Instructions for use

BRCA Plus OncoKitDx

Ref. IMG-313

CE IVD

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

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

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Instructions for Use (IFU) modifications		
Version 14	JAN 2023	Specification of the required sequencing cycles. Indicated in section 7.5
Version 13	NOV 2022	Change of the manufacturer's address: Health in Code S.L., Calle de la Travesía s/n, 15E Base 5, Valencia 46024, Spain.
Version 12	SEP 2022	Change of the manufacturer's identification, going from Imegen to HEALTH IN CODE, S.L.
Version 11	DEC 2021	Section 8.2 update.
Version 10	DEC 2021	Section 7.1 and 7.5 update.
Version 09	DEC 2021	Section 9 update.
Version 08	OCT 2021	Section 8 update.
Version 07	AUG 2021	Section 7 update.
Version 06	AUG 2021	Sections 3 and 8 updates.
Version 05	JUN 2021	Modification of kit contents and sections 7, 8 and 10 updates. Available from batch 31321C003
Version 04	APR 2021	Sections 3, 7 and 8 updates.
Version 03	APR 2021	FFPE DNA analysis included.
Version 02	JUN 2015	Update by certified CE/IVD.

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01 General information

The term cancer refers to a very broad and varied group of diseases characterized by the uncontrolled growth of cells, which can spread to tissues in other parts of the body. The causes that trigger the appearance of cancer are very varied and are often the result of the interaction of many risk factors. These risk factors cause variations in genes and in the genome that give rise to a loss of control over certain biological processes that give rise to uncontrolled cell growth.

Breast cancer is the most common type of cancer in women, with a risk in the general population higher than 10%. Between 5 and 10% of breast/ovarian cancers are hereditary (MIM #604370) and follow a pattern of autosomal dominant inheritance.

Hereditary breast/ovarian cancer is associated with germ-line mutations in the ***BRCA1*** (MIM *113705; NG_005905.2) or ***BRCA2*** (MIM*600185; NG_012772.1) genes, which confer a higher susceptibility to this disease. There have been hundreds of variations in both *BRCA1* and *BRCA2* described, distributed along both genes.

At present, numerous clinical trials have incorporated pharmacogenetic tests to select patients who can best benefit from the pharmacological treatments. Pharmacogenetics is the study of the genetic causes underlying the diversity of responses that are observed for the same dose of a particular medication. This can be understood as the analysis and use of individual or population-wide genetic information to predict the safety, toxicity, or efficacy of medications. In general, chemotherapy agents have a narrow therapeutic range, such that inter-individual variability in their metabolism determines both their efficacy and safety.

References

- *Hereditary breast and ovarian cancer susceptibility genes (review). Kobayashi H1, Ohno S, Sasaki Y, Matsuura M. Oncol Rep. 2013 Sep;30(3):1019-29. doi: 10.3892/or.2013.2541.*
- *Hereditary breast and ovarian cancer due to mutations in BRCA1 and BRCA2. Nancie Petrucelli, Mary B Daly & Gerald L Feldman. Genetics in Medicine volume 12, pages 245–259 (2010)*
- *NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines). Breast Cancer. Version 2.2017*
- *NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines). Genetic/Familial High-Risk Assessment: Breast and Ovarian. Version 2.2017*

02 Intended use

BRCA Plus OncoKitDx has been designed to identify point mutations and small insertions and deletions within coding regions, as well as splice site mutations of the BRCA1 and BRCA2 genes.

BRCA Plus OncoKitDx analyses germline DNA of patients with a predisposition to develop breast and ovarian cancer, by multiplex PCR and, subsequently, by *Next-generation sequencing* (NGS), also known as high-throughput sequencing.

BRCA Plus OncoKitDx allows the analysis of both germline DNA from patients predisposed to breast and ovarian cancer, as well as DNA from tumor tissue from fresh or paraffin-embedded (FFPE) samples. For this, a multiplexed PCR protocol is used and subsequent sequencing with NGS platforms, high-throughput massive sequencing technology.

BRCA Plus OncoKitDx has been designed for *in vitro* diagnostics and it is directed to professionals from the molecular biology sector.

03 Technical characteristics

This kit has been validated using reference DNA samples and samples previously genotyped using other technologies. In this validation, it has been verified that the variants present in the target regions of the product, specified in section 2 of these instructions for use, are detected specifically, and that this analysis is reproducible.

Technical specifications:

- Type of sample: Genomic DNA extracted from peripheral blood and paraffin-embedded tissue.
- Required DNA quantity: 40 ng.
- Limit of detection: 5%.

For samples from peripheral blood:

- Coverage: 100% of the bases covered with minimum sequencing depth 100X.
- Uniform coverage: 100% of amplicons are covered over 20 % of the mean coverage.
- Sensitivity and Specificity: > 99%
- Repeatability and reproducibility: > 99%

For samples from paraffin-embedded tissue:

- Coverage: 99.8% of the bases covered with minimum sequencing depth 1000X.
- Uniform coverage: 99.8% of amplicons are covered over 20 % of the mean coverage.
- Sensitivity and Specificity: > 99%
- Repeatability and reproducibility: > 99%

BRCA Plus OncoKitDx is compatible with Illumina high-throughput sequencing platforms.

Health in Code S.L. is certified under **UNE-EN ISO 13485:2018 Medical Devices: Quality Management Systems - Requirements for regulatory purposes** standard by the SPANISH AGENCY OF MEDICINES AND MEDICAL DEVICES (AEMPS) 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

04 Safety warnings and precautions

- Strictly follow the instructions of this manual, especially regarding the handling and storage conditions.
- Do not pipette by mouth.
- Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.
- You must properly protect any skin condition, as well as cuts, abrasions and other skin lesions.
- Avoid discharge of reagents waste to the sink drinking water. Use waste containers established by the legislation and manage their treatment through an authorized waste manager.
- In case of an accidental release of any of the reagents, avoid contact with skin, eyes and mucous membranes and clean with abundant water.
- The materials safety data sheets (MSDS) of all hazardous components contained in this kit are available on request to Health in Code S.L.
- This product requires the handling of samples and materials of human and animal origin. You should consider all human and animal source materials as potentially infectious and handled in accordance with OSHA Biosafety Level 2 of bloodborne pathogens or must use other relevant biosafety practices for materials containing or suspect that they may contain infectious agents.
- Reagents included in this kit are non-toxic, neither explosive, infectious, radioactive, magnetic, corrosive nor environmental polluters.
- This kit has been validated with specific equipment under certain conditions, which could be different in other laboratories. It is recommended that each laboratory performs an internal validation when the kit is used for the first time.
- The manufacturer is not responsible for the malfunction of the assay when one or more 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 employs reagents not validated by Health in Code S.L., considering them equivalent to those provided in the Kit.
- The manufacturer is not liable for the obtained results when the bioinformatics analysis is carried out on an analysis platform different from **Data Genomics**.

05 Content and storage conditions of the kit

This kit contains sufficient reagents to perform 16 identifications using the following reagents:

- **General Master Mix III**: General PCR master mix with the quantities of enzyme, nucleotides and buffer needed to perform the amplification reactions.
- **BRCA Plus Buffer**: Contains $MgCl_2$ at the concentration needed to perform the amplification reactions.
- Nuclease free water for PCR reactions.
- **Pool A and Pool B PCR**: Contain the oligonucleotides needed to carry out the amplification of the kit's target regions.
- **Index**: Oligonucleotides used in the second PCR reaction with a unique sequence of 8 nucleotides, compatible with the Illumina adapters. These are necessary to mark the libraries of each sample with a unique combination that will allow its analysis and discrimination after sequencing. The kit includes the indexes required for simultaneous sequencing of 32 samples.

The reagents are distributed in two boxes, Box 1 and Box 2, with the reagents necessary for the preparation of the first and second PCR, respectively.

Box 1 of 2			
Reagents	Color indicator	Quantity	Conservation
General Master Mix III	White disc	160 µL	-20°C
BRCA Plus Buffer	Black disc	144 µL	-20°C
Water	Green disc	296 µL	-20°C
Pool A PCR	Purple disc	60 µL	-20°C
Pool B PCR	Yellow disc	60 µL	-20°C

Table 1. Components of box 1 of the BRCA Plus OncoKitDx

Box 2 of 2			
Reagents	Color indicator	Quantity	Conservation
General Master Mix III	White disc	240 µL	-20°C
BRCA Plus Buffer	Black disc	216 µL	-20°C
Water	Green disc	384 µL	-20°C
Index Plate BRCA Plus	Blue disc	32 x 10 µL	-20°C

Table 2. Components of box 2 of the BRCA Plus OncoKitDx

06 Equipment, reagents and material not included in the kit

Equipment:

- Conventional PCR Thermal Cycler
- Micropipettes (10 µL, 20 µL, 200 µL and 1000 µL)
- Vortex
- Centrifuge
- Magnetic rack and shaker (tubes or plates)
- Shaking block (tubes or plates)
- Fluorometer (recommended: *Qubit*; ThermoFisher)
- NGS Sequencer (Illumina)
- Power supply and electrophoresis cuvette

Reagents:

- *Agencourt® AMPure® XP beads* (Beckman Coulter Genomics cat. no. A63880, A63881 or A63882)
- Absolute ethanol
- *Elution Buffer* (Qiagen cat. no. 19086)
- Recommended: *Qubit dsDNA HS Assay* kit (Invitrogen cat. no. Q32854)
- *NaOH 0.2N* (Fluka cat. no. 1091401000)
- *PhiX Control v3* (Illumina cat. no. FC-110-3001)
- Fluorimeter reagents (recommended: *Qubit dsDNA HS Assay* kit; cat. no. Q32853; Invitrogen)
- Agarose

Materials:

- Filter tips for pipettes (10 µL, 20 µL, 200 µL and 1000 µL)
- Sterile 1.5 mL and 0.2 mL tubes
- Powder-free latex gloves
- *Qubit™ assay tubes* (Invitrogen cat. no. Q32856)

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

NOTE

BRCA Plus OncoKitDx is prepared for use in combination with the Health in Code-Sample tracking A y B kit (REF: IMG-234 and IMG-311), allowing each sample to be tracked from the DNA dilution to the bioinformatic analysis of the results. In this way, sample tracking can be assured throughout the entire protocol.

07 Assay protocol

BRCA Plus OncoKitDx includes two primer pools (PCR-Pool A and PCR-Pool B), required to amplify the genomic regions of interest using PCR multiplexing. Therefore, for each sample, two reactions will be carried out, one for Pool A, and another for Pool B.

07.1 | First PCR set up

01 Thaw the *General Master Mix III, BRCA Plus Buffer, Pool A PCR, Pool B PCR*, and DNA sample. Vortex each of the reagents and keep on ice.

02 Add the required volumes of the reagents indicated in table 3, to 1.5 mL tubes, based on the total number of reactions. It is recommended to perform the calculations by adding sufficient reagents to analyse one more reaction, or to add 10% more of each of the reagents.

Reagent	Pool A volume	Pool B volume
General Master Mix III	4 µL	4 µL
BRCA Plus Buffer	3.6 µL	3.6 µL
Water	7.4 µL	7.4 µL
Pool A PCR	3 µL	-
Pool B PCR	-	3 µL

03 Vortex the PCR mixture and dispense 18 µL into the corresponding PCR wells.

Optional: Select one of the twelve sample tracking reagents (*Sample tracking A Kit*, REF. IMG-234) and add it to the sample, in a 1:5 dilution. Example: 1 µL of plasmid + 4 µL of the sample at the concentration indicated in the next step. For cases in which more than twelve samples are being analysed per batch, the *Sample tracking B Kit* has been created, REF. IMG-311.

04 Quantify the DNA samples with the *Qubit* fluorometer (ThermoFisher).

Optional: Quality analysis of the DNA from the FFPE samples using the Agilent's *Genomic DNA ScreenTape* together with the accompanying reagents, following the manufacturer's instructions for the use of the instrument and the kit.

This analysis allows to establish the integrity of the DNA sample, which can influence the results obtained. DIN < 3 can affect the stablished LOD.

05 Add 2 µL of sample DNA at 10 ng/µL or nuclease-free water (negative control) into the corresponding wells. In case the extracted sample has a lower concentration,

adjust the sample volume with the corresponding amount of water so that the reaction contains 20 ng total of DNA.

NOTE: It is recommended to confirm the dilution to 10 ng / µL with a fluorimeter.

06 Place the samples in the thermal cycler and perform the following PCR programme with the appropriate ramp ratio (4°C/s).

Temperature	Time	Cycles
95°C	15 minutes	1
95°C	30 seconds	22
60°C	3 minutes	
72°C	30 seconds	
72°C	5 minutes	1
10°C	∞	1

Table 3. Optimal PCR programme for SimpliAmp Thermal Cycler and GeneAmp PCR System 9700

07 When the amplification program is complete proceed immediately to the next step of the protocol.

07.2 | First PCR products purification

For this process it is necessary to allow the reagents AMPure XP Beads (REF: A63881; Beckman Coulter Inc) and Elution Buffer (REF: 19086; Qiagen) warm at room temperature for 30 minutes before use, and to prepare 400 µL of 80% ethanol per reaction.

01 Shake the tube that contains the magnetic beads vigorously.

02 Incubate the volume of magnetic beads that will be used, based on the number of samples (23.8 µL per reaction), for 30 minutes (room temperature).

03 Prepare one 1.5 mL Eppendorf tube per reaction.

04 Dispense 23.8 µL of magnetic beads into each 1.5 mL Eppendorf tube.

05 Transfer 17 µL from each reaction into its corresponding 1.5 mL tube.

06 Place the tubes on a shaking heat block for 2 minutes, at 1800 rpm and at room temperature.

07 Incubate for 5 minutes at room temperature.

08 Place the tubes on a magnetic plate for 2 minutes so that the magnetic beads adhere to the magnet.

09 Remove the supernatant, pipetting carefully.

10 Add 200 µL of freshly prepared 80% EtOH.

11 Repeat steps 9 and 10 of the protocol.

12 Remove the supernatant. It is very important that no ethanol traces remain.

13 Leave to dry at room temperature for 10 minutes. During this time, remove any remnants that could have remained on the tube walls.

14 Add 17 µL of the *Elution Buffer* reagent (Ref: 19086; Qiagen).

15 Remove the tubes from the magnetic plate and re-suspend vigorously by vortexing until the bead-elution buffer solution is homogenized.

16 Incubate for 2-10 minutes at room temperature.

17 Place the tubes on the magnetic plate again for 2 minutes so that the magnetic beads adhere to the magnet.

18 Transfer the supernatant to a new tube and discard the tube with the magnetic beads.

> The protocol can be stopped at this point. If the second PCR will be carried out within the next 24 hours, keep the PCR product at 4°C. Otherwise, store at -20°C until it is time for their use.

07.3 | Second PCR set up

During the second amplification reaction, the index primers and adapter sequences are added that are needed for subsequent high-throughput sequencing on **Illumina** platforms.

For each sample, two reactions will be carried out, one for Pool A, and another for Pool B, according to the following protocol:

01 Thaw the *General Master Mix III, BRCA Plus Buffer,* the *Index plate*, and the products of the first PCR, if frozen. Vortex each of the reagents and keep on ice.

Each well of the index plate has a unique combination of *Index S* and *N* to sequence up to 32 samples simultaneously. The sequences of the different Indexes, as well as their order on the plate are specified in tables 5 and 6.

02 Add 1 µL of the PCR product and 499 µL of water to a new 1.5 mL tube. (1:500 dilution). Vortex and spin down the tubes of the diluted PCR products.

03 Add the quantities of reagents specified below to 1.5 mL tubes. It is recommended to perform the calculations by adding sufficient reagents to analyze one more sample, or to add 10% more of each of the reagents.

NOTE: When doing the calculations, it must be considered that each sample will require two reactions, one from Pool A of the first PCR and another from Pool B of the second PCR.

Reagent	Volume per reagent
General Master Mix III	6 µL
BRCA Plus Buffer	5.4 µL
Water	9.6 µL

04 Vortex the PCR mixtures and distribute 21 µL in each PCR well.

05 Add 3 µL of selected index to every reaction.

NOTE: Reactions from pool A and pool B of the same sample must be run with the same *Index* combination.

06 Add 6 µL of the diluted PCR product to the corresponding wells.

NOTE: As in this second PCR the template DNA is a product of a previous amplification, to avoid contaminations, we recommend preparing the PCR-mix in the pre-PCR laboratory (a pre-amplification area, PCR-products free) and then perform the steps 2 and 6 of the protocol in a post-PCR zone. We also recommend cleaning the surfaces with DNA decontamination reagents.

07 Place the samples in the thermal cycler and perform the following PCR programme with the appropriate ramp ratio (4°C/s).

Temperature	Time	Cycles
95°C	15 minutes	1
95°C	30 seconds	25
60°C	90 seconds	
72°C	30 seconds	
72°C	5 minutes	1
10°C	∞	1

Table 4. Optimal PCR programme for SimpliAmp Thermal Cycler and GeneAmp PCR System 9700

08 When the amplification program is complete proceed immediately to the next step of the protocol.

07.4 | Libraries purification

For this process it is necessary to allow the reagents *AMPure XP Beads* (REF: A63881; Beckman Coulter Inc) and *Elution Buffer* (REF: 19086; Qiagen) warm at room temperature for 30 minutes before use, and to prepare 400 µL of 80% ethanol per reaction.

01 Shake the tube that contains the magnetic beads vigorously.

02 Incubate the volume of magnetic beads that will be used, based on the number of samples (50 µL per sample), for 30 minutes (room temperature).

03 Prepare one 1.5 mL Eppendorf tube per sample.

04 Dispense 40 µL of magnetic beads into each 1.5 mL Eppendorf tube.

05 Transfer 25 µL of Pool A and 25 µL of Pool B per sample to a single 1.5 mL Eppendorf tube.

06 Place the tubes on a shaking heat block for 2 minutes, at 1800 rpm and at room temperature.

07 Incubate for 5 minutes at room temperature.

08 Place the tubes on a magnetic plate for 2 minutes so that the magnetic beads adhere to the magnet.

09 Remove the supernatant, pipetting carefully.

10 Add 200 µL of freshly prepared 80% ethanol.

11 Repeat steps 8 and 9 of the protocol.

12 Remove the supernatant. It is very important that no ethanol traces remain.

13 Leave to dry at room temperature for 10 minutes. During this time, remove any remnants that could have remained on the tube walls.

14 Add 52 µL of the *Elution Buffer* reagent (Ref: 19086; Qiagen).

15 Remove the tubes from the magnetic plate and re-suspend vigorously by vortexing until the bead-elution buffer solution is homogenised.

16 Incubate for 2-10 minutes at room temperature.

17 Place the tubes on the magnetic plate again for 2 minutes so that the magnetic beads adhere to the magnet.

18 Transfer the supernatant to a new tube and discard the tube with the magnetic beads.

> The protocol can be stopped at this point. Libraries can be stored at 4ºC for the next 24 hours or at -20ºC until it is time for their use.

07.5 | Sequence library preparation

CALCULATION OF THE LIBRARY CONCENTRATIONS

To measure the DNA concentration, it is recommended to use a *Qubit® 2.0* fluorometer, with the commercial *Qubit dsDNA HS Assay* kit (Ref: Q32854) and *Qubit™ assay tubes* (Ref: Q32856) from Invitrogen. The DNA concentration obtained must be within the range 10 - 40 ng / µL.

Health in Code S.L. recommends verifying the size of the libraries by running them on a 3% agarose gel with a voltage of 120 V for 30 minutes. The average size obtained should be close to 300 bp.

> IMPORTANT: **If a second band of approximately 200 bp is observed, it is recommended to repeat the purification step (section 7.4 of this document), to guarantee a higher quality of the sequenced libraries.**

The library concentration is obtained by applying the DNA concentration data and average library size to the following formula.

$$Library\ concentration\ (nM) = (\ Concentration\ \left({}^{ng}/_{\mu L}\right)\ \text{x}\ 5.11\ )$$

NOTE: The 5.11 value included in the formula is a simplification of the amplicon size and the conversion units.

SAMPLE SHEET CREATION

The *Sample Sheet* can be generated using the Illumina *Experiment Manager* software, following these steps.

01 Open the programme and select the option: *Create Sample Sheet*.

02 Select the option *Miseq>Next*.

03 Select *Other>FASTQ Only>Next*.

04 Fill out the fields for each test: *Reagent Cartridge Barcode, Library Prep Workflow (Nextera XT), Index Adapters (Kit D), Index Reads (2, dual), Experiment Name, Investigator Name* and *Description*. The rest of the fields should appear as in the Figure 1 for germ

assays. In case of somatic assays, to improve the depth, it is recommended to increase the number of cycles as follows.

Germ assay	
Read 1	150
Read 2	150
Index 1 (i7)	8
Index 2 (i5)	8

Somatic assay	
Read 1	160
Read 2	160
Index 1 (i7)	8
Index 2 (i5)	8

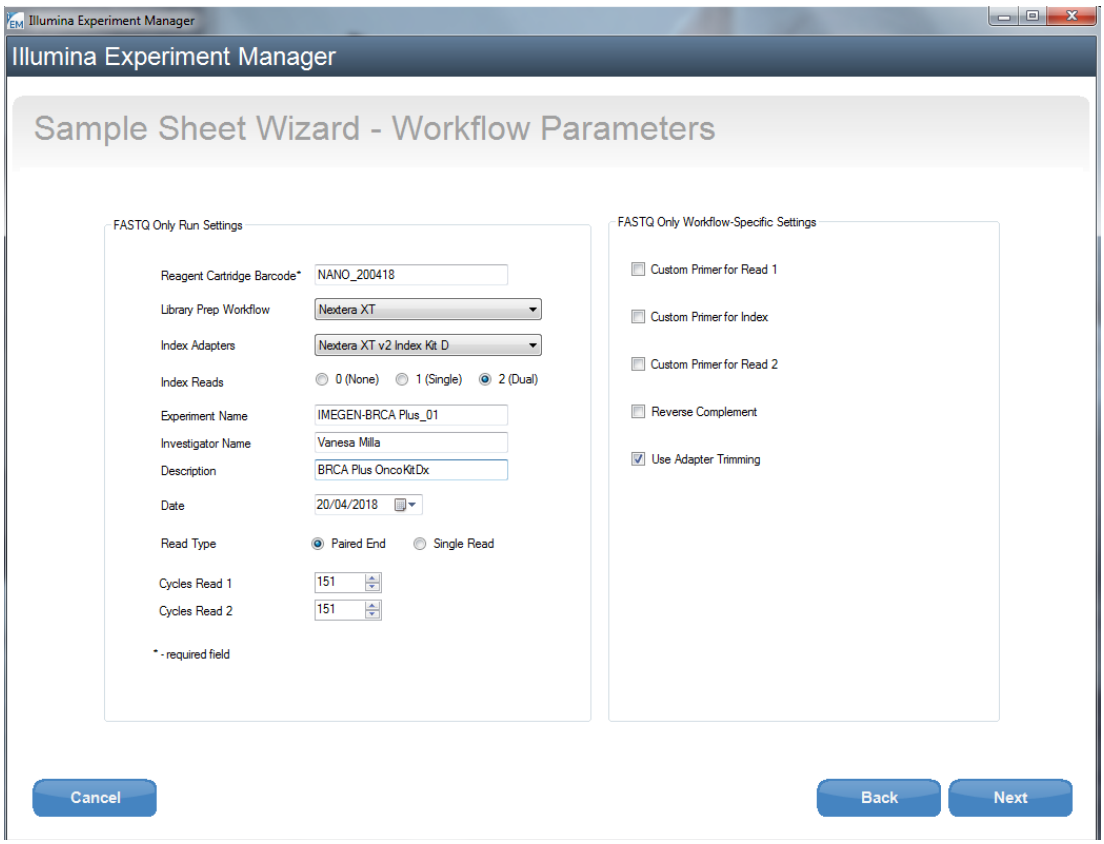

Figure 1: Sample Sheet creation with Illumina Experiment Manager

05 Select *Next*

06 On the following screen, you must add a row for each sample included in the test (by selecting *Add Blank Row*)

07 Fill in the fields for each sample.

08 Select *Finish*.

09 The option to store the *Sample Sheet* created will appear. Save as .csv.

	1	2	3	4
A	S513/N718	S517/N718	S518/N718	S520/N718
B	S513/N719	S517/N719	S518/N719	S520/N719
C	S513/N720	S517/N720	S518/N720	S520/N720
D	S513/N721	S517/N721	S518/N721	S520/N721
E	S513/N722	S517/N722	S518/N722	S520/N722
F	S513/N723	S517/N723	S518/N723	S520/N723
G	S513/N724	S517/N724	S518/N724	S520/N724
H	S513/N726	S517/N726	S518/N726	S520/N726

Table 5. Location of the index pairs on the Plate-Index

Name	Sequence
S513	TCGACTAG
S517	GCGTAAGA
S518	CTATTAAG
S520	AAGGCTAT
N718	GGAGCTAC
N719	GCGTAGTA
N720	CGGAGCCT
N721	TACGCTGC
H04	AGTCACTA
N723	TAGCGCTC
N724	ACTGAGCG
N726	CCTAAGAC

Table 6. Sequences for each index

DENATURATION AND LOADING OF THE LIBRARIES

01 Thaw the *HT1 reagent* (included in the Illumina reagent kit for the platform to be used for sequencing); for example: *MiSeq Reagents nano Kit v2* (300 cycles). Ref: MS-103-1001) and keep on ice until its use.

02 Thaw the PhiX control and keep on ice until its use. The PhiX control must be denatured and diluted to 12.5 pM (follow the *PhiX Control v3* denaturing protocol provided with the reagent by Illumina).

03 Dilute each library to a concentration of 2 nM using the *Elution Buffer* reagent.

04 Add all the libraries together that will be loaded onto the same run in a single pool. For this, add 10 µL of each of them into a new 1.5 mL tube. Vortex and spin down.

05 Add 5 µL of the pooled libraries to a 1.5 mL tube and 5 µL of *NaOH 0.2N* (not provided with the kit). Vortex and spin down.

06 Incubate for 5 minutes at room temperature.

07 Add 990 µL of HT1 and vortex.

08 To this mix, add 10 µL of denatured PhiX control diluted to 12.5 pM. At this time, the libraries will be at 10 pM concentrations.

09 Load the volume contained in the 1.5 mL tube onto the sample loading reservoir on the cartridge.

The following tables specifies the maximum recommended number of samples per run, depending on the sequencing kit used, to guarantee a minimum number of clusters:

- **Samples from peripheral blood:** 100.000 clusters per sample, and a minimum depth of 100X.

MiSeq Reagents Kit	Maximum no. of samples
MiSeq Reagents nano Kit v2 (300 cycles). Ref: MS-103-1001	10
MiSeq Reagents micro Kit v2 (300 cycles). Ref: MS-103-1002	40

Table 7. Samples recommended for analysis, allowing a minimum depth of 100X

- **Samples from paraffin-embedded tissue (FFPE)**: 700.000 clusters per sample, and a minimum depth of 1000X.

MiSeq Reagents Kit	Maximum no. of samples
MiSeq Reagents Kit v2 (500 cycles). Ref: MS-102-2003	16

Table 8. Samples recommended for analysis, allowing a minimum depth of 1000X

If other larger capacity kits are used, the BRCA Plus OncoKitDx libraries can be loaded with others, so long as they are tagged with an 8-nucleotide index.

NOTE: Due to the variable quality of ffpeDNAs, the use of *MiSeq Reagents Kit v3 (600 cycles)*, Ref: TG-142-3003, is recommended to obtain greater coverage in all sequenced libraries.

Once the *Sample Sheet* is created and the libraries are denatured, the steps indicated for the sequencer should be followed to start the sequencing process (*Miseq Control Software*).

08 Analysis of results

The bioinformatic analysis of the results is performed using an analysis pipeline designed especially for BRCA Plus OncoKitDx with the **Data Genomics** analytical platform. Access the analysis pipeline via www.datagenomics.es.

The software can analyze different samples and, consequently, get all the resulting files generated by the bioinformatics analysis.

The limit of detection may be affected in samples with a DIN <3 and a percentage of tumor infiltration with non-tumorous cells greater than 30%. In these samples the limit of detection may be not corresponding to that established in the technical specifications.

08.1 | Request for analysis

01 Select the "*Import Samples*" on the main screen (*Orders* tab) to begin the analysis of the sequenced samples. This way, you will access the file import screen (Figure 2). On this screen, the fastq files associated with each sample must be imported; optionally, the *Sample Sheet* file, which will allow us to import simultaneously all the files from the same sequencing run, can also be imported.

02 Once the files are uploaded, the sequencing run name must be indicated, and the study modality (*BRCA Plus OncoKitDx* for germ analysis, and *BRCA Plus FFPE OncoKitDx* for somatic analysis) and the STID (*Sample Tracking ID*) used for each sample (or "*no stid*" if none was used) must be selected.

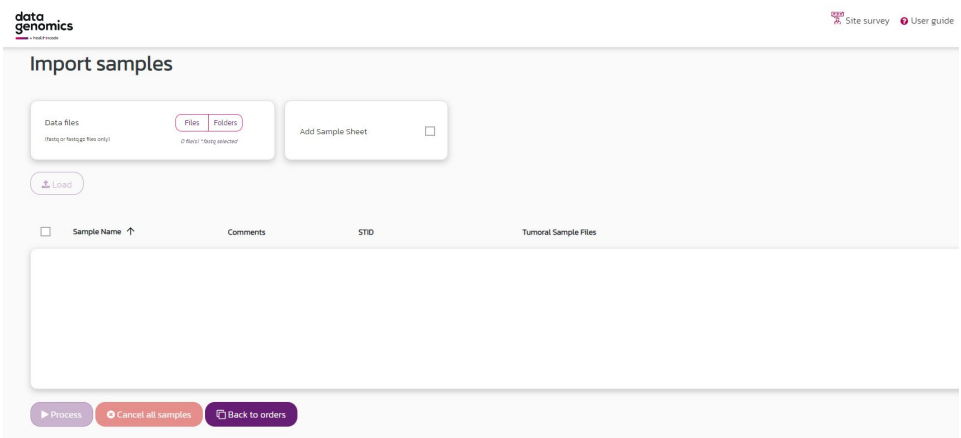

Figure 2. Screen for importing the fastq files and the sample sheet and commence the analysis request

03 To carry out the analysis request, select the samples that you want to be analyzed and click the "*Process*" button. Once the process has finished successfully, the following message will appear: **√ The import has been performed correctly.**

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

08.2 | Management of orders

All requests created will appear on the "*Order*" tab, along with their associated applications and their status (*In bioinformatic process, Pending, In review, Finished, Cancelled*), in the tab it will also be shown the sample name, the modality and the status of the analysis.

Clicking on the sample takes you to a screen where you can add and save notes on certain characteristics of each sample.

Through the "*Show results*" application, you can access the "*Workspace*" tab, which makes all uploaded files and files resulting from bioinformatic analysis available to the user: the alignment files (BAM and BAI), the variants list (VCF) and others files containing the information about the coverage and the report with the calculation of sequencing quality statistics.

The parameters taken into account in the different generated files of the sequencing, for a sample to pass the bioinformatic quality control established for the BRCA Plus OncoKitDx assay are:

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

  NOTE: When the ROIs capture is performed by amplicons in NGS assays, such as BRCA Plus OncoKitDx, the quality of the last bases may be poor, which can lead to a *Fastq Fail Alert*. In these cases, check the QC Report.

- STIDs: Verification that the tracking reagent obtained matches the expected one (if it has been used), as shown in Figure 3.

If the sequencing data do not pass the quality controls established, an alert will be displayed once the "Bioinformatics" tab once the analysis is completed. Clicking on the alert icon will download the quality control report.

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

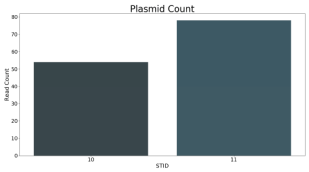

Figure 3. Quality control of the sample tracking system (optional)

To access the variants filter, the "*Filtering*" request will apply the *BRCA Plus OncoKitDx* filter for germ analysis and *BRCA Plus FFPE OncoKitDx* for somatic analysis.

- BRCA Plus OncoKitDx:
  - Quality variants: ***PASS; consensus_variant, primers_variant, d100, only_samtools_variant AMPBIAS*** (*Fault summary*).
  - Depth: ***≥ 20X*** (*Clean total count*).
  - Allele frequency: ***≥ 30%*** (*Variant Freq*).

- BRCA Plus FFPE OncoKitDx:
  - Quality variants: ***PASS; consensus_variant, primers_variant, d1000, only_samtools_variant AMPBIAS*** (*Fault summary*).
  - Depth: ***≥ 500X*** (*Clean total count*).
  - Allele frequency: ***≥ 5%*** (*Variant Freq*). Limit of detection established for samples with DIN >3 and tumor cellularity ≥30%.

08.3 | Variants filtering

By selecting the "*Filtering*" button, you will access to the "*Variants Filtering*" tab, that shows all the variants found in the BRCA1 and BRCA2 genes with all the data related to such variants.

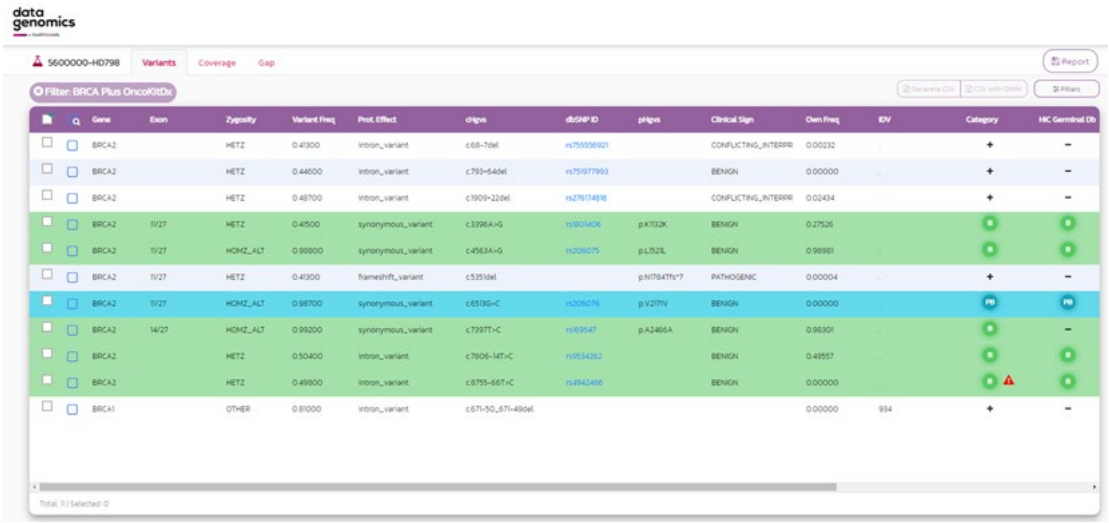

Figure 4. Variants filtering with Data Genomics

Once variant analysis is opened, the variants that have met the filter criteria are shown. For a user to evaluate whether a variant shall be validated as pathogenic even if it has not met the filter criteria, it is recommended to clean the previous filter and set up a new one.

New filters can be created by clicking the "*Filters*" button on the "*Variants*" page. A pop-up window where the new filter can be created appears. The user shall 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*".

It is recommended to review the d100 / d1000 variants. These variants are less coverage than expected but if they are real, they could be relevant to the patient's clinic.

Each variant found 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.es.

Below are shown the quality tags exclusive to BRCA Plus OncoKitDx:

- **Only_samtools_variant**: Variants called only with one of the two callers used.
- **Primer_variant**: To improve alignment and variant calling, these are done in two phases. The first phase includes the sequences of the primers. The second phase includes only

the ROIs of the panel. The primer_variant quality tag will appear on variants that have been called only during the alignment with primers. It is recommended to carefully review these variants as they can be artifactual changes.

- **Consensus_variant**: Variants called with two callers used, Vardict and Samtools.
- **LOW_QUAL**: Variants with a quality score less than 50, according to the two callers used.

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

08.4 | Variants categorization

Once the desired filters have been set up by the user, each variant found can be categorized.

By clicking on the "*Category*" column, a drop-down menu appears for each variant, showing the different categories to which, the variant can be assigned. The different categories are pathogenic (P), probably pathogenic (PP), variant of uncertain significance (VUS), probably benign (PB), or benign (B).

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

In the *Hic Germinal Db*, Health in Code S.L. provides the categorization of variants considering both the functional impact that the variant would cause at the biological level.

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

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

09 Troubleshooting

Listed below are the possible unexpected results occurring throughout the amplification and sequencing protocol of the BRCA Plus OncoKitDx.

- **Library concentration too low:**

  Concentrations lower than 10 ng/µL may be due to a fault in the amplification protocol or in the purification of the libraries. In this case, it is recommended to analyze the library by agarose gel electrophoresis.

  - If there is one single peak that roughly corresponds to an average size of 300 bp, continuing with the sequencing protocol is appropriate.
  - If several bands appear, review the different steps of the amplification and purification protocol.
  - In samples with a DIN <3 and an insufficient concentration to carry out the sequencing, it is recommended to modify step 3 of section 7.3 of this document, diluting the PCR product 100 times instead of 500 times.

- **Cluster density different from expected:**

  In this case, it is recommended to review the quantification of the libraries and the pool generation protocol prior to sequencing:

- **The sample has not passed the quality controls established in the analysis software:**

  In these cases, it is recommended to analyze the libraries with the TapeStation 2200.

  - If the result is like that obtained in the Figure 1, you are advised to review the denaturation and sequencer loading protocol.
  - If instead of a single 300 bp peak, there are several that appear, review the different steps of the amplification and purification protocol.

- **Errors in the STIDs assignment:**

  If using the sample tracking reagents supplied by Health in Code S.L., it is possible that the STID will not match the expected one. In this case, check that the STIDs specified in the *Sample Sheet* are correct.

- **Non-uniform coverage:**

  In this case it is recommended to review the Bam files of the affected samples. A sample without coverage problems should have a pattern like the one shown in figure 5, A. If on the other hand, a pattern like the one shown in Figure 5, B is observed, review the amplification protocol for the first PCR, as well as the purifications of the PCR products.

Figure 5. Coverage along exon 11 of BRCA2, A correct coverage pattern, B incorrect coverage pattern

10 Limitations

10.1 | Analytical

- The allelic drop-out is an intrinsic limitation of this technique. In this way, the presence of variants located in the design regions of the primers can cause the loss of an allele during amplification.
- The 5 'splicing region of exon 5 of BRCA2 (NM_000059.3) is covered at -7 bp instead of -10 bp.
- NGS is not yet considered the "Gold Standard" for some types of mutation, so it is recommended, whenever possible, to confirm positive results using a complementary and standardized technique.
- All the obtained data and information must be clinically evaluated and interpreted in an integrated way, together with the rest of the patient's clinical information.

10.2 | Equipment

BRCA Plus OncoKitDx has been validated using the following PCR Thermal Cyclers:

- **SimpliAmp Thermal Cycler (ThermoFisher Scientific)**
- **GeneAmp PCR System 9700 (ThermoFisher Scientific)**

If you use another brand or model of thermal cycler, you may need to adjust the amplification programme. Please contact our technical support team for any query or clarification.

BRCA Plus OncoKitDx has been validated using the following high-throughput sequencing platform:

- ***MiSeq System*** **(Illumina)**

This kit is only compatible with Illumina high-throughput sequencing platforms. Should high-throughput sequencing equipment other than the *MiSeq* system be used, the final concentration of the libraries will have to be adjusted to the protocol specifications for these platforms.

10.3 | Reagents

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

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

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

10.4 | Product stability

Optimal performance for this product is validated so long as the recommended storage conditions specified are maintained, within the optimal shelf life for the product, associated with each production batch.

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

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