five necessary strips, must be sealed when placed inside the instrument.



Step 1/10: Carga del recipiente desechable en el cajón de desechos.
Step 2/10: Carga de Magnis Deep-Well HSM.
Step 3/10: Carga de Magnis Thermal Cycler Seal en la ranura del módulo termociclador.
Step 4/10: Carga de Magnis 96-Well PCR Plate en el bloque del módulo termociclador.
Step 5/10: Carga de 3 cajas de puntas completamente llenas.
Step 6/10: Carga de Beads and Buffers Plate.
Step 7/10: Comprobación de temperatura óptima en el módulo de enfriamiento.
Step 8/10: Carga de Reagent Plate en el módulo de enfriamiento.
Step 9/10: Carga de las tiras de tubos en el módulo de enfriamiento (QC Strip opcional).
Step 10/10: Cerrar la puerta del equipo Magnis.

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

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

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

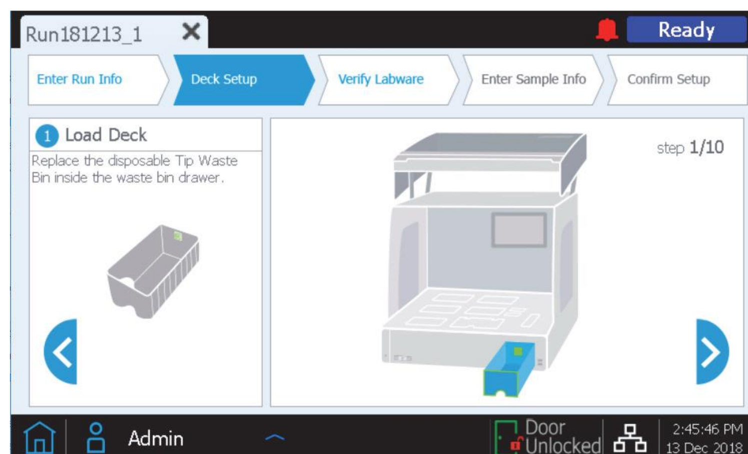


Figure 6. Step 1 of 10 on the *Deck Setup* screen of the Magnis NGS Prep System

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

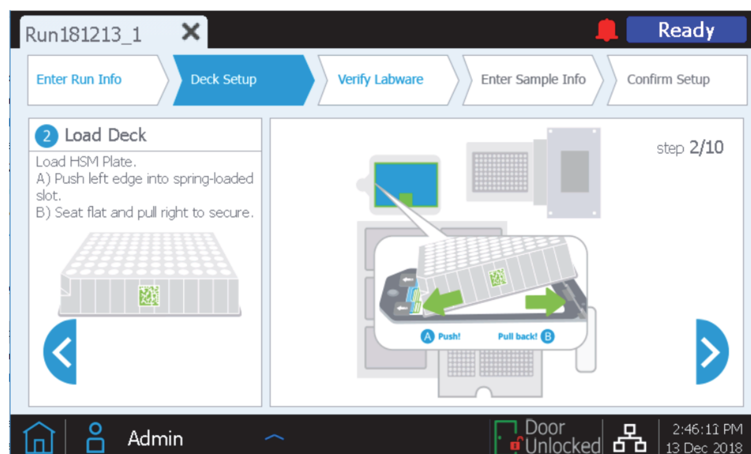


Figure 7. Step 2 of 10 of the *Deck Setup* screen of the Magnis NGS Prep System

3. Place the *Magnis Thermal Cycler Seal* (included in the "*Magnis Empty Consumables*" box) as shown on the instrument's touchscreen. To do this, peel the protective film from the white foam pad located below the metal plate. After removing all the film, insert the Thermal Cycler Seal in the thermal cycler slot, with the *barcode* facing up, and slide until it is fixed in its 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.

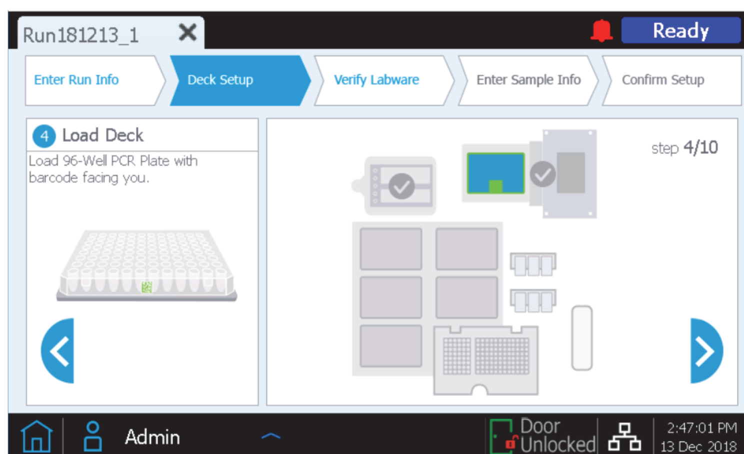


Figure 9. Step 4 of 10 of the *Deck Setup* screen of the Magnis NGS Prep instrument.

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.

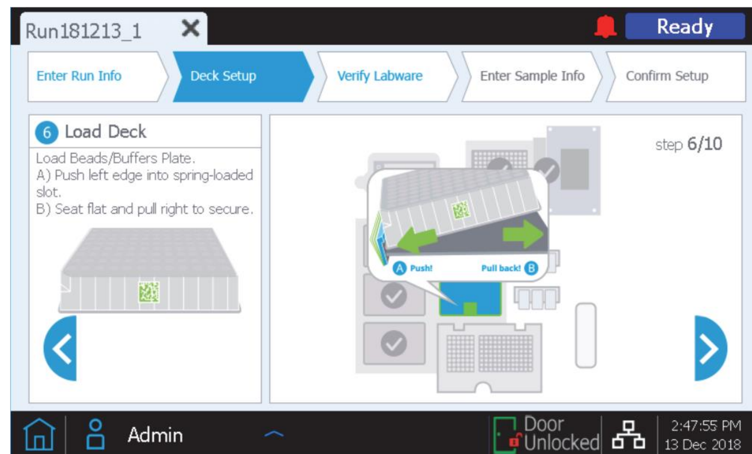


Figure 11. Step 6 of 10 of the *Deck Setup* screen of the Magnis NGS Prep instrument.

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.

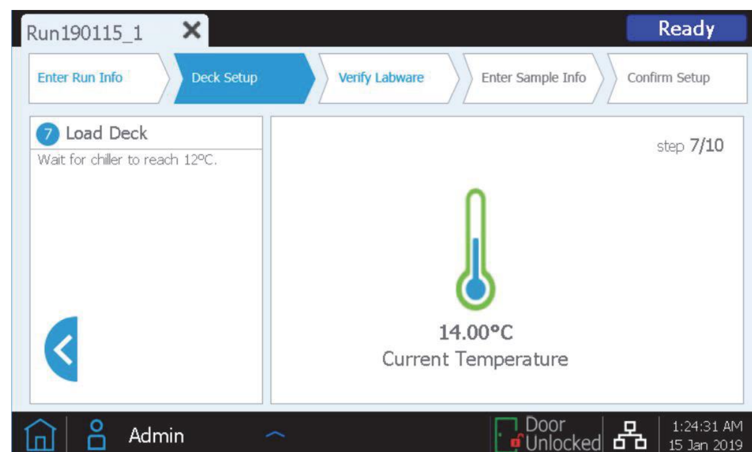


Figure 12. Step 7 of 10 of the *Deck Setup* screen of the Magnis NGS Prep instrument.

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. 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 *Nephropathies Probe Strip* (white strip), prepared in section 7.3 of this document, into the chiller holder position labeled with **P**.
    - Load the *Magnis Library Output Strip* (green strip), included in the “Magnis Empty Consumables” box, into the chiller holder position labeled with **L**.
    - Optional: If the run will include the collection of aliquots of the pre-capture libraries for quality control, as recommended by Health in Code, load the *QC Strip* (blue strip), included in the “Magnis Empty Consumables” box, into the chiller holder position labeled with **Q**.

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

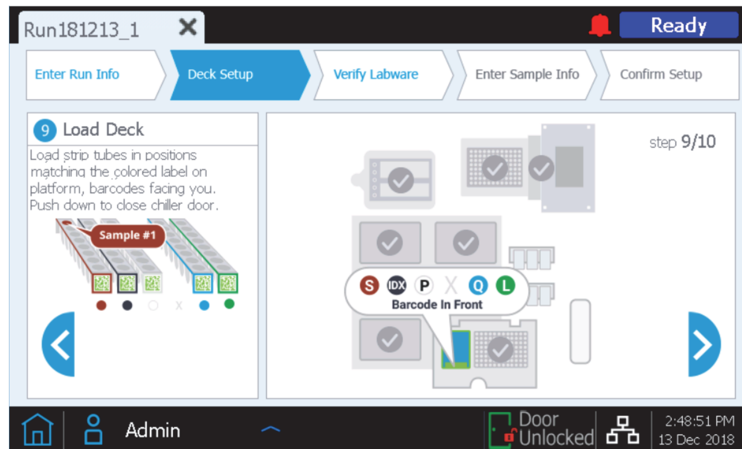
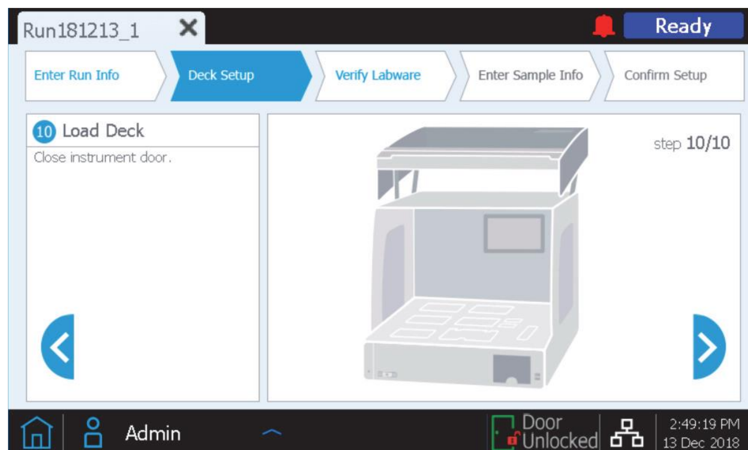


Figure 14. Step 9 of 10 of the *Deck Setup* screen of the Magnis NGS Prep instrument.

10. Close the instrument door.

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



7.4.3 Labware verification

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

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

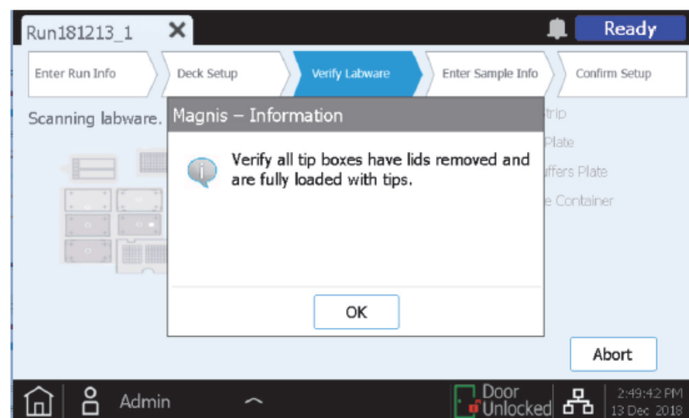


Figure 16. *Verify Labware* pop-up window of the Magnis NGS Prep System.

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

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

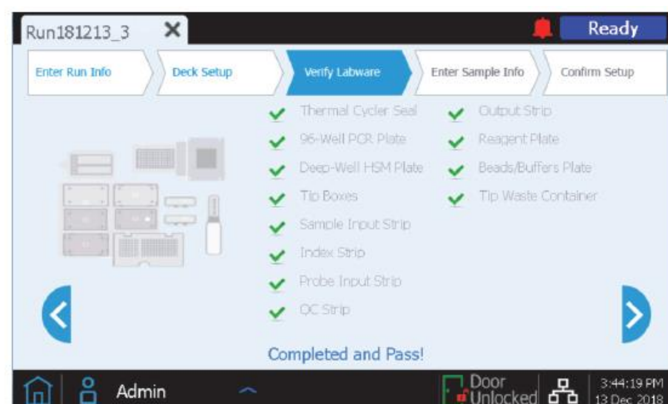


Figure 17. *Verify Labware*screen of the Magnis NGS Prep System after correct labware verification.

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

7.4.4 Entering sample information

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

1. Manual sample assignment:
    - On the *Enter Sample Info* screen, select a specific sample position shown on the touchscreen.

- Use the *Edit Sample ID* tool to enter the desired text.
- Press *Change* to save the new text for the selected sample position.

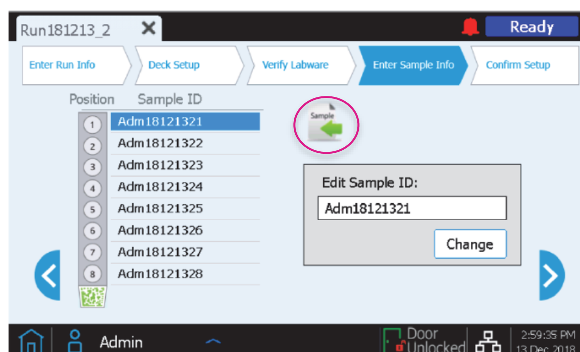


Figure 18. *Edit Sample Info* screen of the Magnis NGS Prep System; the Load samples button is highlighted with a circle.

2. Import of sample assignments using a .csv file:

- Create a .csv (comma-separated value) file containing sample names in the correct order. To enter the new name of the sample, you may use Microsoft Excel and later save the file in .csv format.
- Write the header *sample_id* in cell A1, as shown in Figure 19.

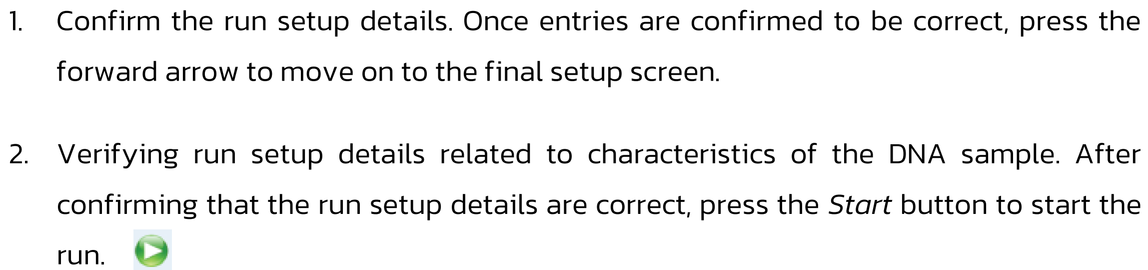
	A
1	sample_id
2	HD18060701
3	HD18060702
4	HD18060703
5	HD18060704
6	HD18060705
7	HD18060706
8	empty1
9	empty2

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

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

7.4.5 Confirm setup and start the run.

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

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

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

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

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

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

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

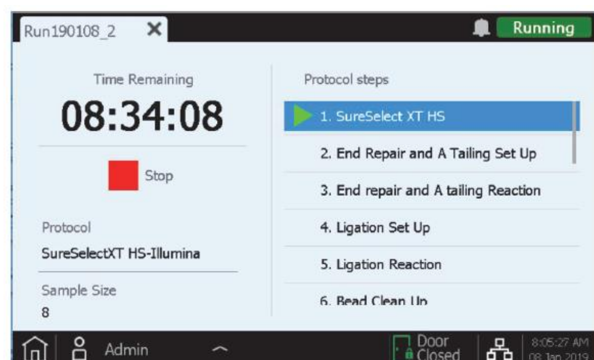


Figure 20. *Running* screen during a run.

7.4.6 Collecting libraries from the instrument

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

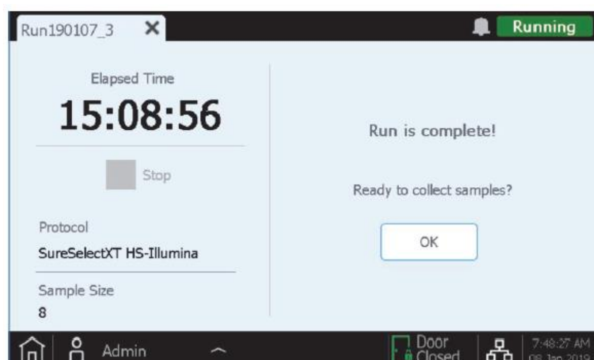


Figure 21. *Running* screen after a run.

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

The chiller module will be held at 12°C for up to 2 hours from the moment 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 24 hours or at -20 °C for longer storage periods.

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

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

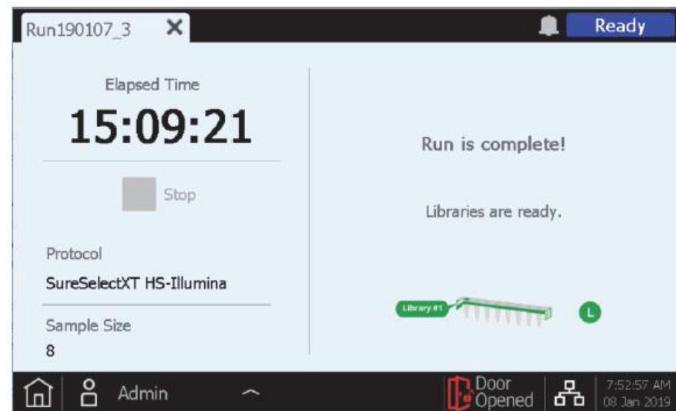


Figure 22. The *Running* screen after a run and after removing the libraries.

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

7.5 Instrument cleaning after a run

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

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

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

7.6 Library validation and quantification

7.6.1 Optional quality control of the pre-capture library

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

After adding 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 300-350 bp should be obtained (Figure 23). In case unexpected sizes are obtained, please review the protocol or contact Health in Code's technical support team.

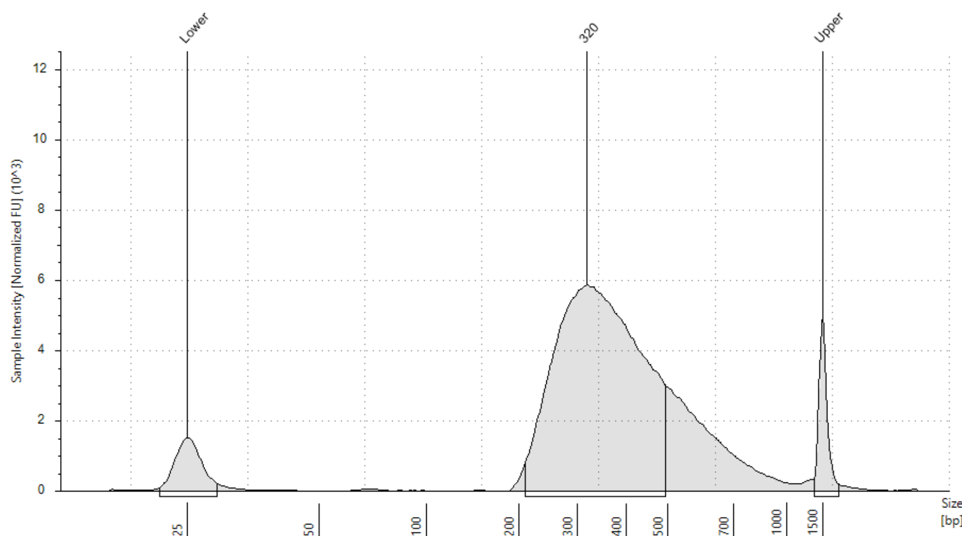


Figure 23. Expected result after analyzing pre-capture library size with TapeStation 2200.

To determine DNA concentration, the peak area corresponding to the expected library size must be integrated. The amount of library DNA obtained will vary depending on the concentration of input DNA, ranging from 30 to 100 ng/µL. The overall pre-capture library yield can be calculated as the amount of DNA in 1 µL of the reconstituted QC sample x 36 (this value includes dilution adjustments).

7.6.2 Quality control of the post-capture library

Prior to pooling the libraries for multiplexed sequencing, it is necessary to analyze the quantity and quality of each one 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™ assay tubes (cat. no. Q32856) by Invitrogen.

The concentration of post-capture libraries will vary between 2 and 10 ng/µL.

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

The expected mean fragment size ranges from 325 to 370 bp. If unexpected sizes are obtained, review the protocol and pre-capture library quality control, carefully read section 9, or contact Health in Code's technical support team.

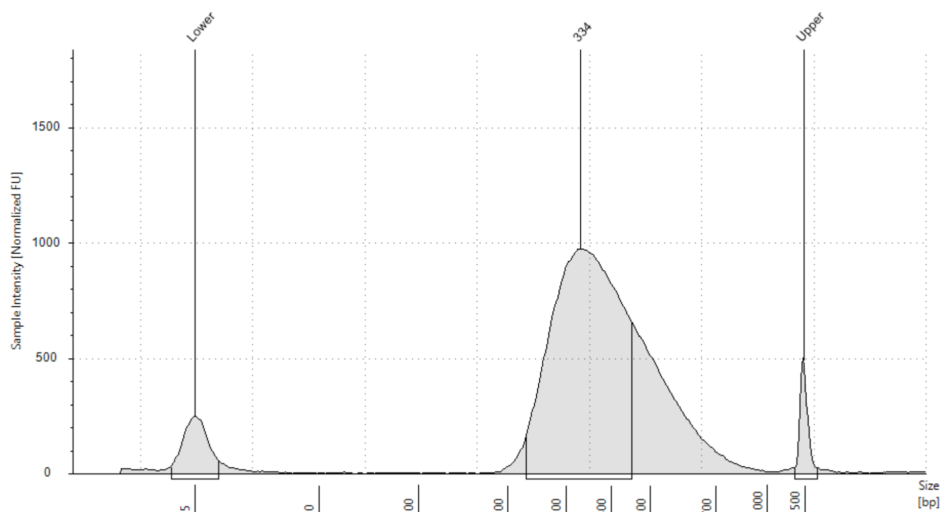


Figure 24. Expected result after the analysis of post-capture library size with TapeStation 2200

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

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

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

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 24 hours or at -20 °C for longer storage periods.

7.7 Library denaturation

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

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 denaturation protocol for libraries 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 1,222 µL of HT1.
9. To this mix add 1.2 5 µL of denatured PhiX control diluted to 20 pM. At this point, the library should be diluted to 1.2 pM.
10. Load the entire volume contained in the 1.5 mL tube into the cartridge.

The following table specifies the maximum number of samples per run, depending on the sequencing kit used, to guarantee a minimum number of *PF clusters* of approximately 20 millions per sample:

NextSeq Reagents Kit	Maximum no. of samples
NextSeq 500/550 Mid Output v2.5 kit (300 cycles) Ref: 20024905.	8
NextSeq 500/550 High Output v2.5 kit (300 cycles) Ref: 20024908.	24

Table 8. NextSeq Illumina kit and maximum number of samples to be analyzed with Inherited NephroKitDx.

7.8 Configuration of the NextSeq platform

1. Configure the platform by executing the independent mode ("*Standalone*"), since *BaseSpace* currently does not admit the sequencing of the "*molecular barcode*" as an *index*.
2. Follow the equipment's loading instructions.
3. When the loading has finished, the run configuration will appear on the screen. Select the following parameters:
    - *Read Type: Paired End.*
    - *Cycles: Read 1: 150*
      Read 2: 150
      Index 1 (i7): 8

8 Analysis of results

Bioinformatic analysis of the results is done through an analysis pipeline designed especially for **Inhereted NephroKitDx**, through the Data Genomics platform. To access this tool, visit www.datagenomics.es.

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

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

8.1 Request for analysis

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

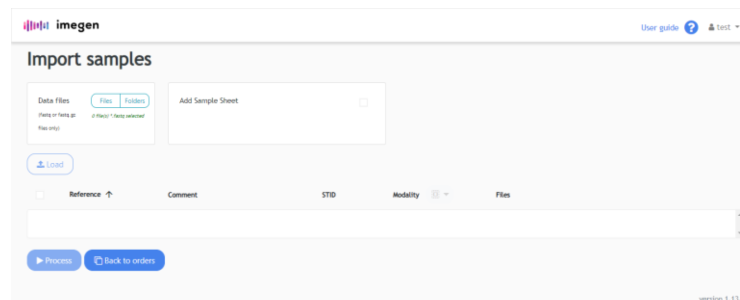


Figure 25. Screen for importing the fastq files and the sample sheet and commence the analysis request.

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

3. 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.

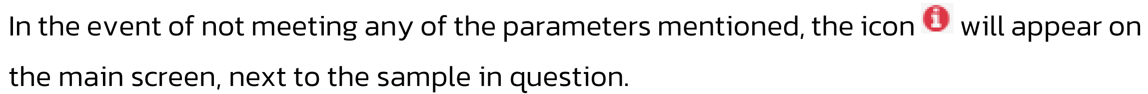
By clicking on the sample, you will access a window where you can enter and save certain characteristics of each sample, such as received date, clinical indications, etc.

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: alignment files (bam and bai), list of variants (vcf),and 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).

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

- FASTQ: The established acceptance criteria are detailed in the *Data Genomics* instructions for use, available at: www.datagenomics.es.
- BAMs:
  - *On-target (%)*
    - *Fail: ≤ 82*
    - *Warn: 82– 83.5*
    - *Pass: ≥ 83.5*
  - *DP50 (%)*
    - *Fail: ≤ 98*
    - *Warn: 98 – 99.3*
    - *Pass: ≥ 99.3*
  - *Uniformity 50% (%):*
    - *Fail: ≤ 81.5*
    - *Warn: 81.5 –84.5*
    - *Pass: ≥ 84.5*

- STIDs: Verification that the tracking reagent obtained matches the expected one (if it has been used), as shown in Figure 26.


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

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

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

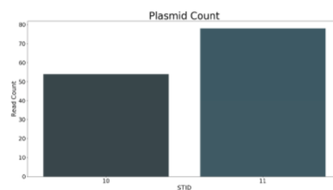


Figure 26. Quality control of the integrated tracking system

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

- Quality variants: **PASS; d200; pseudogenic homology**, **Hotspot, LowMappabilityRegion** (*Fault summary*).
- Depth: ≥ **20X** (*Clean total count*).
- Allele frequency: ≥ **20%** *(Variant Freq)*.
- Distance to the exon: **20** (*Exon distance*).

Once the variants shown by the aforementioned filter have been reviewed and categorized, applying a second filter is recommended to display all variants classified as pathogenic or possibly pathogenic in ClinVar.

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 6 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.

Inherited NephroKitDx 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 Data Genomics, access the results of the "Filtering" request and, specifically, the CNVs tab.

Data Genomics integrates an alert system to warn the user about the reliability of the results based on sample quality parameters. According to these parameters, the 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, all PASS variants will be shown by default. PASS variants are good quality varaitns defined by a p-value ≤0.0005 and a ratio ≤0.7 or ≥1.3. In some cases, PASS variants may be tagged with one or both of the following flags:

- "High Controls Variation": indicates a high coverage variation within the reference samples set used for a specific sample and the target region interrogated.
- "Low Zeta score": a z-score values is a measure of how many standard deviations away from the population mean a raw score is. This flag indicates that the event identified has a low average z-score and, thus, that its standard deviation falls

close to the mean. Specifically, in CNVs analysis the values compared are the depth of coverage obtained for each region.

As aforementioned, CNVs identification use the depth of coverage of the captured reagions as a measure of copy number status. NGS technology holds intrinsic limitations, for instance, pseudogenes and homology regions affect the mappeability of the targeted regions. In this sense, the **Inheirted NephroKitDx** comprises genes with high homology such as *PKD1, PKD2, C4A* and *C4B.* Moreover, a frequent deletion encompassing the genes *CFHR1* and *CFHR3,* which are also included in this panel, has been described in population studies. Taking into account the nature and panel design, true positives CNVs calls from genes affected by frequent CNVs or with low mapeability rates may prompt CNVs tagged with the "High Controls Variation" and "Low Zeta score" flags.

On the other hand, users also have the choice of checking all CNVs called regardless their quality by selecting the "No Pass" option.

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

Data Genomics provides graphic representation (Figure 27) of the sample's coverage profiles vs. those of the reference samples. This plot allows viewing any SNPs and INDELs present in the analyzed regions.

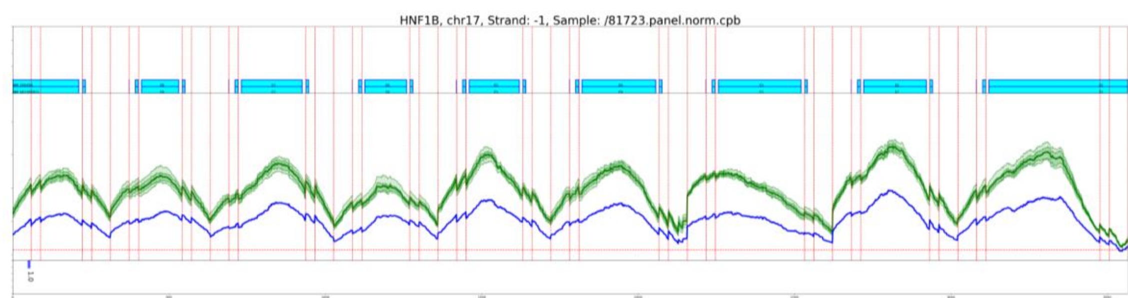


Figure 27. Results of CNVs shown by the Data Genomics platform

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.

8.4 Variant filtering

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

Figure 28. Variant filtering with *Data Genomics*

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 filter characteristics have been selected, they can be saved (Save) and used on the current sample by clicking "Apply".

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

8.4.1 Categorization of SNV, INDEL, CNV, and SV 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 Germline Db column, Health in Code 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 questions regarding results analysis, please contact Health in Code's technical support team.

Category

Category: Benigna

User Modifier

Commentary

Reference

Date

Verified

Verifier user

Date of verification: dd/mm/aaaa --:--

Verification comment

Historical

Cancel | ✔ Accept

Figure 29. Drop-down of the "Category" column.

9 Troubleshooting

A list of possible unexpected results throughout the library preparation and sequencing protocol using **Inherited NephroKitDx** 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 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* teachpoint 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 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 shouldbe restarted after a failed IHC, following the steps below:

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

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

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

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

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

    Verify that the labware is present at the required deck position and oriented correctly (review section 7 of this document for complete deck loading steps). Should there be any positioning errors, correct them and repeat the *Verify Labware step.* If the failed components are present and correctly positioned, visually inspect the barcode for integrity. For successful scanning, barcodes

must be free of scratches, smudges, condensation, obstruction by foil seals, 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.

- **Library fragment size is larger than expected in electropherograms:**

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

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

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

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

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

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

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

10 Limitations

10.1 Analytical

- The employed technique cannot distinguish between regions with high sequence homology, such as homologous genes, pseudogenes, etc., which can lead to false positives or negatives. A list of pseudogenic regions can be found in Table 9. In the analysis of results, the label "Pseudogenic_homology" will appear in the column "Fault summary" when a variant is detected in a region of homology with pseudogenes.

Chromosome	Initial position	Final position	Gene	Exon	Reference sequence
6	31976379	31976502	*CYP21A1P*	EX8	NR_040090.1
6	31976379	31976502	*TNXA*	EX13	NR_001284.2
6	31976801	31976986	*TNXA*	EX12	NR_001284.2
6	31977042	31977225	*TNXA*	EX11	NR_001284.2
6	31977296	31977414	*TNXA*	EX10	NR_001284.2
6	31977486	31977659	*TNXA*	EX9	NR_001284.2
6	31977730	31977884	*TNXA*	EX8	NR_001284.2
6	31977982	31978134	*TNXA*	EX7	NR_001284.2
6	31978206	31978371	*TNXA*	EX6	NR_001284.2
6	31978468	31978610	*TNXA*	EX5	NR_001284.2
6	31978750	31978825	*TNXA*	EX4	NR_001284.2
6	31978918	31979062	*TNXA*	EX3	NR_001284.2
6	31979292	31979649	*TNXA*	EX2	NR_001284.2
6	31979917	31980152	*TNXA*	EX1	NR_001284.2
6	32009114	32009237	*CYP21A2*	EX10	NM_000500.9
6	32009114	32009237	*TNXB*	EX44	NM_019105.8
6	32009536	32009721	*TNXB*	EX43	NM_019105.8
6	32009777	32009960	*TNXB*	EX42	NM_019105.8
6	32010031	32010149	*TNXB*	EX41	NM_019105.8
6	32010296	32010393	*TNXB*	EX40	NM_019105.8
6	32010464	32010618	*TNXB*	EX39	NM_019105.8
6	32010716	32010868	*TNXB*	EX38	NM_019105.8
6	32010940	32011105	*TNXB*	EX37	NM_019105.8
6	32011202	32011343	*TNXB*	EX36	NM_019105.8
6	32011782	32011916	*TNXB*	EX34	NM_019105.8
6	32012146	32012503	*TNXB*	EX33	NM_019105.8
6	32012771	32012846	*TNXB*	EX32	NM_019105.8

Chromosome	Initial position	Final position	Gene	Exon	Reference sequence
6	32012941	32013113	*TNXB*	EX32	NM_019105.8
16	2151985	2151987	*PKD1*	IN26	NM_000296.4
16	2152226	2152307	*PKD1*	EX26	NM_000296.4
16	2167646	2167996	*PKD1*	EX6	NM_000296.4
16	75579261	75579403	*TMEM231*	EX3	NM_001077416.2
16	75579712	75579862	*TMEM231*	EX2	NM_001077416.2

Table 9. List of pseudogenic regions *IN=Intron

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

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

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

Inherited NephroKitDx has been validated using the following massive sequencing platform

- NextSeq System (Illumina)

This kit is compatible with Illumina's massive sequencing platforms only. 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

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

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

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

10.4 Bioinformatics analysis platform

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

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

10.5 Product stability

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