Instructions for use

Inherited NephroKitDx

Ref. IMG-370

CE IVD

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All the products marketed by Health in Code, S.L. undergo rigorous quality control. Inherited NephroKitDx has passed all internal validation tests, which guarantee the reliability and reproducibility of each batch manufactured.

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

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Modifications to the instructions for use (IFU)		
Version 09	DEC 2023	Revision and update of section "3. Technical characteristics".
Version 08	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"*. Correction of index number (section 5) Change of *"Sample input strip"* material from box 1 to 4 (section 5). Update of sections 8.2 and 8.4 to clarify how filtering works.
Version 07	OCT 2022	Update of sections 3, 5 and 10. Update of quality control for pre-capture and post-capture libraries (sections 7.6.1 and 7.6.2).
Version 06	OCT 2022	Change of manufacturer identification: from Imegen to HEALTH IN CODE, S.L and update of variant filtering (section 8.2)
Version 05	JUL 2022	Update of variant filtering (section 8.3)
Version 04	JUL 2022	Update of sections 5 and 7.2.1.
Version 03	JUN 2022	Modification of fragmentation program
Version 02	MAY 2022	Update with product CE-IVD marking

index

01 **General information** 4

02 **Intended use** 5

03 **Technical characteristics** 7

04 **Safety warnings and precautions** 8

05 **Kit contents and storage conditions** 9

06 **Equipment, reagents and material not included in the kit** 11

07 **Assay protocol** 13

- 07.1 | Preparing the Magnis instrument to execute a protocol 13
- 07.2 | Preparing and fragmenting template DNA 14
- 07.3 | Preparing the reagents and consumables used in the Magnis system 16
- 07.4 | Executing the library preparation protocol 18
- 07.5 | Cleaning the equipment after an assay 26
- 07.6 | Validating and quantifying libraries 27
- 07.7 | Denaturing libraries 29
- 07.8 | Setting up the NextSeq platform 30

08 **Analysis of results** 31

- 08.1 | Requesting an analysis 31
- 08.2 | Managing requests 32
- 08.3 | Analyzing large rearrangements (CNVs) 33
- 08.4 | Variant filtering 35

09 **Troubleshooting** 37

10 **Limitations** 40

- 10.1 | Analytics 40
- 10.2 | Equipment 41
- 10.3 | Reagents 42
- 10.4 | Bioinformatic analysis platform 42
- 10.5 | Product stability 42

01 General information

Renal diseases comprise a wide spectrum of diseases of diverse origins, diagnoses and prognoses that affect millions of people all over the globe, and are currently among the causes of death with the greatest impact. In Spain, it is estimated that the prevalence of chronic kidney disease affects one in seven individuals, so these diseases are a matter of great interest to the country's public health system. Within the realm of renal diseases, hereditary nephropathies account for more than 5% of all known genetic diseases. Furthermore, around 10% of nephropathies requiring transplantation in adults, and practically all of those in children, are hereditary. Their prevalence is extremely variable, ranging from some 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 the pathogenic variants, genetic testing is of great clinical utility, facilitates genetic counseling and, on numerous occasions, contributes to disease prognosis.

Thanks to the latest breakthroughs in the field of genetics and the use of high-throughput sequencing technologies, also known as next-generation sequencing (NGS), clinicians and researchers in the field of nephrology have a test—necessary in some contexts—for accurate diagnosis of the disease, optimization of patient management, prognosis of disease progression and impact on genetic counseling at their disposal.

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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*

02 Intended use

Inherited NephroKitDx has been designed to analyze the sequences of the coding regions of 529 genes that have been selected for the study of most renal diseases. The diseases addressed by sequencing this panel would be 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 diseases of the kidney.
- **Cystic kidney diseases**: Polycystic kidney disease, nephronophthisis, syndromes with the development of renal and/or hepatic cysts.
- **CAKUT and other syndromes with congenital anomalies of the kidneys, mostly corresponding to diseases in children**.
- **Hereditary kidney cancer**.

This kit serves as a tool to help healthcare professionals in their approach to hereditary nephropathies, including genes associated with the development of glomerular diseases, tubulointerstitial diseases, cystic diseases and polymalformative syndromes of the kidney, based on the current guidelines for diagnosis and management of hereditary nephropathies, recommendations of scientific societies and relevant scientific literature.

The 529 genes selected for the study of renal diseases addressed by sequencing in this panel are listed below:

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, ATP6VOA4, 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, ZNF423

In addition, 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, ZAP70

Inherited NephroKitDx is based on probe capture technology to capture target regions of these 529 genes including all the necessary reagents for the complete preparation of the libraries. The results obtained in this assay will provide the clinician with the variants in the coding regions and the splice variants of the genes included in the panel. This information will determine the patient's susceptibility to renal disease.

By using Inherited NephroKitDx, SNVs, INDELs and CNVs can be detected across the 529 genes included. CNV detection will fall outside the scope of CE/IVD marking.

Inherited NephroKitDx is for *in vitro* diagnostic use only and is intended for professionals in the molecular biology sector.

03 Technical characteristics

Inherited NephroKitDx has been validated on Illumina's *NextSeq 500/550 System* platform, through the analysis of reference DNA samples from the *Coriell Institute* and clinically relevant samples previously genotyped with other technologies. This validation process has been used to verify specific detection of the variants in the selected genes, as well as to determine repeatability and reproducibility.

Technical specifications:

- Type of sample: DNA from peripheral blood.
- Required quantity of DNA: 50-100 ng.
- Average coverage: 1200X.
- Coverage: 99.7% of the bases covered at a depth of 50X.
- Uniformity: 97.5% of the bases covered at > 20% of the average coverage.
- Specificity: > 99 %
- Sensitivity: > 99 %
- Repeatability: > 99 %
- Reproducibility: > 99 %

04 Warnings and precautions

- We recommend strictly following the instructions in this manual, especially regarding the handling and storage conditions for the reagents.
- Do not pipette by mouth.
- Do not smoke, eat, drink or apply cosmetics in the areas where kits and samples are handled.
- Any skin conditions, as well as cuts, abrasions and other skin lesions should be properly protected.
- Do not pour reagent residues into the drinking water system. It is recommended to use the waste containers set out by the legal regulations and to manage them via an authorized waste manager.
- In the case of accidental spillage of any of the reagents, avoid contact with skin, eyes and mucous membranes and clean with plenty of water.
- Material safety data sheets (MSDS) for all hazardous components contained in this kit are available upon request.
- This product requires the handling of samples and materials of human origin. We recommend all human-sourced materials be considered potentially infectious and handled in accordance with the OSHA Biosafety Level 2 standard for bloodborne pathogens or other relevant biosafety practices should be used for materials that contain or are suspected of containing infectious agents.
- This kit has been validated with specific equipment and under specific conditions that may vary significantly in other laboratories. It is therefore recommended that each laboratory perform an internal validation when using the kit for the first time.
- The manufacturer is not responsible for the assay failing to work properly when the reagents included in the kit are replaced by other reagents not supplied by Health in Code, S.L.
- The manufacturer does not guarantee the reproducibility of the assay when the user includes reagents not validated by Health in Code S.L., considering them equivalent to those supplied in the kit.
- The manufacturer is not responsible for the results obtained when the bioinformatic analysis is performed on an analysis platform other than **Data Genomics**.

05 Kit contents and storage conditions

This kit contains enough reagents to prepare 16 libraries. The list of reagents included in the kit is as follows:

- ***Fragmentation Buffer:*** Buffer required for DNA fragmentation before preparation of the NGS libraries.
- ***Fragmentation Enzyme:*** Enzyme required for DNA fragmentation and preparation for adapter ligation.
- ***Elution Buffer:*** Buffer for eluting the DNA.
- ***Reagent Plate:*** Plate with all the reagents required to perform DNA fragment end repair reactions, Illumina adapter ligation, and amplifications carried out during the library preparation protocol.
- ***Beads and Buffers Plate:*** Plate with the magnetic particles and wash buffers necessary to perform the required capture and purification processes during the library preparation protocol.
- ***Index Strip:*** Oligonucleotides with a unique sequence of 8 nucleotides that are compatible with Illumina adapters. They are necessary for marking the libraries of each sample to produce a unique combination that will enable analysis after sequencing. The kit includes 16 different indexes on various single-use strips.
- ***Nephropathies Probes Strips:*** Biotinylated synthetic oligonucleotides complementary to the target regions of the kit, which allow hybridization with these regions and are then captured with streptavidin magnetic particles thanks to the biological binding properties between the biotin-streptavidin molecules.
- ***Sample Input Strips:*** Strips with eight empty wells to hold the sample DNA.
- ***Magnis Library Output Strips, QC Strips and Foil Seals:*** 8-well strips for collecting the generated libraries; strips for collecting the pre-capture libraries, which can be used to perform an optional quality check; and seals for the well strips included in the kit.
- ***Magnis 96-Well PCR Plate:*** Plate where the amplification reactions will take place.
- ***Magnis Deep-Well HSM Plate:*** Plate for the capture and the purifications required in the library preparation protocol.
- ***Magnis Thermal Cycler Seal:*** Seal for the for 96-well plate.
- ***Magnis Tip Waste Bin:*** Container for disposing of the tips used during the protocol.

All the components contained in the kit are listed below:

Box 1 of 4			
Reagents	Support color	Quantity	Storage
Beads and buffer plates	White	2 plates	4°C
Elution Buffer	Green pad	3 x 1 mL	4°C

Table 1. Reagents from Inherited NephroKitDx box 1

Box 2 of 4			
Reagents	Support color	Quantity	Storage
Fragmentation Buffer	Green cap	32 µL	-20°C
Fragmentation Enzyme	White cap	16 µL	-20°C
Reagent Plate	Black/White	2 plates	-20°C
Index Strip*	Black	2 strips	-20°C

Table 2. Reagents from Inherited NephroKitDx box 2

NOTE: Each kit will include two of the four possible index combinations: A1, A2, A3 and A4.

Box 3 of 4			
Reagents	Support color	Quantity	Storage
Nephropathies Probes strips	White	2 strips	-80°C

Table 3. Reagents from Inherited NephroKitDx box 3

Box 4 of 4			
Reagents	Support color	Quantity	Storage
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

Table 4. Reagents from Inherited NephroKitDx box 4

NOTE: Each kit shall include two units of Box 4, one for each 8-sample run with the Magnis equipment.

06 Equipment, reagents and materials not supplied

Equipment:

- Thermal cycler with temperature adjustable lid
- 10 µL, 20 µL, 200 µL and 1000 µL micropipettes
- Vortex (compatible with 1.5-mL tubes; with adjustable speed range of 300 to 3000 rpm)
- Centrifuge (compatible with 1.5-mL tubes and 0.2-mL strips; with minimum adjustable speed of 1000 rpm)
- Plate centrifuge
- Fluorometer (recommended: *Qubit*; Thermo Fisher Scientific)
- Fragment analyzer (optional: *TapeStation System* from Agilent Technologies; *LabChip GX Touch/GXII Touch* from PerkinElmer)
- *Magnis NGS Prep System* automated library preparation equipment from Agilent Technologies (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 to perform NGS sequencing.

Materials:

- Pipette tips with filter (10 µL, 20 µL, 200 µL and 1000 µL)
- Sterile tips with filters, compatible with *Magnis NGS Prep System* (Ref: 19477-022; Agilent)
- 1.5 mL sterile tubes
- 0.2-mL sterile 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 for use in combination with ***Imegen®-Sample Tracking Component*** kits (REF: IMG-340), which enable each sample to be monitored from the DNA dilution to the bioinformatic analysis of the results with an integrated sample identification system. This enables us to ensure traceability of samples throughout the protocol. These references are available upon request.

07 Assay protocol

The reagents included in Inherited NephroKitDx for use by the automated *Magnis NGS Prep System* equipment are supplied pre-dosed for producing 16 libraries over two assays with 8 libraries each, to optimize the use of the equipment.

The steps for preparing 8 libraries with Inherited NephroKitDx are listed below.

07.1 | Preparing the Magnis instrument to execute a protocol

01 Make sure that there is no material from previous assays remaining on the instrument deck, as this could interfere with the startup and configuration processes.

02 Close the instrument door.

03 Turn on the instrument by pressing the power button on the front of the device (the LEDs on the instrument will light up). Wait for the system to perform a series of startup actions, which could take a few minutes.

04 Before each assay, UV decontamination, as described below, is recommended:

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

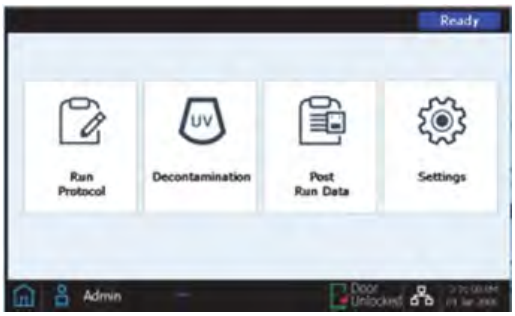


Figure 1. Home screen of the Magnis NGS Prep system

- On the *Decontamination* screen, press *Quick cycle*, then *Start* (the LEDs will turn off during UV decontamination to allow UV emission).

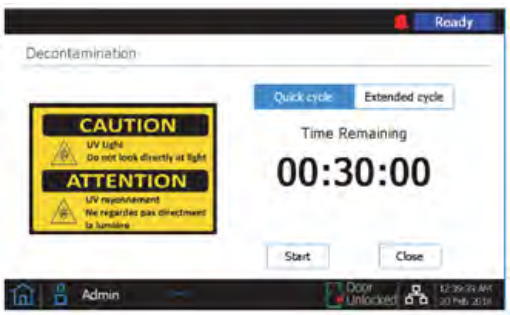


Figure 2. Decontamination screen of the Magnis NGS Prep system

WARNING: Do not gaze directly at the UV light while the decontamination process is active.

NOTE: While the 30-minute decontamination process is running, you can continue with the protocol.

05 Once the decontamination cycle has finished (indicated by blue LEDs on the instrument), press *Close* to return to the *Home* screen.

07.2 | Preparing and fragmenting DNA template strand

The steps for preparing and fragmenting 8 samples with Inherited NephroKitDx are listed below.

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

07.2.1 | Quantifying and diluting DNA samples

01 Thaw DNA samples at room temperature.

02 Stir and quantify the DNA samples using a fluorometer, such as a *Qubit* device.

03 Dilute each DNA sample to 30 ng/µL using nuclease-free water to a final volume of 25 µL.

Optional: If the integrated traceability system from Health in Code, S.L. (*Sample Tracking Components*; Ref. IMG-340) will be used, perform this step replacing 1.5 µL of nuclease-free water with the same amount of a unique monitoring reagent for each sample.

04 Stir in a vortex mixer and quantify each sample again using a fluorometer, such as a *Qubit* device.

05 Dilute each DNA sample with nuclease-free water until you obtain a total amount of 100 ng, at a final volume of 7 µL, in a strip with 0.2-mL wells.

If you do not have a total of 100 ng at a volume of 7 µL, choose one of the following options:

- Double the final volume, also doubling the other reagents used in the fragmentation.
- Lower the total concentration to a total of 50 ng. If this option is chosen, it should be applied to all samples of the run that do not reach a total of 100 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

Both options have disadvantages, which should be evaluated before making a choice. Doubling the final volume involves using more reagents than are included in the kit, so additional fragmentation reagents will be

required. 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.

06 Stir all the dilutions in a vortex mixer, spin them and keep them cold until they are used in the following section.

07.2.2 | DNA fragmentation

In this section, DNA is fragmented enzymatically, in order to obtain DNA fragments with a size between 200 and 300 bp.

Reagents to be used in this section:

Reagent	Color	Storage
Fragmentation Buffer	Green cap	-20°C
Fragmentation Enzyme	White cap	-20°C

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

02 Prepare the appropriate fragmentation mix volume under cold conditions, as described below, stirring each reagent before use. The *Fragmentation Buffer* reagent should be vortexed vigorously, while the *Fragmentation Enzyme* reagent should be mixed by inversion several times. When 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

03 Stir vigorously in a vortex mixer.

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

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

- Lid preheated 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: For this protocol, the lid must be preheated to 100°C. In thermal cyclers with fast ramps, such as the one used in the validation of this protocol, *GeneAmp PCR System 9700* (Thermo Fisher Scientific), it is not necessary to preheat the lid. In all other cases, preheat the lid for a few minutes before beginning the protocol.

06 When the fragmentation program finishes, take the samples out of the thermal cycler, spin them, add nuclease-free water to each sample for a final total volume of 50 µL, transfer the entire volume to a *Sample Input Strip*, seal with the aluminum seals provided, and keep them cold until they are used in the following step.

NOTE: If the fragmentation volume is doubled, this should be taken into account when bringing the final volume up to the total of 50 µL.

NOTE: In the *Magnis NGS Prep System*, the sample should be placed as shown in Figure 3, with Sample 1 loaded in the well farthest from the barcode.

NOTE: Do not add any text or labels that could hide the barcode on the *Sample Input Strip*.

Figure 3. Required orientation of the samples on the Sample Input Strip

07.3 | Preparing the reagents and consumables for use in the Magnis system

Reagents to be used in this section:

Reagent	Color	Storage
Reagent Plate	Blue plate	-20°C
Beads and Buffers Plate	White plate	4°C
Index Strip	Black strip	-20°C
Nephropathies Probe Strip	White strip	-80°C
Box 4	N/A	15-25°C

01 Preparing the *Reagents Plate* reagent:

- ◇ Thaw the plate at room temperature, leaving it inside its white cardboard sleeve.
- ◇ Once the contents of all the wells have thawed, vortex the plate, keeping it inside its cardboard sleeve. Begin by pressing the long side of the plate against the vortex head for 10 seconds. Afterwards, turn the plate 90° and press the short side against the vortex head for an additional 10 seconds. Continue this rotation/mixing sequence on all four sides of the plate.

- Centrifuge the plate wrapped in cardboard at 250 x g for 1 minute.
- Make sure that there are no bubbles at the bottom of the wells in the plate. If there are, repeat the centrifugation process.
- Store the plate, keeping it in its packaging, at cold temperature for later use the same day.

02 Preparing the *Beads and Buffers Plate* reagent:

- Temper for at least 30 minutes before use, keeping it in its white cardboard sleeve.
- Vortex the plate, keeping it in its cardboard sleeve. Begin by pressing the long side of the plate against the vortex head for 10 seconds, Afterwards, turn the plate 90° and press the short side against the vortex head for an additional 10 seconds. Continue this rotation/mixing sequence on all four sides of the plate.
- Centrifuge the plate wrapped in cardboard at 150 x g for 10 seconds. Do not exceed the recommended centrifuging speed and duration as this could cause pelleting of the magnetic particles.
- Keep the plate, in its sleeve, at room temperature for use later the same day.

03 Preparing the *Index Strip* reagent:

- Determine and record the index set to be used in the assay. The supplied strips bear the combination they include—A1, A2, A3 or A4—on the end opposite the barcode. The table below shows the order of the indexes on 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 7. Sequences of the indexes included in the kit.

- Thaw the selected index strip at cold temperature, vortex for 5 seconds and then spin.
- Check the wells in the strip to ensure that the liquid has filled the bottom of the wells, and that there are no bubbles.

IMPORTANT: If the index strip used for an assay has not been recorded, this can be checked using the *Magnis Prep System* on the *Post-Run Data* screen. From this screen, open the *Labware Info* tab and look for the *Index Strip* row. The strip number will be shown as a value from 1 to 12 on the right side of the screen, in the *Index Strip* column. The specific indexes associated with each number from 1 to 12 are shown in the table below.

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

Table 8. Correlation of the indexes between the Post-Run Data screen and the strip inscription

04 Immediately before use, thaw the *Nephropathies Probe Strip* at cold temperature. Vortex for 5 seconds and spin. It is important to make sure that no bubbles have formed at the bottom of the well.

NOTE: The probe is pre-dosed in the first well on the strip, which does not include readable labels showing the specific identity of the probe design. Great care should be taken to ensure traceability of this reagent in storage and during the protocol.

05 Finally, prepare one unit of Box 4 to be used during deck setup.

07.4 | Executing the library preparation protocol

07.4.1 | Initiating the protocol

01 On the Home screen on the touchscreen, press *"Run Protocol"*. The system will lock the instrument door and perform an instrument health check (IHC), which may take several minutes.

02 Once the check has been completed, the *Enter Run Info* screen will appear. On the *Protocol* menu, select *SSEL XTHS-RevB-ILM*.

03 **Recommended**: Tick the *Aliquot sample for QC* verification box to make the system collect an aliquot of each pre-capture library for subsequent quality control analysis.

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

Figure 4. Enter Run Info screen on the Magnis NGS Prep system

04 Move on to the next screen.

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

06 Select the starting DNA amount on the *Input Amount* menu. Although you will see options for 10 ng, 50 ng, 100 ng and 200 ng, when preparing libraries using Inherited NephroKitDx, 100 ng is recommended. Change the amount of DNA if starting with a different amount (minimum of 50 ng).

NOTE: The quality and quantity settings for template DNA will determine the number of cycles performed by the equipment in subsequent amplifications. Therefore, it is important to enter this information correctly and for all the samples to have the same quantity of starting DNA.

07.4.2 | Deck setup

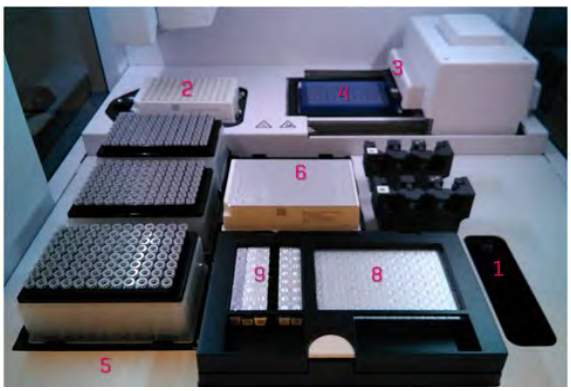
The deck can be set up easily by following the steps indicated on the Magnis touchscreen.

For each deck loading step when loading, the loading position will be shown in blue on the touchscreen. Once each step has been completed, move on to the next screen.

To ensure that the reagents and consumables have been correctly placed in the Magnis instrument, make sure that the barcode for each element is facing the user, i.e. facing the front of the instrument. An exception to this is the *Magnus Thermal Cycler Seal*, whose barcode should be facing backwards, and the 3 tip boxes required, which are not included in the kit and do not have barcodes.

After removing the lid on the full boxes of new tips, it is important to check that the boxes are well secured on the platform.

The figure below shows a fully loaded deck, with each material numbered from 1 to 10, following the steps indicated by the Magnis equipment. As can be seen, the two reagent plates, as well as the five strips required, must be placed in the instrument sealed.



Step 1/10: Load the waste container in the waste bin.
Step 2/10: Load the *Magnis Deep-Well HSM*
Step 3/10: Load the *Magnis Thermal Cycler Seal* in the thermal cycler module slot
Step 4/10: Load the *Magnis 96-Well PCR Plate* in the thermal cycler module block
Step 5/10: Load 3 full tip boxes
Step 6/10: Load the *Beads and Buffers Plate*
Step 7/10: Check the optimal temperature in the cooling module
Step 8/10: Load the *Reagent Plate* in the cooling module
Step 9/10: Load the tube strips in the cooling module (*QC Strip* optional)
Step 10/10: Close the Magnis instrument door

Figure 5. Magnis NGS Prep instrument deck loaded for the assay and quickloading guide.

The setup steps indicated on the Magnis touchscreen are as follows:

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

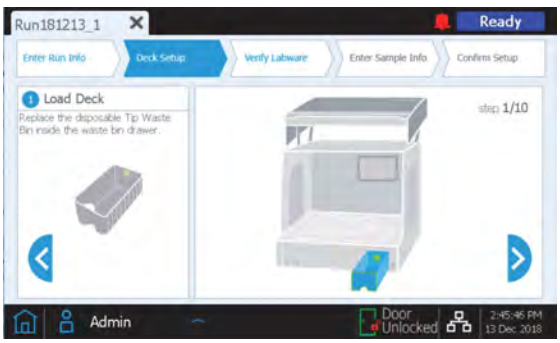


Figure 6. Step 1 of 10 on the Magnis NGS Prep Deck Setup screen

02 Position the *Magnis Deep-Well HSM Plate* (included in Box 4) as shown on the instrument's touchscreen. To do so, first insert the left side of the plate into the spring-loaded slot, and then lower the right side of the plate until it is in line with the platform. Once the plate is lined up, shift it slightly to the right, until it is properly secured inside the holder.

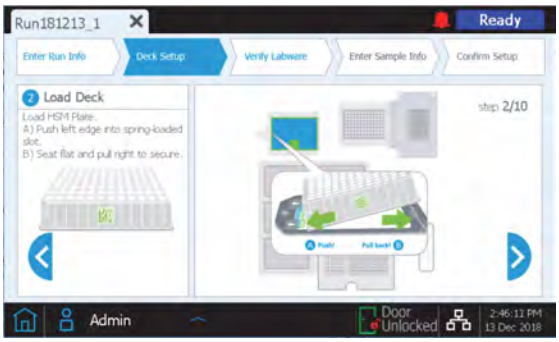


Figure 7. Step 2 of 10 on the Magnis NGS Prep Deck Setup screen

03 Position the *Magnis Thermal Cycler Seal* (included in Box 4) as shown on the instrument's touchscreen. To do so, remove the protective film from the white pad under the metal plate. After removing the entire sheet of film, insert the *Thermal Cycler Seal* into the thermal cycler slot, with the barcode facing up, and slide it in until it clicks into place.

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

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

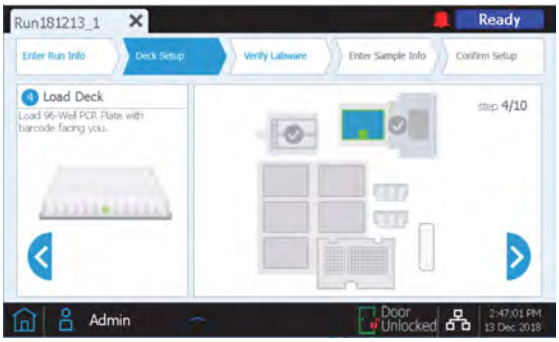


Figure 9. Step 4 of 10 on the Magnis NGS Prep Deck Setup screen

05 Load a full box of new tips in each deck position indicated on the instrument's touchscreen (three boxes in total). After removing the lid, make sure that each box of tips is properly secured on its platform.

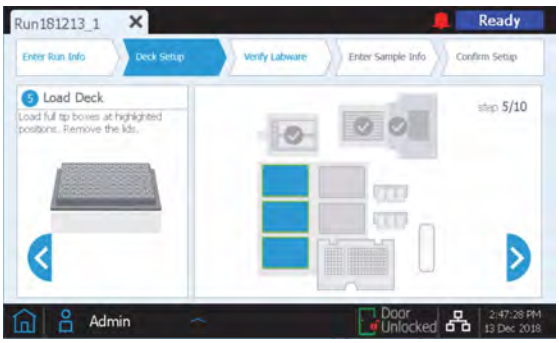


Figure 10. Step 5 of 10 on the Magnis NGS Prep Deck Setup screen

06 Place the *Beads and Buffers Plate* (prepared in section 7.3 of this document). Remove the white cardboard sleeve and then position 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 into the spring-loaded slot, and then lower the right edge of the plate until it is in line with the platform. Once the plate is lined up, shift it slightly to the right, until it is properly secured inside the holder.

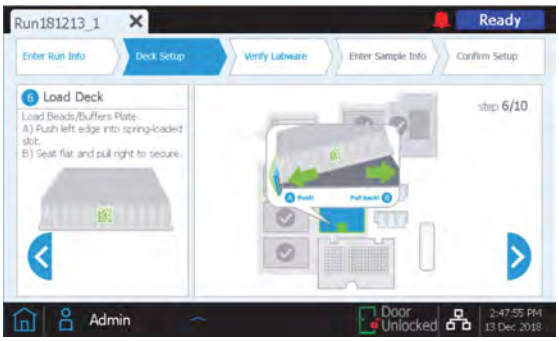


Figure 11. Step 6 of 10 on the Magnis NGS Prep Deck Setup screen

07 Before loading the Magnis instrument, the instrument's cooling module should be at 12°C. If this temperature has not been reached by this step, the touchscreen display will appear as shown in Figure 12. However, if the cooler has already reached the required temperature, this screen will not appear.

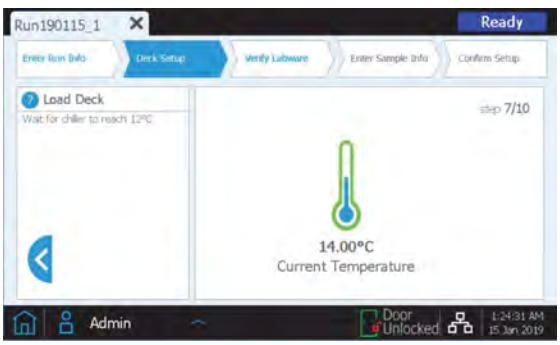


Figure 12. Step 7 of 10 on the Magnis NGS Prep Deck Setup screen

08 Open the cooler module door by pressing 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 cooling module. Remove the white cardboard sleeve and then load the plate as shown on the instrument's touchscreen, with the barcode facing the user. Press down firmly, applying pressure evenly across the length of the plate.

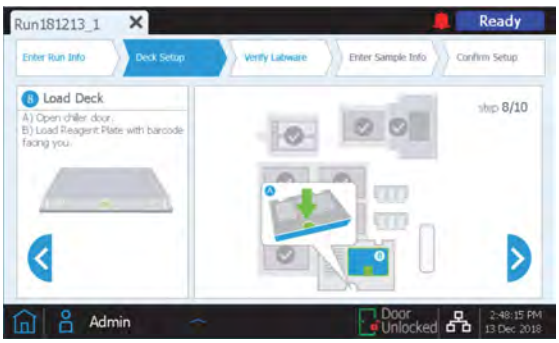


Figure 13. Step 8 of 10 on the Magnis NGS Prep Deck Setup screen

09 Load the tube strips for the assay in the positions indicated in the chiller module, as shown on the touchscreen. Press each strip firmly in place by applying pressure evenly along the edges of the tube strip. Avoid touching or damaging the aluminum seals. The barcodes on all the tube strips should be facing the user.

- Load the *Sample Input Strip* (red strip), with the DNA samples prepared as per section 7.2 of this document, in the chiller holder position labeled with **S**.
- Load the *Index Strip* (black strip), prepared as per section 7.3 of this document, in the chiller holder position labeled with **IDX**.
- Load the *Nephropathies Probe Strip* (white strip), prepared as per section 7.3 of this document, in the chiller holder position labeled with **P**.
- Load the *Magnis Library Output Strip* (green strip), included in Box 4, in the chiller holder position labeled with **L.**
- **Optional**: If aliquots from the pre-capture libraries will be collected during the assay for quality control, as recommended by Health in Code, S.L., load the *QC Strip* (blue strip), included in Box 4, in the chiller holder position labeled with **Q**.

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

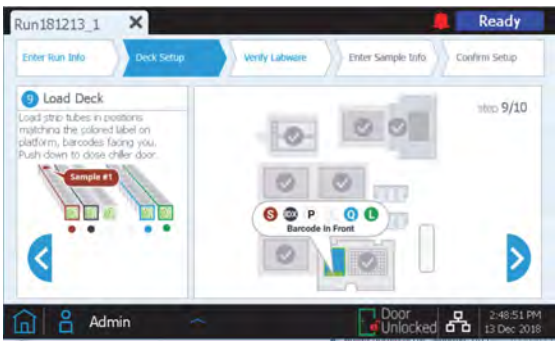


Figure 14. Step 9 of 10 on the Magnis NGS Prep Deck Setup screen

10 Close the instrument door.

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

07.4.3 | Labware verification

Once the equipment has been loaded, complete the *Verify Labware* stage, where the instrument scans the barcode of each component in the unit.

Before initiating the automatic verification, check that the lids have been removed from all the tip boxes and that they are all full, as shown in the figure below. Once this has been checked, press OK to start labware verification.

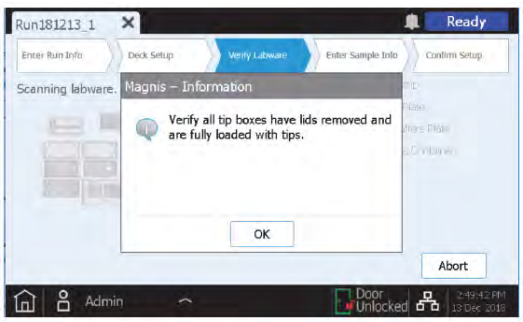


Figure 16. Pop-up window on the Magnis NGS Prep Verify Labware screen.

During labware verification, the instrument will ensure that all the components needed for the assay are present, in the correct position and facing the right direction, and it will also ensure they have not passed their expiration date.

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

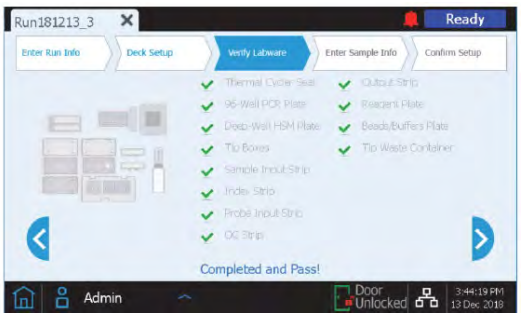


Figure 17. Magnis NGS Prep Verify Labware screen after a correct verification of labware.

The final Verify Labware screen makes it possible to check the probe details. Move on to the next screen.

07.4.4 | Assigning the sample information

The Magnis software will automatically assign a preset *Sample ID* for the position of each sample, which may be replaced with a sample name chosen by the user, using either of the two methods below:

01 Manually assigning sample information:

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

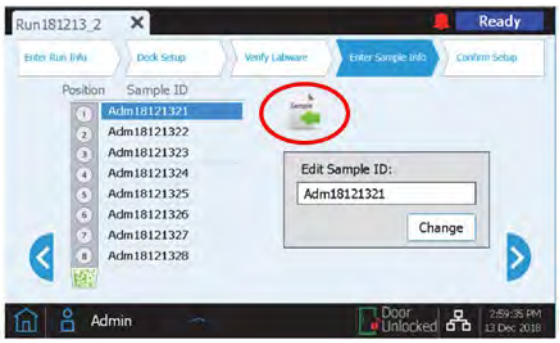


Figure 18. Edit Sample Info screen on Magnis NGS Prep (sample loading button circled in red).

02 Importing sample assignments using a .csv file:

- Create a .csv (comma-separated values) file containing the sample names in the correct order. You can use *Microsoft Excel* to enter the sample names and save in .csv format.
- Enter the heading "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 contents (shown in spreadsheet format) for loading sample assignments.

- Enter the name of each sample in cells A2 to A9. The sample entry file must contain 8 unique sample IDs. If the protocol will be carried out with less than 8 samples, fill in those positions in the file as shown in Figure 19 (empty1 and empty2).
- Save the file in .csv format.
- Download the .csv file onto an unencrypted USB drive and then insert it into one of the ports on the Magnis instrument.
- When setting up the assay, on the *Enter Sample Info* screen, press the sample loading button (circled in red on Figure 18).
- Follow the protocol setup assistant instructions to transfer the sample IDs from the USB drive.

07.4.5 | Confirming the setup and beginning the assay

01 Check the general characteristics of the assay. Once you have confirmed that everything is correct, press the forward arrow to move on to the final setup screen.

02 Check the assay details related to the DNA sample characteristics. After confirming that the setup details are correct, press *Start* button to begin the assay.

> IMPORTANT: **The pre- and post-capture PCR cycle numbers have been set according to the DNA quality and quantity. Changing them would affect the sensitivity, specificity and LOD of *Inherited NephroKitDx*.**

Once the assay has been initiated, the LED will glow green and the touchscreen will show the assay status, as well as the estimated time remaining before the end of the assay.

The *SSEL XTHS-RevB-ILM* protocol lasts approximately 9 hours and may run overnight if this is more convenient. Once the protocol is complete, the libraries prepared will be automatically conserved at 12°C. Collect the instrument libraries within a maximum period of 24 hours.

If necessary, the assay may be aborted by pressing the red *Stop* square on the *Running* screen. A warning message will appear, asking you to confirm that you would like to abort the assay. Once an assay has been stopped, it cannot be restarted, and the labware used cannot be reloaded for another assay.

The *Running* screen should stay open throughout the assay and the screen closure button (x) and other browsing buttons will be deactivated while the assay is in progress. You cannot use the touchscreen for other purposes during an assay.

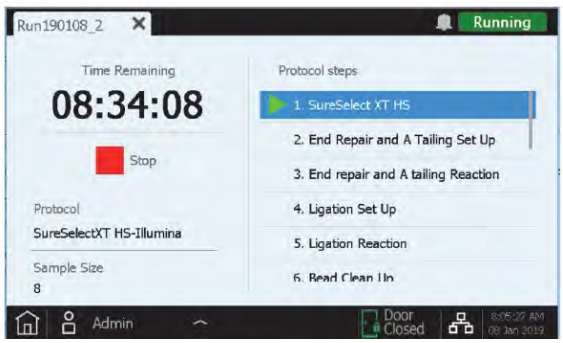


Figure 20. Running screen during an assay

07.4.6 | Collecting libraries from the instrument

Once the assay has finished, the touchscreen will appear as shown in the figure below. Press *OK* and the instrument will transfer 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 cooling module.

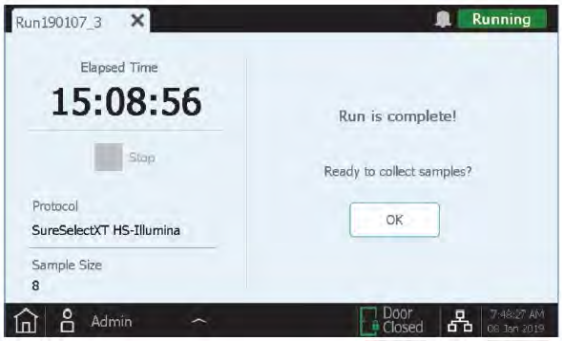


Figure 21. Running screen after an assay

Before opening the instrument door, wait for the LEDs to turn blue, which indicates that all the sample processing steps being performed by the instrument have been completed.

The cooling module will be kept at 12°C for a maximum of 2 hours from the moment when the libraries are placed on the green *Library Output Strip*, provided that the instrument door is kept closed.

Open the instrument door (until the LED glows white), then collect and seal the libraries from the green *Library Output Strip*.

> It is possible to stop the protocol at this point by keeping the libraries at 4°C if they are to be used in the next 24 hours, or at –20°C if they are to be stored for longer.

If optional samples were collected for quality control of pre-capture assay libraries, remove the blue *QC Strip* from the cooling module and allow to dry at room temperature unsealed, if the protocol is to be continued in the next 24 hours, or sealed for longer storage.

Once the door has been opened for collection of the libraries, the unit touchscreen will appear as below:

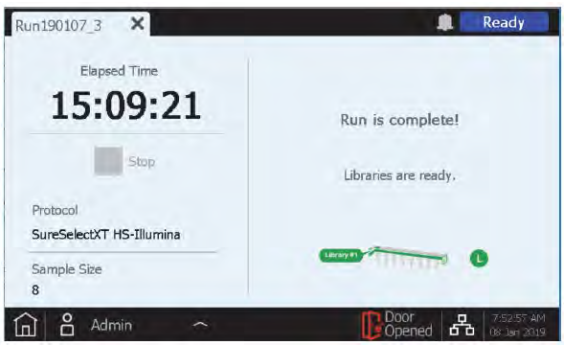


Figure 22. Running screen after an assay, with libraries already collected

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

07.5 | Cleaning the equipment after an assay

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

- The disposable container with the tips used over the course of the assay.
- The *Magnis Deep-Well HSM*.
- The *Magnis Thermal Cycler Seal*.
- The *Magnis 96-Well PCR Plate*.
- All the tip boxes, including partially full ones.
- The *Beads and Buffers Plate*.
- The *Reagent Plate*.
- The red, black and white strips used during the assay.

If you notice any material spillage on the instrument deck, it is recommended that the extended-cycle UV decontamination procedure be followed. Clean up the spillage by following the instructions provided in the instrument user guide.

07.6 | Validating and quantifying libraries

07.6.1 | Optional quality control of the pre-capture library

If an analysis of the pre-capture libraries is needed, resuspend the dry libraries in 6 µL of nuclease-free water to obtain a suitable concentration for the analysis, using the *TapeStation System* and the *D1000 Reagents* (cat. no. 5067-5583) and *D1000 ScreenTape* (cat. no. 5067-5582) commercial kits from Agilent Technologies as instructed.

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

After analyzing the samples with *TapeStation*, a library size of **300-400 bp** should be obtained (Figure 23). If an unexpected size is obtained, review the protocol or contact technical support at Health in Code, S.L.

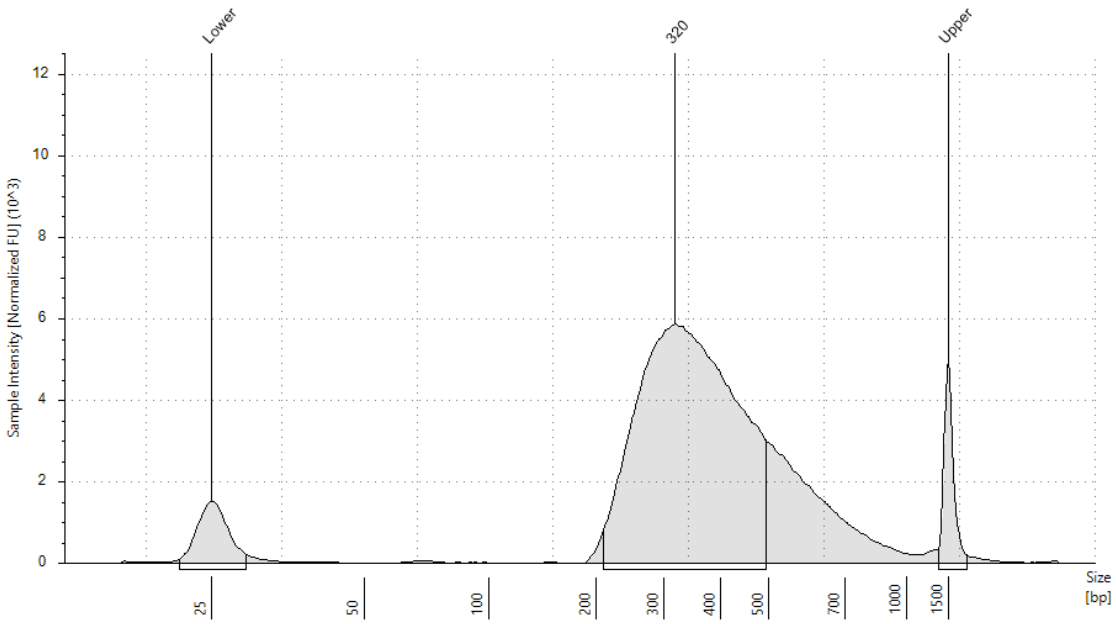


Figure 23. Expected result after analyzing pre-capture library size with the TapeStation System

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

07.6.2 | Quality control of post-capture library

Before pooling the libraries for multiplex sequencing, the quantity and quality of each library must be analyzed.

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

The concentration of post-capture libraries ranges from 2 to 10 ng/µL.

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

The average expected fragment size will vary from **310 to 390 bp**. If an unexpected size is obtained, review the protocol and the pre-capture library quality analysis, read section 9 carefully, or contact technical support at Health in Code, S.L.

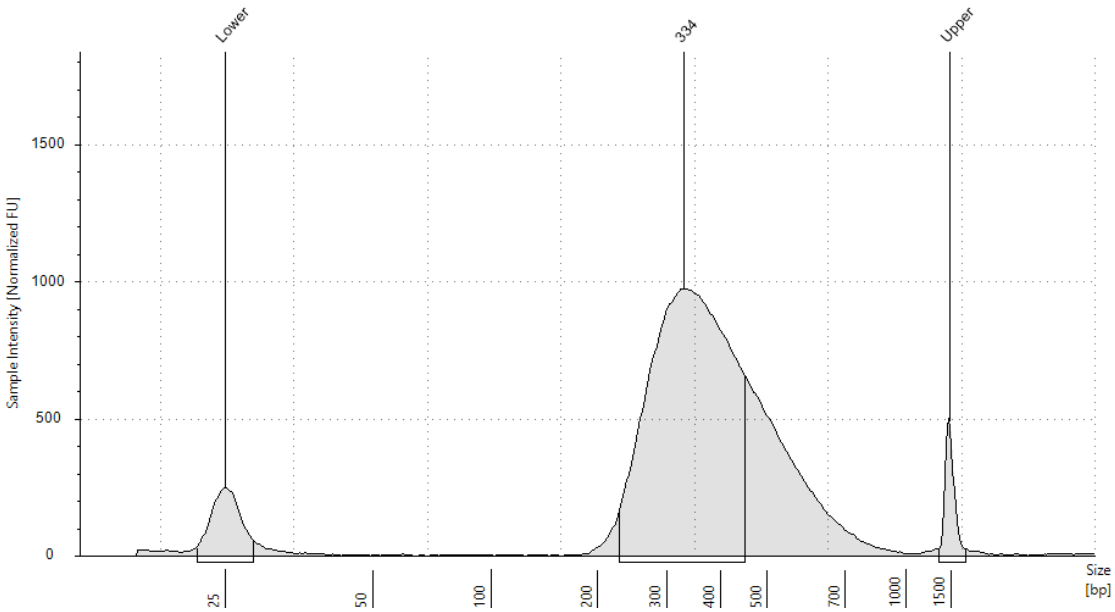


Figure 24. Expected result after analyzing pre-capture library size with the TapeStation System

The library concentration can be determined from the DNA concentration data and the library peak size with the following formula:

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

Finally, dilute each library at 4 nM with the *Elution Buffer* reagent and prepare an equimolar pool of all the libraries to be included in a run.

It is possible to stop the protocol at this point by keeping the libraries at 4°C if they are to be used in the next 24 hours, or at -20°C if they are to be stored for longer.

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

Next, follow the steps below to carry out the denaturation protocol before loading into an Illumina *NextSeq 500/550* sequencer:

01 Thaw the *HT1* reagent (included in the Illumina reagent kit that will be used for the sequencing) and keep cold until use.

02 Thaw *PhiX Control* and keep cold until use. *PhiX Control* should be denatured and diluted to 20 pM.

> IMPORTANT: *PhiX Control* should be denatured and diluted to 20 pM, so *PhiX* makes up 1% in the reaction. To denature *PhiX Control*, follow the *PhiX Control v3* denaturation protocol for each instrument, as provided by Illumina.

03 Add *5* µL of the library pool (diluted to 4 nM) to a 1.5-mL tube and 5 µL of *NaOH 0.2N*. Vortex and spin.

04 Incubate for 5 minutes at room temperature.

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

06 Add 985 µL of *HT1* and vortex. At this point, the library will be at 20 pM.

07 Transfer 78 µL of the library at 20 pM to a fresh 1.5-mL tube.

08 Add 1222 µL of *HT1*.

09 Add 1.2 µL of *Phix Control* to this mixture, denatured and diluted to 20 pM. At this point, the library will be diluted to 1.2 pM.

10 Load the entire contents of the 1.5-mL tube into the cartridge.

The table below specifies the maximum number of samples recommended per run, according to the sequencing kit being used, in order to guarantee a minimum of approximately 20 million PF clusters 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 9. Illumina NextSeq kit and maximum number of samples to analyze with Inherited NephroKitDx

IMPORTANT: Sequencing fewer samples than recommended will increase the read depth, and may decrease the sensitivity of this kit in the analysis of ALUs, fusions and CNVs as more false positives appear. Reviewing the .bam alignment file is recommended in these cases.

07.8 | Setting up the *NextSeq* platform

01 Set up the platform by running the standalone mode, as *BaseSpace* currently does not support molecular barcode sequencing as an index.

02 Following the loading instruction for the instrument.

03 When loading is complete, the run setup screen will appear. Enter the following parameters:

- *Read type*: *Paired end*.
- *Cycles*:
  - *Read* 1: 150
  - *Read* 2: 150
  - *Index* 1 (i7): 8

08 Analysis of results

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

This tool allows us to carry out the analysis of the different samples and to obtain all the files generated after the bioinformatic analysis of the samples.

As NGS technology is still not considered the *Gold Standard* for certain mutation types, wherever possible, it is recommended to confirm the positive results using complementary and standardized technology.

08.1 | Requesting an analysis

01 Select *"Import Samples"* on the main screen (Orders tab) to launch the analysis of the sequenced samples. This will take you to the file import screen (Figure 25). On this screen, you must import the 12 *FastQ* files associated with each sample, and, optionally, the *SampleSheet* file, which would enable you to simultaneously import all the files from the same sequencing run.

Figure 25. Screen to import FastQ files and the SampleSheet and launch the analysis request

02 Once the files have been loaded, indicate the name of the sequencing run and select the study mode, *Inherited NephroKitDx*, and the *STID (Sample Tracking ID)* used in each sample (or *"no STID"*, if none is used).

03 To carry out the analysis request, select the samples to be analyzed and click on the "Process" button. When the process has been successfully completed, the following message will appear: **√ Import has been completed successfully.**

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

08.2 | Managing requests

All the requests created will appear in the *Orders* tab in the corresponding section according to their status *(In bioinformatic process*, *Pending*, *In review*, *Finished*, *Canceled)*. The request will show the name of the sample, the mode and the status of the analysis.

Clicking on the sample will take you to a screen where you can note and save certain characteristics of each sample, such as dates of receipt, clinical indication, etc.

To access the results of the bioinformatic analysis, click on the bioinformatics request and select *"Show results"* to open the *"Workspace"* window. This screen provides the user with the files resulting from the bioinformatic analysis: alignment files (bam and bai), list of variants (vcf), as well as other files with information on coverage and the sequencing quality report after the bioinformatic analysis. In the *"CNV"* request, by selecting *"Show results"*, you can access the files resulting from the analysis of CNVs per gene (_calls.tsv, images_cnv.zip and _sample_QC.tsv).

The parameters taken into account in the different files generated from sequencing for a sample to pass the bioinformatics quality control set out for the Inherited NephroKitDx assay are:

- *FASTQ*: The established acceptance criteria are described 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: Check that the traceability reagent obtained matches the expected one (if used), as shown in Figure 26.

If any of the parameters above are not exceeded, the icon will appear on the main screen next to the sample in question.

In the Inherited NephroKitDx assay, VCF files are not taken into account for quality control, as the panel is too small to be representative and consistent.

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

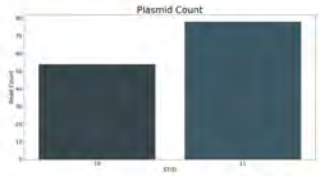


Figure 26. Quality control of the integrated traceability system.

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

- Quality variants: ***PASS; d50; pseudogenic_homology; LowMappeabilityRegion; hotspot*** *(Fault summary).*
- Depth: ≥ 20X *(Clean total count).*
- Allele frequency: ≥ 20% *(Variant Freq).*
- Exon distance: 20 *(Exon distance).*

NOTE: Intronic variants present in regions designed for their clinical implication will have the Hotspots flag and can be observed with this filter even if they are more than 20 bp away from the exon.

08.3 | Analyzing large rearrangements (CNVs)

The analysis of large rearrangements or CNVs from NGS sequencing data consists of a correlation between the number of normalized reads of a region with regard to the number of DNA copies for that region.

Since the number of readings should be normalized between different samples, variability between samples will result in poorer identification of CNVs, which means, wherever possible, that it is important that we try to standardize experiment conditions between the different samples and the different genomic regions of the same sample. To reduce variability and ensure correct analysis of CNVs, the following recommendations are suggested:

- The library preparation and capture process conditions should be consistent. To achieve this, the various steps should be carried out simultaneously with the samples from the same sequencing assay, using the same equipment at the same time and following the instructions outlined in section 7 of this document.
- Starting DNA is another source of variability. As such, all the DNA analyzed should be extracted according to the same extraction protocols.

Inherited NephroKitDx offers an analysis of CNVs that may affect one or several exons of a gene or a complete gene 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 the sample quality parameters. Based on these parameters, the results

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

With regard to the the CNVs result, the PASS variants will be displayed by default. These will be the highest-quality variants, having a p-value ≤ 0.0005 and a ratio ≤ 0.7 or ≥ 1.3. Some of these variants may come up with one or more of the following flags:

- *"High Controls Variation":* indicates that there is a high variation in coverage within the reference group compared to the problem sample.
- *"Low Zeta score":* the *z-score* value indicates how many standard deviations the identified event is away from the population mean. This flag indicates that the identified event has a low average *z-score* value and, therefore, that its standard deviation is not too far from the mean. As these are CNVs, these values are based on the coverage obtained for each region.

As mentioned previously, the calculation of CNVs lies in the depth of coverage of each sample. NGS technology has certain intrinsic limitations, for example, homology between regions and the existence of pseudogenes can affect the mappability of the regions of interest. In this regard, Inherited NephroKitDx contains genes that are complex to analyze due to their homology to other genes or regions such as *PKD1*, *PKD2*, *C4A* and *C4B*. Likewise, this kit contains the *CFHR1* and *CFHR3* genes, which are frequently deleted in the Western population. Taking into account the design and nature of the panel, genes with population CNVs or with mapping problems can generate the above-mentioned flags, giving rise to true positives.

Furthermore, through the filters, the user will be able to choose the option to show all the variants called without any pre-filtering by quality by clicking on the "No Pass" option.

If the CNV analysis could not be performed, an alert will appear in **Data Genomics** indicating the reason.

Data Genomics provides a graphical representation (Figure 27) of the sample coverage profiles against the reference samples. This graph enables visualization of the SNPs and INDELs present in the analyzed regions.

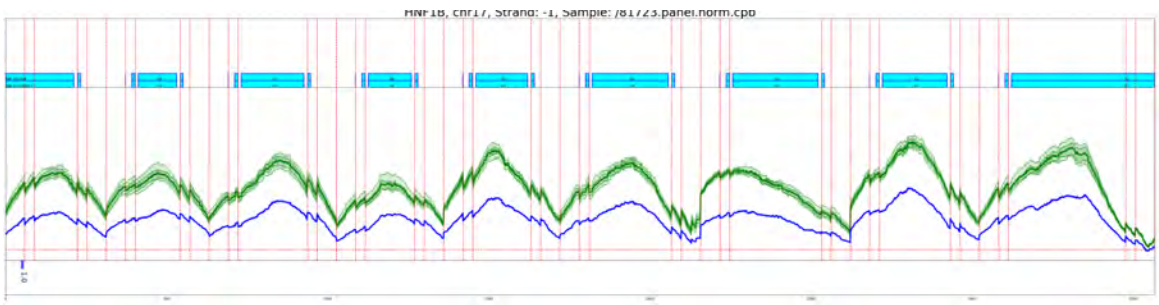


*Figure 27. CNV results shown on the **Data Genomics** platform.*

08.4 | Variant filtering

By selecting the *"Request: Filtering"* button, you will access a pop-up screen with the different variant analyses generated so far.

Figure 28. Variant filtering with Data Genomics

When accessing the variant analysis, the variants that have passed the criteria of the *Inherited NephroKitDx Default* filter, which aims to show the best-quality variants within the target regions of Inherited NephroKitDx, will be displayed.

Once the variants displayed with the previous filter have been reviewed and categorized, it is advisable to apply a second filter, to be generated by the user. This will display all variants cataloged by *Clinvar* as pathogenic or possibly pathogenic, even though they do not meet the criteria specified in the *Inherited NephroKitDx Default* filter. This will show variants that, regardless of their quality, have clinical implications. If, by using this filter, low-quality variants of interest are detected, Health in Code, S.L. recommends confirming them using another technology.

New filters are created by clicking on the *"Filters"* button from the *"Variants"* screen. A pop-up window appears from which the new filter can be created. To do this, the user should adjust the different options accordingly. Once the filter characteristics have been chosen, they can be saved and applied to the current sample by selecting *"Apply"*.

Each variant found will have a quality label associated with it in the *"Fault summary"* column. The possible labels, as well as their description and all the information provided by variant filtering, are described in the **Data Genomics** Instructions for Use, available at: www.datagenomics.es.

08.6.1 | Categorization of SNV variants and InDels, CNVs and SVs

Once the filters have been applied by the user, each variant found—whether point mutations, small deletions and insertions or CNVs—can be categorized.

By clicking on the *"Category"* column above each variant, a drop-down menu appears with the different categories with which the variant can be associated. These include:

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

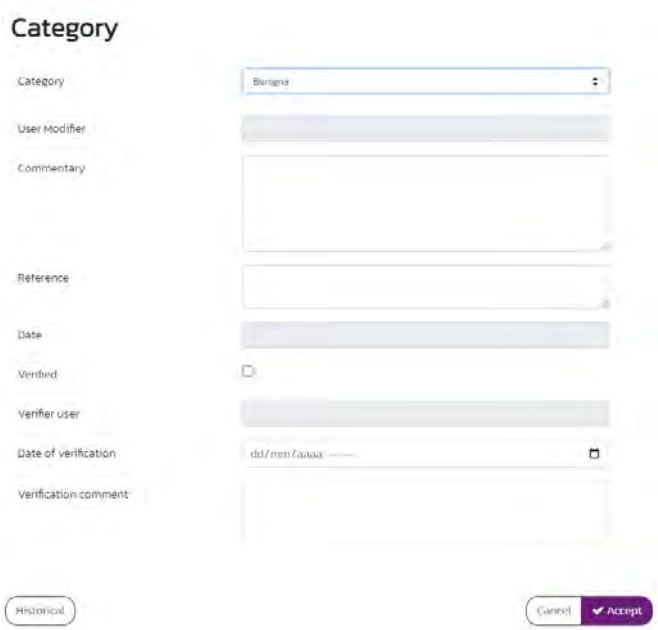


Figure 29. Drop-down menu of the "Category" column

If you do not wish to evaluate the variant or if it is suspected to be a false positive, it is advisable to categorize it as *"not evaluable/artifact"* which, if selected, would prevent any further selection.

Health in Code, S.L. will provide, in the *"HIC Germinal Db"* column, the categorization of the variants considering the functional impact that the variant would cause at the biological level.

After analyzing the samples, it is possible to generate a file of the selected variants, either as a .csv file or issuing an automatic report in pdf format. To do this, press the *"Report"* button and the analysis will be completed after a final review of the selected variants for inclusion in the report. If you have any questions about the analysis of results, please contact Health in Code, S.L.'s technical support, and your issue will be resolved within 24 hours.

If you have any questions about the analysis of results, please contact Health in Code S.L.'s technical department.

09 Troubleshooting

Below is a list of potential unexpected results and guidelines for resolving them for the library preparation and sequencing protocol using Inherited NephroKitDx. To resolve any other general problems with the Magnis instrument that are not included in this section, check the instrument user guide.

The touchscreen for assay setup has functionality problems:

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 touchscreen interface.

To reset touchscreen functionality, the system must be rebooted.

The LEDs on the instrument are glowing red and the touchscreen is showing the error message *"Teach points are shifted. Please perform auto teaching from the Settings screen"*:

This message appears when one of the control points has not passed the instrument health check (IHC), meaning that this point may be hidden or that the instrument needs to perform an auto-teaching programming routine before an assay can be configured. To prepare the instrument for an assay, follow these steps:

01 Make sure that there is no consumable material or other residue remaining on any of the positions of the instrument. If there is any material on the instrument, this may prevent detection of all the verified control points.

02 Clean the barcode scanner window according to the cleaning instructions in the Magnis instrument user guide. Residue or fingerprints on the scanner may be obscuring the verified control points, causing a verification failure.

03 Reboot the system. After you log in, the instrument will perform another IHC. If this status check is completed satisfactorily, the setup process may be resumed without carrying out the auto-teaching routine.

If the IHC is not completed satisfactorily, the auto-teaching routine should be carried out as follows:

01 On the *Home* screen, open the *Settings* menu and press *Auto Teaching*. Follow the instructions on the touchscreen. The auto-teaching process will take approximately 30 minutes and the user will need to be present to put the labware in the instrument.

02 Once the auto-teaching process is complete, you can begin assay setup by pressing *"Run Protocol"* on the *"Home"* screen.

The LEDs on the instrument are glowing red and the touchscreen is showing the error message instrument health check (IHC):

We recommend resetting the instrument after an IHC error, following the steps below:

01 In the error message box, press *"Cancel"* to refuse a diagnostic test.

02 Press the error icon at the bottom of the screen and make a note of the error code in case you need it to resolve the problem with Agilent technical support.

03 Turn off the instrument by pressing the power button on the front.

04 Make sure that there is no consumable material or other residue remaining on any of the positions of the instrument. Any material on the instrument unit may interfere with the IHC performed upon restarting.

05 Turn on the instrument by pressing the power button.

06 After you log in, the instrument will perform another IHC. If this status check is completed satisfactorily, you can begin the assay configuration. If the IHC fails again, get in touch with the Agilent technical support service to request assistance.

The Verify Labware screen states that there is a problem with one or more labware components after performing the automated labware verification:

If all or most of the labware does not pass the verification, you may need to clean the scanner window. Check the instrument user guide for cleaning instructions. After you have finished cleaning, repeat the *Verify Labware* step.

If only one or just a few labware components fail the verification, press the error icon at the bottom of the screen to see more information about the position with the error, so you can see the reason for the fault.

- ***If the barcode scanner cannot scan a particular labware component:***

  Make sure that the labware is present, in the required position and facing the correct direction (see section 7 of this document for complete equipment loading instructions). If there are any errors, resolve them and repeat the *Verify Labware* step. If all the components are present and facing the correct direction, visually inspect the barcode to make sure it is readable. For barcodes to scan properly, they should be free of scratches, stains and condensation, and they should not be obstructed by aluminum seals, writing or any other markings on the plastic material. If there is a damaged barcode, replace the component and repeat the *Verify Labware* step.

- ***If the scanned labware has expired:***

  Replace any expired component with non-expired components, then repeat the *Verify Labware* step.

- ***If the scanned labware is in an incorrect position:***

  Replace the unsuitable labware with the correct component and repeat the *Verify Labware* step.

The touchscreen shows a Time Remaining value of 0:00 at the end of the assay for a certain amount of time, without moving on to the assay/sample collection screens:

The Time Remaining value displayed on the touchscreen is simply an estimation of the remaining time of the assay, and it may remain at 0:00 for several minutes

before the system is ready to begin sample collection. This does not mean that there are problems in the assay or the instrument.

- **Larger sizes than expected after DNA 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, as if not, you will not achieve optimum performance in the process.

- **The size of the libraries is larger than expected in the electropherograms:**

  Ensure that the enzymatic fragmentation protocol (section 7 of this document) was carried out properly.

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

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

  Make sure that the starting DNA sample meets the specified quality and concentration guidelines.

  Verify that the assay has been set up for the appropriate input DNA concentration and quality. On the *Run Setup* tab on the *Post Run Data* screen, you can review the setup of completed assays.

  Check that the assays are being carried out in humidity conditions of 30% to 70% (without condensation). If the humidity is outside of this range, the performance can be affected.

  Very low or zero yield on one or more assay samples may be indicative of a problem with the points used in the assay. To carry out the protocol correctly, the tip boxes should be completely full, properly seated and within the frames of their platforms.

10 Limitations

10.1 | Analytical

- Inherited NephroKitDx 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 technology used does not make it possible to distinguish between regions with high levels of homology in their sequences, as they may be homologous genes, pseudogenes, etc., which can lead to false positives or negatives. The Inherited NephroKitDx regions with 100% homology to pseudogenic regions are listed below. In the *"Fault summary"* column of the results analysis, the *"Pseudogenic_homology"* label will appear when a variant is detected in the region that is homologous to pseudogenes.

Chromosome	Start position	End 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
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 10. List of pseudogenic regions. *IN=Intron*

- We cannot guarantee the results obtained if the established quality parameters are not met.
- NGS technology is still not considered the gold standard for certain mutation types, so it is recommended that, wherever possible, the positive results are confirmed using complementary and standardized technology.
- All the data and information obtained should be clinically assessed and interpreted by a clinician in conjunction with all the patient's clinical information and other complementary analytical or imaging test results.

10.2 | Equipment

Inherited NephroKitDx has been validated using the following thermal cycler for DNA fragmentation.

- *GeneAmp PCR System 9700* (Applied Biosystems)

If you use another make or model of thermal cycler, you may need to adjust the amplification program. Please contact our technical support for any questions or clarifications.

Inherited NephroKitDx has been validated using the following automated equipment for library preparation.

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

Inherited NephroKitDx has been validated using the following massive sequencing platform:

- *NextSeq 500/550 Dx System* (Illumina)

This kit is only compatible with Illumina massive sequencing platforms. When using any massive sequencing equipment other than the *NextSeq 500/550Dx System*, the final concentration of the libraries will need to be adjusted to the specifications of the specific protocols of the 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 (Equipment, reagents and materials required but not supplied).

For NGS, we suggest using the reagents recommended by the supplier: **Illumina**.

If in doubt, please contact Health in Code, S.L.'s technical department.

10.4 | Bioinformatics analysis platform

Inherited NephroKitDx was validated using **Data Genomics**, a bioinformatic analysis platform for *in vitro* diagnosis. This platform includes an analysis pipeline designed especially for Inherited NephroKitDx. It allows us to detect all the targets specified in section 2 of this document.

If a different analysis platform is used, Health in Code, S.L. accepts no liability for the results obtained.

10.5 | Product stability

The optimum performance of this product is confirmed provided that the recommended storage conditions according to the optimum product date for each production batch are followed.

Contact our Technical Department for any questions about the applications of this product or its protocols:

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healthincode