



# Instructions for use

## TP53 OncoKitDx

Ref. IMG-317



Manufactured by:

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

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**Our products are intended for *in vitro* diagnostic use.** Health in Code S.L. does not offer any other warranty, express or implied, that extends beyond the correct functioning of the components of this kit. Health in Code S.L. only obligation, with respect to the guarantees, will be to replace the products, or to return the purchase price thereof, at the customer's discretion, provided that the existence of a defect in the materials or in the development of the products is identified. Health in Code S.L. will not be responsible for any damage, direct or indirect, that results in economic losses or damages that may occur due to the use of this product by the buyer or user.

All products sold by Health in Code S.L. are subjected to rigorous quality control. The **TP53 OncoKitDx** kit has passed all internal validation tests, ensuring the reliability and reproducibility of each assay.

For any questions regarding the application of this product or its protocol, please contact our Technical Department:

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Instructions for Use (IFU) modifications		
Version 08	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 07	SEP 2022	Change of the manufacturer's identification, going from Imegen to Health in Code S.L.
Version 06	NOV 2021	Revision content
Version 05	APR 2021	Modification of the content of the reagent TP53 Index.
Version 04	JAN 2021	Update of section 7.4.
Version 03	MAR 2020	Update of section 4, 7.4 and 10.
Version 02	JUL 2018	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. There are multiple causes that trigger the appearance of cancer which is often the result of the interaction of a large number of 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.

The transcription factor p53 responds to diverse cellular stresses to regulate target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. In unstressed cells, p53 is kept inactive essentially through the actions of the ubiquitin ligase MDM2. Numerous post-translational modifications modulate p53 activity, most notably phosphorylation and acetylation. Activity of p53 is ubiquitously lost in human cancer either by mutation of the p53 gene itself or by loss of cell signaling upstream or downstream of p53.

Somatic mutations in the TP53 gene are one of the most frequent alterations in human cancers, in chronic lymphocytic leukemia (CLL) *TP53* is a strong prognostic factor and the evaluation of *TP53* mutation status and cytogenetic abnormalities by are highly recommended prior to initiating treatment. Furthermore, germline mutations are the underlying cause of *Li-Fraumeni syndrome*, which predisposes to a wide spectrum of early-onset cancers.

More than 75% of the mutations result in the expression of a p53 protein that has, in most cases, lost the wild-type functions and may exert a dominant-negative regulation over any remaining wild-type p53.

Most mutations are single-base substitutions distributed throughout the coding sequence. Their diverse types and positions may inform on the nature of mutagenic mechanisms involved in cancer aetiology. *TP53* mutations are also potential prognostic and predictive markers, as well as targets for pharmacological intervention.

## References

- > Muller PAJ et al. *Nat Cell Biol.* (2013)
- > Strano S et al. *Head Neck.* (2007)
- > Petijean A et al. *Hum Mutat.* (2007)
- > Liu DP et al. *Oncogene* (2010)
- > *NCCN guidelines CLL.* (2018)

## 02 Intended use

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**TP53 OncoKitDx** has been designed to identify point mutations and small insertions and deletions within coding regions, the promoter (5'UTR) and the non-coding part of exon 1 and 2, as well as splice site mutations of the TP53 gene.

As the tumor suppressor gene TP53 is the most frequently mutated gene in human cancer and the target gene of *Li Fraumeni Syndrome*. **TP53 OncoKitDx** is optimised to detect germline and somatic mutations in genomic DNA extracted from peripheral blood or tissue biopsies, by conventional multiplex PCR and *Next-generation sequencing* (NGS).

**TP53 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 PCR product, specified in Section 2 of this manual (Intended use), are detected specifically, and that this analysis is reproducible.

### Technical specifications:

- ◇ Type of sample: Genomic DNA extracted from peripheral blood as well as DNA extracted from paraffin-embedded, fresh or frozen tissues.
- ◇ Required DNA quantity: 40 ng
- ◇ Coverage: 99.4% of the bases covered with minimum sequencing depth 500X and 98.5% at 1000X.
- ◇ Uniform coverage: 100% of amplicons are covered over 20 % of the mean coverage.
- ◇ Sensitivity and specificity: >99.9%
- ◇ Detection threshold: 2.5%.

**NOTE:** Out of the CE mark **TP53 OncoKitDx** can detect mutations with allelic frequency of 1% with a specificity of 98%. In these cases we recommend that the analysis should be carried out in triplicate, in order to avoid false positives and to assure the specificity of the detected variants with frequencies lower than 2.5%.

**TP53 OncoKitDx** is compatible with Illumina 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.
- ◇ The reagents included in this kit are not toxic, explosive, infectious, radioactive, magnetic, corrosive not biological or environmental pollutants.
- ◇ This kit has been validated using specific equipment and conditions which might vary from the conditions in other laboratories. Thus, it is recommended that each laboratory performs an internal validation prior to the utilization of the kit.
- ◇ 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 responsible for the results obtained when the bioinformatic analysis is performed using an analytical platform other than **Data Genomics**.

# 05 Content and storage conditions of the kit

This kit contains sufficient reagents to perform 48 identifications. The list of reagents included in the kit is the following:

- **General Master Mix III:** General PCR master mix with the quantities of enzyme, nucleotides and buffer needed to perform the amplification reactions.
- **TP53 Buffer:** Contains  $MgCl_2$  at the concentration needed to perform the PCR amplification reactions.
- Nuclease free water for PCR reactions.
- **Pool A PCR and Pool B PCR:** Contain the oligonucleotides needed to carry out the amplification of the kit's target regions.
- **TP53 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 48 samples.

Reagents	Color indicator	Quantity	Conservation
<b>General Master Mix III</b>	White disc	2 x 336 $\mu$ L	-20°C
<b>TP53 Buffer</b>	Black disc	1 x 375 $\mu$ L	-20°C
<b>Water</b>	Green disc	2 x 833 $\mu$ L	-20°C
<b>Pool A PCR</b>	Purple disc	1 x 144 $\mu$ L	-20°C
<b>Pool B PCR</b>	Yellow disc	1 x 144 $\mu$ L	-20°C
<b>TP53 Index</b>	Purple plate	48 x 6 $\mu$ L	-20°C

Table 1. Reagents of the TP53 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)
- *Qubit Fluorometer* (ThermoFisher)
- NGS Sequencer (Illumina)
- *TapeStation System* by Agilent Technologies (optional)

**Reagents:**

- *Agencourt® AMPure® XP beads* (Beckman Coulter Genomics cat. no. A63880, A63881 or A63882)
- Absolute ethanol
- Nuclease free water
- *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)
- *TapeStation* (optional): *High Sensitivity D1000 Reagents* (cat. no. 5067-5585; Agilent)

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

**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)
- *High Sensitivity D1000 ScreenTape* (cat. no. 5067-5584) (optional)

**NOTE**

*TP53 OncoKitDx* is prepared for use in combination with the *Sample tracking A kit* (REF: IMG-234), and *Sample tracking B kit* (REF: 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

## 07.1 | Preparation of the first amplification reaction

TP53 OncoKitDx includes two primer pools (*Pool A-PCR* and *Pool B-PCR*), 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. Below is detailed the procedure to follow to perform this assay.

- 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 For one sample, add the required volumes of the reagents indicated in the following table to two fresh 1.5 mL tubes, and scale up accordingly based on the total number of samples. It is recommended to perform the calculations by adding sufficient reagents to analyze 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
TP53 Buffer	1.2 µL	1.2 µL
Water	9.8 µL	9.8 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 analyzed per batch, the *Sample tracking B Kit* has been created, REF. IMG-311. Tracking kits allow to analyze up to 24 samples for sequencing run.
- 04 Add 2 µL of sample DNA at 10 ng/µL (quantified by *Qubit fluorometer*, ThermoFisher) 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 each reaction contains 20 ng total of DNA.
- 05 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	
60°C	1 minute	22
72°C	30 seconds	
72°C	5 minutes	1
10°C	∞	1

Table 2. Optimal PCR programme for SimpliAmp Thermal Cycler and GeneAmp PCR System 9700

↘ 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.

## 07.2 | Preparation of the second amplification reaction

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

**NOTE:** For each sample, one reaction will be carried out.

- 01 Thaw the *General Master-Mix III*, *TP53 Buffer*, the required index primers, and the products of the first PCR, if frozen. Vortex each of the reagents and keep on ice. For each sample, two unique Index primers (already pre-mixed in the purple plate) are required. In case of running several samples on the same run, each sample must have a unique combination of Index primers. The position of the index primers, as well as their sequence, are reported in the Tables 1 and 2 of the Appendix I of this manual.
- 02 Thaw the product of the first PCR in case it is frozen. Vortex and spin down.
- 03 Add the quantities of reagents specified in in the following table 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.

Reagent	Volume per reagent
General Master Mix III	6 µL
TP53 Buffer	5.4 µL
Water	15.1 µL

- 04 Vortex the PCR mixtures and distribute 26.5 µL in each PCR well.
- 05 Add 1.5 µL of a unique pre-mixed Index primers to each sample.
- 06 Add 1 µL of the PCR product of Pool A and 1 µL of Pool B of the same sample.

**NOTE:** As in this second PCR the template DNA is a product of a previous amplification, in order to avoid contaminations, we recommend to prepare the PCR-mix in the pre-PCR laboratory (a pre-amplification area, PCR-products free) and then perform the step 5 of the protocol in a post-PCR zone. We also recommend to clean the surfaces with DNA decontamination reagents.

- 07 Place the samples in a 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	
60°C	90 seconds	25
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

↘ The protocol can be stopped at this point. PCR products can be stored at 4°C for the next 24 hours or at -20°C until it is time for their use.

## 07.3 | Purification of the products of the second PCR

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 Prepare one 1.5 mL Eppendorf tube per sample.
- 03 Dispense 25 µL of magnetic beads into each 1.5 mL Eppendorf tube (Ratio 1:1).
- 04 Transfer 25 µL of PCR reaction to a corresponding 1.5 mL Eppendorf tube.
- 05 Place the tubes on a shaking block for 2 minutes, at 1800 rpm and at room temperature.
- 06 Incubate for 5 minutes at room temperature.
- 07 Place the tubes on a magnetic plate for 2 minutes so that the magnetic beads adhere to the magnet.
- 08 Remove the supernatant, pipetting carefully.
- 09 Add 200 µL of freshly prepared 80% ethanol.
- 10 Repeat steps 8 and 9 of the protocol.
- 11 Remove the supernatant. It is very important that no ethanol traces remain.
- 12 Leave to dry at room temperature for 10 minutes. During this time, remove any remnants that could have remained on the tube walls.
- 13 Add 25 µL of the *Elution Buffer* reagent (Ref: 19086; Qiagen).
- 14 Remove the tubes from the magnetic plate and re-suspend vigorously by vortexing until the bead-elution buffer solution is homogenized.
- 15 Incubate for 2-10 minutes at room temperature.
- 16 Place the tubes on the magnetic plate again for 2 minutes so that the magnetic beads adhere to the magnet.
- 17 Transfer the supernatant to a new tube and discard the tube with the magnetic beads.

↘ The protocol can be stopped at this point. PCR products can be stored at 4°C for the next 24 hours or at -20°C until it is time for their use.

## 07.4 | Preparation of the libraries for sequencing

### ➔ CALCULATION OF THE LIBRARY CONCENTRATIONS

First, Health in Code S.L. recommends verifying the size of the libraries by using the *TapeStation 2200* and the commercial *High Sensitivity D1000* (Ref:5067-5585) and *High Sensitivity D1000 ScreenTape* (Ref:5067-5584) kits from Agilent Technologies or running them on a 3% agarose gel with a voltage of 120 V for 30 minutes. The average size obtained should be around 280-300 bp as shown in the image below. If the average size is not the expected one, check the protocol or contact our Technical Support team.

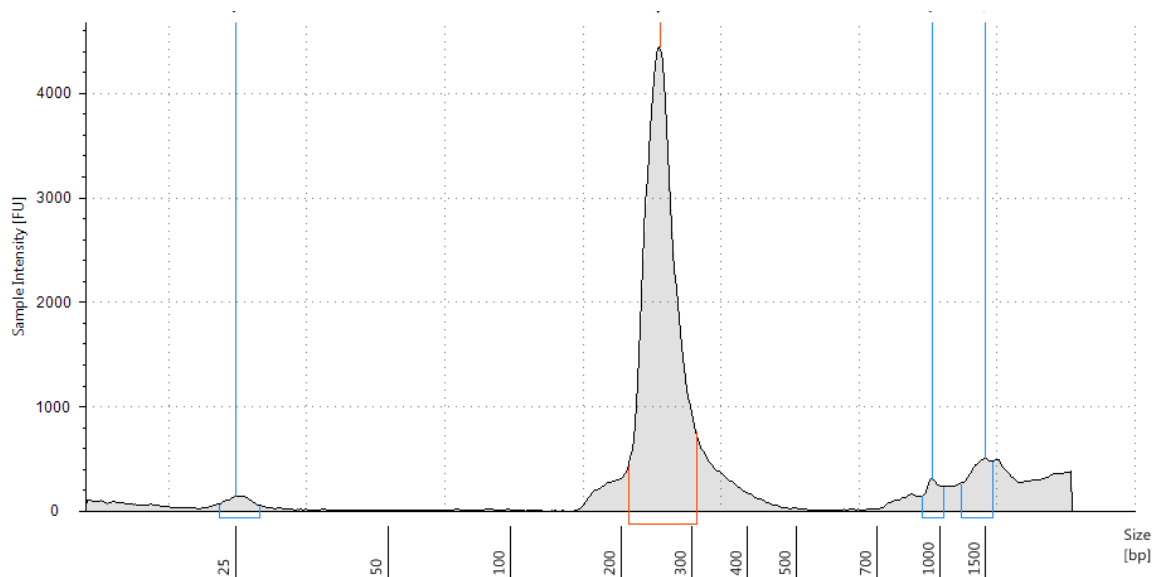


Figure 1. Expected size result from the analysis of the library by TapeStation 2200

Then, 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 library concentration is obtained using the concentration value obtained by Qubit according to the following formula:

$$\text{Library concentration (nM)} = (\text{Concentration (ng/}\mu\text{L)}) \times 5.36$$

**NOTE:** The 5.36 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:

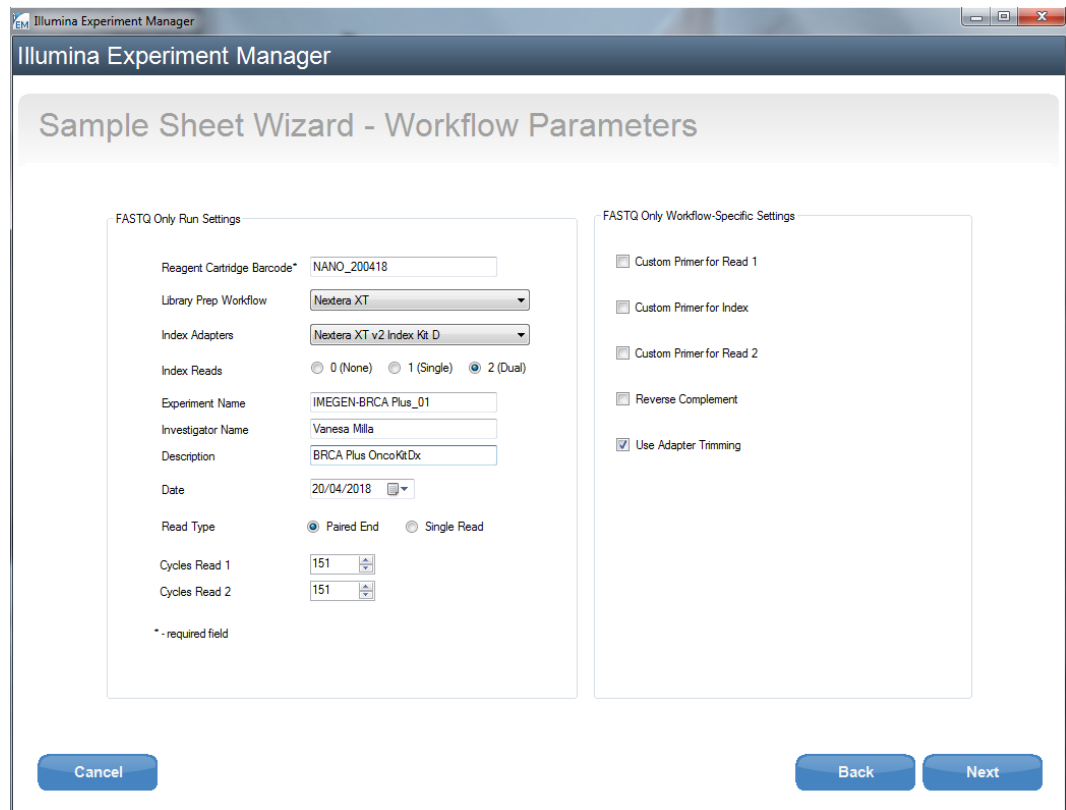


Figure 2. Sample Sheet creation with Illumina Experiment Manager

- 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.

## ➤ DENATURATION AND LOADING OF THE LIBRARIES

In order to load the sample in the Illumina *MiSeq* the following denaturing protocol has to be performed:

- 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 into a fresh 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 60 µL of denatured PhiX control diluted to 12.5 pM. At this time, the libraries will be at 9.4 pM concentration.
- 09 Load the volume contained in the 1.5 mL tube onto the sample loading reservoir on the cartridge.

The following table specifies the maximum recommended number of samples per run, depending on the sequencing kit used, to guarantee a minimum number of clusters of approximately 20,000 per sample, and a minimum depth of 500x:

MiSeq Reagents Kit	Maximum no. of samples
MiSeq Reagents nano Kit v2 (300 cycles). Ref: MS-103-1001	48
MiSeq Reagents micro Kit v2 (300 cycles). Ref: MS-103-1002	160

Table 4. Samples recommended for analysis using TP53 OncoKitDx

**NOTE:** The kit includes enough indexes to analyze 48 samples in a single sequencing run. If more than 48 samples are analyzed in a single run, please contact our technical support team.

If other Illumina sequencing reagents are used, the **TP53 OncoKitDx** libraries can be loaded with other libraries as long as they are individually tagged with specific 8-nucleotide indexes.

If a different Illumina NGS sequencer is used, the final concentration of the libraries will need to be adjusted according to the protocol specifications of that platform.

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 **TP53 OncoKitDx** with the **Data Genomics** analytical platform. Access the analysis pipeline via [www.datagenomics.es](http://www.datagenomics.es).

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

## 08.1 | Request for analysis

- 01** Select "*Import Sample Sheet*" from the main screen (*Order* screen) in order to begin the analysis of the sample. This will take you to the file import tab (image shown below). From this tab you can import the samples fastq files and, optionally, the *Sample Sheet* file, that will allow to import all the samples of the same run simultaneously.
- 02** Once you have loaded the files, you will select the name of the sequencing run and the study method, *TP53 OncoKitDx* in this case, and the *STID* (*Sample Tracking ID*) used in each sample, or "*does not contain*" in case of not having used any.
- 03** To carry out the request, select "*Process*". When the process has terminated successfully a message will appear "**✓ samples load performed correctly**".

The screenshot shows the 'Import samples' interface. At the top left is the 'data genomics' logo. At the top right are links for 'Site survey' and 'User guide'. The main area is titled 'Import samples' and contains a 'Data Files' section with 'Files' and 'Folders' buttons, and an 'Add Sample Sheet' button. Below this is a 'Load' button. A table with columns 'Sample Name', 'Comments', 'STID', and 'Tumoral Sample Files' is shown. At the bottom, there are buttons for 'Process', 'Cancel all samples', and 'Back to orders'.

Figure 3. Screen to import fastq and Sample Sheet files



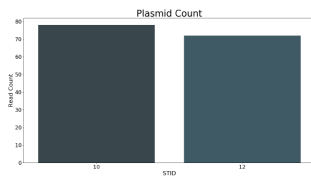
## 08.2 | Management of orders

All requests created will appear on the "Order" tab, along with their associated applications and their status (*Pending, In review, Finalized, 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.

In the event that the sequencing has not passed the quality controls established or that the tracking reagent does not match that expected, an alert on the button corresponding to the quality control will be displayed, once the bioinformatic analysis is completed. By clicking this icon, you will be able to access directly to the quality control report.

Through the "Bioinformatics" 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, which includes verification that the tracking reagent obtained the expected match, as shown in the following example:



Counts of each plasmid detected.

### QC of the tracking sample

Feature	Obtained	Expected	Status
STID	1012	1210	MATCH

Figure 4. Quality control of the sample tracking system

## 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 TP53 gene with all the data related to such variants.

Figure 5. Variants Filtering in Data Genomics

- + In the "Action" column, selecting the IGV option, you can visualize the sequence of each variant.
- + The "HEALTH IN CODE DB" application calculates the allelic frequency of each variant found within the population of samples analyzed by the client with this tool. These calculations appear in the *Variants Filtering* screen in three columns:
  - ◇ **Frq Own**: Allelic frequency of the variant in the client's samples in which it has been sought.
  - ◇ **Frac Freq Own**: Fraction of the number of occurrences of the variant and the number of total client samples for which this region has been analyzed.
- + It is possible to generate a file of the selected variants for each sample, either as a .csv or as a report in pdf. These reports are stored along with the rest of the information associated with each sample.

In case you require more information about processing data with the bioinformatic analysis tool, you can access the **Data Genomics** manual via the link [www.datagenomics.es](http://www.datagenomics.es).

## 09 Troubleshooting

Listed below are the possible unexpected results throughout the protocol for the amplification and sequencing of the targets of the **TP53 OncoKitDx**.

### + Library concentration too low:

Concentrations lower than 10 ng/μL may be due to an error in the amplification protocol or in the purification of the libraries. In this case, it is recommended to analyze the library using the *TapeStation 2200*, with commercial *High Sensitivity D1000* reagents (Ref:5067–5585) and the *High Sensitivity D1000 ScreenTape* (Ref:5067–5584) from Agilent Technologies (Figure 1).

- ◇ If there is one single peak that roughly corresponds to the expected size, average size of 280 bp, continuing with the sequencing protocol is appropriate.
- ◇ If instead of a single 280 bp fragment there are several amplicones, review the different steps of the amplification and purification protocol.

### + Cluster density different from expected:

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 (if you use Health in Code S.L bioinformatics software):

In these cases, it is recommended to analyze the libraries with the *TapeStation 2200* (Figure 1).

- ◇ If the average size of the library is 280–300bp, it is recommended to revise the denaturing protocol and the sequencer loading protocol.
- ◇ If instead of a single 280 bp peak, several amplification products have been produced, review the different steps of the amplification and purification protocol.

### + Errors in the STIDs:

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.

# 10 Limitations

## 10.1 | Equipment

TP53 **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 for any query or clarification.

TP53 **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.

## 10.2 | Reagents

TP53 **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 recommended to use the reagents recommended by the sequencer supplier: **Illumina**.

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

## 10.3 | Bioinformatic analysis platform

TP53 **OncoKitDx** has been validated using **Data Genomics**, a bioinformatic analysis platform for in vitro diagnosis. This platform includes an analysis pipeline specially designed for **TP53 OncoKitDx**, which allows the detection of all the targets specified in section 2 of this document.

In case of using another analysis platform, Health in Code S.L. is not responsible for the obtained results.

## 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.

appendix  
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	1	2	3	4	5	6
A	S513 N716	S517 N716	S518 N716	S520 N716	S521 N716	S522 N716
B	S513 N718	S517 N718	S518 N718	S520 N718	S521 N718	S522 N718
C	S513 N719	S517 N719	S518 N719	S520 N719	S521 N719	S522 N719
D	S513 N720	S517 N720	S518 N720	S520 N720	S521 N720	S522 N720
E	S513 N721	S517 N721	S518 N721	S520 N721	S521 N721	S522 N721
F	S513 N722	S517 N722	S518 N722	S520 N722	S521 N722	S522 N722
G	S513 N723	S517 N723	S518 N723	S520 N723	S521 N723	S522 N723
H	S513 N724	S517 N724	S518 N724	S520 N724	S521 N724	S522 N724

Table A1. Pair-Index position in the Index plate

Name	Sequence
S513	TCGACTAG
S517	GCGTAAGA
S518	CTATTAAG
S520	AAGGCTAT
S521	GAGCCTTA
S522	TTATGCGA
N716	ACTCGCTA
N718	GGAGCTAC
N719	GCGTAGTA
N720	CGGAGCCT
N721	TACGCTGC
N722	ATGCGCAG
N723	TAGCGCTC
N724	ACTGAGCG

Table A2. Sequences for each index

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

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