

INSTRUCTIONS FOR THE USE OF OG-TP53

OG-TP53

**READY-TO-USE KIT FOR NGS LIBRARY GENERATION BY AMPLICONS FOR TP53
GENE FOR ILLUMINA® SYSTEMS**









RUO
Research Use Only

Protocol version 2 , April 2019

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1. GLOSSARY OF TERMS

	Stable up to YYYY/MM		Storage temperature
	Maker		Instructions for use
	Reference	RUO	Research Use Only
	Lot code		

Ordering information:

Online: www.seqplexing.com

Email: info@seqplexing.com

Related references

- OG-TP53-24, OG-TP53-96 (kits for 24 and 96 samples, respectively; BARDOCES INCLUDED)
- OG-TP53-NB-24, OG-TP53-NB-96 (kits for 24 and 96 samples, respectively; BARDOCES NOT INCLUDED)
- OG-BC-01, OG-BC-02, OG-BC-03, OG-BC-04 (BARCODES: 24 combinations for each reference; for higher number of barcode combinations, ask to info@seqplexing.com).

2. BACKGROUND AND INTENDED USE

In recent years, many genetic alterations have been identified in tumors (somatic mutations) of different nature that affect the treatment response, evolution and prognosis of the disease. Recently, anticancer drugs have been developed whose efficacy increases or decreases considerably depending on the presence of certain somatic mutations in different tumors. Therefore, the need to know if a tumor has mutations important for treatment has made the study of these mutations essential to establish such treatment and, many times, is mandatory. So, many drugs require specific genetic studies to be used. To carry out these studies, DNA must be obtained from tumor tissue, usually included in paraffin. The inclusion in paraffin produces the alteration and fragmentation of DNA, so specific techniques are required to work with this genetic material.

On the other hand, somatic mutations in tumors can be found in a low proportion with respect to normal DNA. This may be due to the presence of a small proportion of tumor cells in the sample, a small proportion of cells with the mutation, or both. Being able to be detected with our kits from 1% of the copies present in the analyzed DNA.

Our OncoGenBasic (OGB) kits are based on the generation of amplicon libraries for NGS, which Seqplexing has optimized (Figure 1). OGB protocols allow the use of the first PCR product directly in the second, without purification or quantification of the PCR product. Our kit OGB for TP53 (OG-TP53) allows the amplification of all regions of interest for TP53 gene analysis in only two consecutive PCR reactions. The protocol produces specific amplification free of artefacts that can interfere with data analysis. The protocol is simple and requires little manual work. The final product is ready to perform the sequencing reaction with an NGS system from Illumina® that allows paired-end (PE) reactions of 150 bp, dual barcoding and using Nextera oligos for sequencing (Figure 2). Our kits produce practically only specific products (more than 98%), avoiding different problems in the analysis that may be produced by nonspecific or artefactual products (products in unwanted regions, small-sized PCR products or PCR products that include several amplicons).

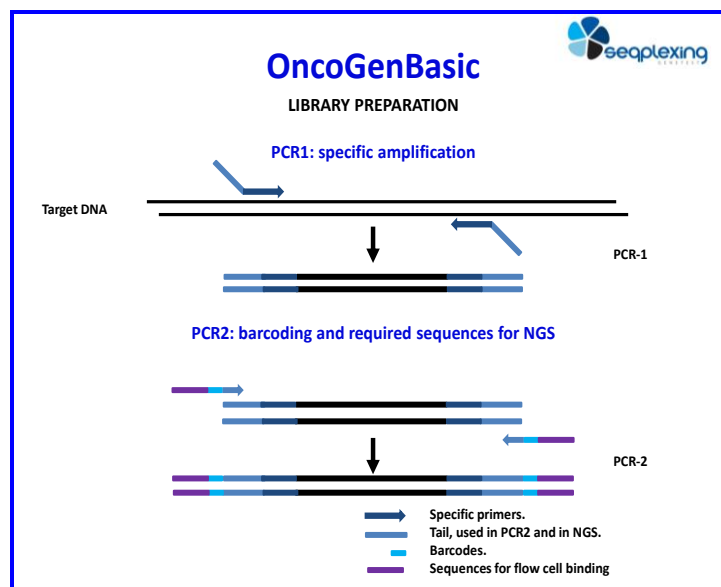


Figure 1: Diagram of the process used for libraries generation by amplicons used in OGB kits. The products of PCR1 are used directly in PCR2, without the need to purify.

Our OG kits allow detecting all point mutations in more than 1% of the DNA of the copies present in the regions analyzed. The analysis of the libraries is done by sequencing the amplicons by the MiSeq system or other Illumina® systems by 150x2 sequencing (PE sequencing and dual barcoding). Therefore, the kit can be used for the detection of somatic and germline mutations, requiring different depth for each application: 1000x minimum for somatic and 100x for germline mutations. The OG-TP53 kit allows detecting the point mutations present in all the coding regions of the TP53 gene and the important regions for the splicing at the ends of the exons and introns that may have scientific and/or clinical interest (Figure 2). The reference sequences used are SwissProt P04637, NC_000017.10, NM_000546 and ENST00000269305.8 (GRCh38.p12).

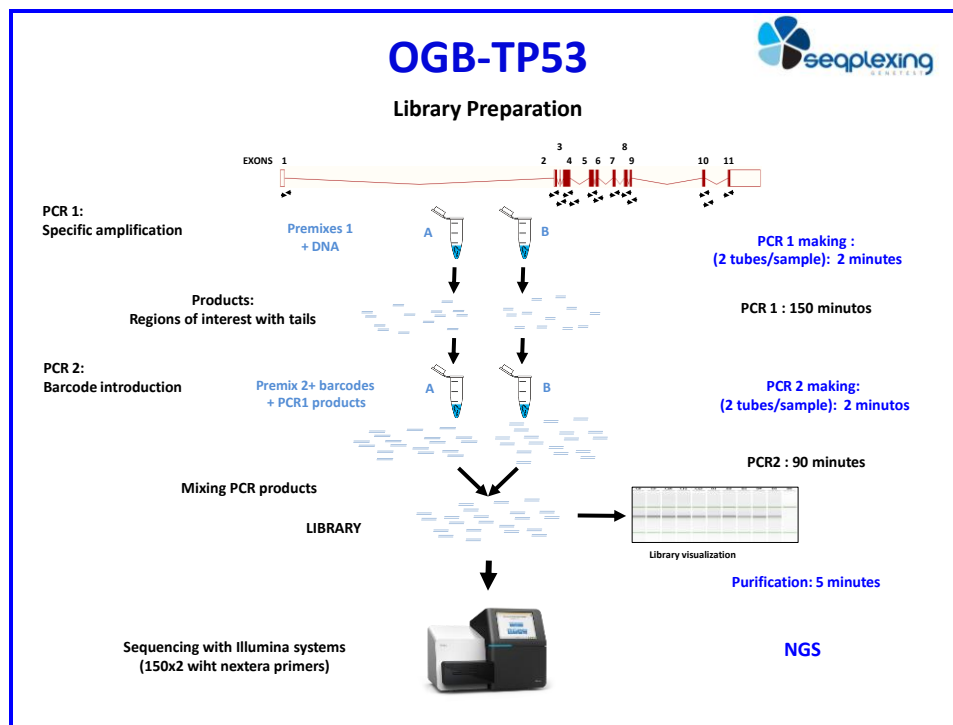


Figure 2: Schematic representation of the TP53 gene, the regions analyzed and the process necessary to obtain the NGS libraries.

3. CONTENT AND CONSERVATION

The OG-TP53 kit includes reagents for 24 or 96 samples that allow the amplification and generation of libraries for the analysis of the regions of interest. The reagents are sufficient to perform:

- Amplification reaction (two reactions): the specific amplification reaction that generates the amplicons for all regions of interest in TP53 and includes specific tails at the ends.
- Labelling reaction or "barcoding": reaction using the introduced tails to generate complete libraries for Illumina® sequencing and including barcodes for the sample identification. The barcodes allow the identification of 24 or 96 samples, there are kits that include the barcodes (ref: OG-TP53-24, OG-TP53-96) and others that require that they be purchased separately (ref: OG-TP53-NB- 24, OG-TP53-NB-96).

The final products include the regions necessary to carry out the sequencing with the Illumina® System (using Nextera primers).

The kit consists of:

- PCR1-Premix A: 1 or 4 tubes (kits for 24 or 96 samples, respectively) with all the necessary reagents to amplify half of the regions of interest. Each tube contains a volume of Premix 1 sufficient to dispense 12 µl per sample for 24 samples.
- PCR1-Premix B: 1 or 4 tubes (kits for 24 or 96 samples, respectively) with all the necessary reagents to amplify the other half of the regions of interest. Each tube contains a volume of Premix B sufficient to dispense 12 µl per sample, for 24 samples.
- Premix of PCR2: 1 or 4 tubes (kits for 24 or 96 samples, respectively) with all the reagents necessary for the labelling reaction except the barcodes (must be purchased separately).

The kit should be stored at -20°C from reception.

4. REQUIRED MATERIAL NOT SUPPLIED IN THE KIT

This item shows the list of all the necessary materials and equipment that are not supplied with the kit.

4.1. Required reagents not supplied

- Set of barcodes available for the Labeling Reaction (See references: OG-BC-01, OG-BC-02, OG-BC-03, OG-BC-04), required for references OG-TP53-NB.
- DNA extraction kits for different types of samples. For paraffin extraction, some kits have been tested: the cobas® DNA sample preparation kit (cobas® DNA Sample Preparation Kit, Roche), the FFPE DNA isolation kit from High Pure (High Pure FFPE DNA Isolation Kit, Roche) or the kit of purification Maxwell® 16 LEV RNA FFPE (Maxwell® 16 LEV RNA FFPE Purification Kit, Promega). For the extraction of blood or cells, Maxwell® protocols and specific Qiagen kits have offered good results. Any other extractions system suitable for NGS can be used but we recommend to determine or verify if they can cause alterations in the DNA that may affect the result.
- Purification kits MagSi-NGS PREP (MagnaMedics, Ref: MD60021,) or Ampure (AgencourtAMPure XP, BeckmanCoulter, Ref: A63880).
- Kits for quantification by fluorometry with the QuantiFluor™ dsDNA System (Promega).
- MiSeq reagents for sequencing with Nextera oligos: 300 bp sequence kits that allow the sequencing of PE of 150 bpx2 plus the reading of barcodes at both ends.

4.2. Equipment

- PCR-machines (96-well or for 200 µl tubes) with heated lid for tubes or plates. The protocol has been carried out in thermocyclers from AppliedBiosystems-ThermoFisher and Eppendorf.
- Micropipettes for volumes between 1 and 100 µl (0.5 to 10 is recommended, another of 2-20 and another of 10-100 or up to 200 µl).
- Centrifuge for 200 µl strips, tubes or plates.

- Electrophoresis system that allows analyzing the libraries obtained: agarose gel, Bioanalyzer (Agilent Technologies), Qiaxcel® (Qiagen), etc.
- Fluorimeter (necessary to calculate the concentration of double-stranded DNA with QuantiFluor™ or similar before continuing with the sequencing procedure).
- MiSeq system (Illumina®) or other Illumina® systems that allow the sequencing of PE of 150 bp and the dual barcode (readings of 150x2). Barcodes are ready for their use with the different illumine systems.

5. GENERAL RECOMENDATIONS FOR WORKING WITH PCR.

The system developed is very sensitive to allow the detection of variants present in low proportions and work with very small amounts of DNA. Therefore, it can detect any contamination between samples or with reaction products. Therefore, any source of contamination should always be avoided and the samples should not be contaminated between them.

- Verify that everything necessary is available.
- Pre-PCR area: will never be used with PCR products to avoid contaminating the samples or PCR reactions with these products. There should be specific micropipettes for this area (they should never be used with PCR products).
- Post-PCR area: PCR products should be handled in specific areas that are easy to clean and different from the PCR preparation area. The micropipettes should be specific to this area and the gloves and clothing used in this area should not be used in other laboratory areas, especially in the Pre-PCR.
- Before starting any experiment, the work area and all the surfaces of the material present in this area (pipettes, bench, hood, frames, etc.) must be cleaned with products for the DNA elimination.
- Identify the plates, tubes or strips that will be used. They can be identified by the coordinates of the plates and identifying the plate.
- Always use pipette tips with filter and free of DNA and DNAses.
- Use clean gloves in each amplification procedure (Pre-PCR area), if reagents or material from another area of the laboratory is touched, the gloves should be changed.
- Avoid touching the inside of the caps of tubes and covers, samples, dilutions, etc.
- The procedure for using the entire kit in a single experiment is explained, but it can be subdivided using fewer samples. For this, you have to take into account including at least one negative control in each experiment.
- Sequencing of the libraries obtained with this kit can be done in combination with libraries obtained with other OncoGenBasic kits from Seqplexing.

6. RECOMENDATIONS FOR LIBRARY HANDLING AND SEQUENCING

Follow the instructions of the Illumina® sequencing system selected for sequencing reaction. Our recommendations are:

- Discard those libraries that have intense bands of 150 bp or lower size (possibly primer dimers and have not been amplified correctly). These libraries will produce a reduction in the useful readings and will modify the concentration of the pool.
- Purify the libraries obtained with MagSi-NGS PREP (MagnaMedics) or Ampure (AgencourtAMPure XP, BeckmanCoulter) following their instructions.

- It is important to place the same amount of library of each sample in NGS reaction, so it is necessary to quantify the purified product. QuantiFluor™ dsDNA System (Promega) can be used, according to standard procedures. The GloMax®-Multi Detection System (Promega) can also be used as a quantization method.
- Adjust the concentration to 10 nM (1.9 ng/μl) of each sample with 0.1% Tris-Tween 20 (prepared according to the indications of Illumina®). In the calculation of the readings that will be obtained per sample, it should be considered that 20 amplicons are analyzed.
- The samples amplified with other OG kits can be sequenced simultaneously. To facilitate this procedure, Seqplexing can provide calculation pages to improve the distribution of accurate reading between samples.
- Add 5 μl of each sample in a clean tube (do not include negative control or samples that have not been amplified correctly).
- Vortex for 1 minute to mix well. This library can be stored frozen at -20°C to use it another day.
- To carry out the sequencing, the indications of the Illumina® System are used.

ALL THE PURIFICATION AND PREPARATION OF THE SAMPLES PROCESS FOR SEQUENCING MUST FOLLOW THE INDICATIONS OF THE PROTOCOLS OF ILLUMINA® AND THE REACTION OF THE SEQUENCING WITH PRIMERS NEXTERA. THE ANALYSIS MUST BE CARRIED OUT IN ACCORDANCE WITH THESE PROTOCOLS. THE BOATS OF OUR KITS CORRESPOND TO THE NUMBERING AND SEQUENCE USED BY ILLUMINA®.

7. SAMPLES

The kit is designed for the analysis of DNA from samples included in paraffin and from other sources, such as saliva or blood samples. DNA fragmentation is especially relevant, so the more complete the DNA, the smaller amount of DNA is necessary, since there are more effective copies of DNA. An extremely fragmented DNA may not be amplifiable. Our studies with numerous samples indicate that the minimum amount of DNA required is 5 ng / reaction but could be lower / higher depending on the state of the DNA.

We recommend the quantification of the starting DNA with the QuantiFluor™ dsDNA System (Promega) or a similar fluorometric method. The GloMax®-Multi Detection System (Promega) can also be used as a quantization method. The kit accepts a wide range of concentrations; we recommend working with concentrations between 5 and 100 ng / μl.

8. PROTOCOL

8.1. Amplification of target regions

Use the necessary tubes, wells or plates for the desired number of reactions. Each sample should be initially amplified in 2 separate tubes (one with PCR1-Premix A and the other with PCR1-Premix B), these 2 tubes should also be used for the controls.

IMPORTANT: NEVER MIX THE CONTENTS OF THE PCR1-PREMIX 1 AND PCR1-PREMIX 2 TUBES!

If you are going to analyze many samples, it is convenient to place several negative controls.

- 8.1.1. Thaw the tubes with PCR1-Premix A and B. Vortex for 5 seconds and centrifuge for 5 seconds (verify that the entire volume is on the bottom).
- 8.1.2. Prepare and label tubes, strips or PCR plates. 2 tubes / wells will be used for each sample (one for each Premix of PCR1).
 - 8.1.2.1. Distribute 12 µl of PCR1-Premix A in as many tubes as samples plus controls.
 - 8.1.2.2. Distribute 12 µl of PCR1-Premix B in as many tubes as samples plus controls.
- 8.1.3. Add 3 µl of genomic DNA (between 5 and 100 ng total) to the tubes/wells with PCR1-Premix A and B.
- 8.1.4. Add 3 µl of water in the negative control reactions (PCR1-Premix A and B).
- 8.1.5. Close the tubes, strips or plates with the corresponding lid.
- 8.1.6. Centrifuge 10 seconds. Check that the entire volume is on the bottom and that there are no bubbles.
- 8.1.7. Place in the thermal cycler and use the following PCR program

STEP	Temp. (°C)	Time (seconds)	Cycles
Activation	98	900	1
Amplification	98	30	25
	55	60	
	60	60	
	65	60	
	70	60	
Final extension	70	1200	1
Stop-conservation	4-10	∞	1

Once the first amplification reaction is completed, the product obtained will be used as a template for the barcoding reaction WITHOUT PURIFICATION (protocol is optimized to use the product of this PCR without purifying).

The PCR product obtained can be conserved at 4° (hours-days) or -20°C (days-months) up to its use.

8.2. Labeling reaction with barcodes and the remaining sequences required for NGS reaction of with Illumina® Systems.

THE SECOND PCR REACTION IS ALSO CARRIED OUT IN 2 SEPARATE REACTIONS, SO EACH SAMPLE MUST BE AMPLIFIED IN TWO TUBES USING THE SAME COMBINATION OF BARCODES FOR EACH SAMPLE. See figure 2.

- 8.2.1. Thaw the PCR2-TP53 premix tube. Mix well and centrifuge at the maximum speed for 5 seconds (check that the entire volume is at the bottom and that there are no bubbles).
- 8.2.2. Dispense 12 µl of the Premix of PCR2-TP53 in each tube or well where a sample will be analyzed (1 for reaction A of PCR1 and another for reaction B of each sample and controls).
- 8.2.3. Add 2 µl of the selected barcodes mix for each sample (includes 5 'and 3' combination) to each tube or well. ATTENTION: THE SAME COMBINATION MUST BE USED FOR A SAMPLE IN THE TWO WELLS AND MUST BE DIFFERENT FROM THE OTHER SAMPLES. See recommendation for Barcode Combinations (point 8.3).
- 8.2.4. Add 1 µl of the product of PCR1-Premix A to the well / tube of the PCR2-TP53-Premix A reaction.
- 8.2.5. Add 1 µl of the PCR1-Premix B product to the PCR2-TP53-premix B reaction well / tube.
- 8.2.6. Add 1 µl of PCR1-Premix water in the negative control reactions.

8.2.7. Close the tubes, strips or plates with the corresponding lid.

8.2.8. Centrifuge 5 seconds. Check that the entire volume is on the bottom and that there are no bubbles.

8.2.9. Place in the thermal cycler and use the following PCR program

STEP	Temp. (°C)	Time (seconds)	Cycles
Activation	95	900	1
Amplification	98	20	38
	60	30	
	72	60	
Final extension	72	600	1
Stop-conservation	15	∞	1

8.2.10. Once the amplification reaction ends, the products of each sample are visualized by combining the two reactions (A + B of PCR2) or independently. Samples and amplified controls can be analyzed by different systems (QiAxcel, Bioanalyzer, agarose gels, etc.).

Briefly: for the QiAxcel® system (Qiagen) mix 2 µl of the PCR 2 product for one sample plus 8 µl of dilution buffer. For a high sensitivity agarose gel: prepare 2-3% high sensitivity agarose and 1% normal agarose, charge 2 + 2 µl of each PCR product (containing the libraries) generated for each sample, 4 µl of buffer of charge and a DNA size marker between 50 and 400. It should be used in a separate well. Visualize the amplification results and continue with the instructions. Figure 3 shows the appearance of the libraries.

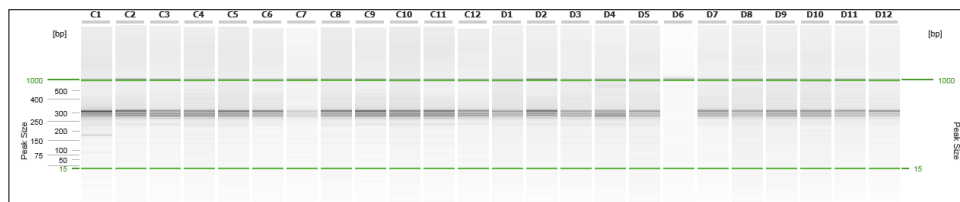


Figure 3: Example of the results obtained from the amplification with real samples. Each well shows the result of one of the 23 samples and the negative control (well D6). Well C1 has a small amount of dimer, well C7 has low amplification by having a much smaller amount of start DNA than recommended, both can be analyzed by NGS. The rest of the samples have been amplified correctly.

The libraries obtained can be conserved at 4° (hours-days) or -20°C (days-months) up to its use.

8.3. Barcode use.

The barcodes allow the identification of 24 or 96 samples, we have kits that include barcodes (ref: OG-TP53-24, OG-TP53-96) and others that require their purchase separately (ref: OG-TP53-NB -24, OG-TP53-NB-96). In these kits (labelled by NB) for 24 samples, the additional acquisition of a barcode kit is required for 24 samples (reference OG-BC-01). In the case of needing more than 24 barcodes, Seqplexing offers combinations of barcodes up to 96 samples (OG-BC-02, OG-BC-03, OG-BC-04); if a larger number is required, ask at info@seqplexing.com.

8.4. Comments

- We use two separate reactions for each sample and specific reagents to reduce nonspecific amplifications.

- If the DNA is in the right conditions, a wide band of approximately 250-300 bp or several nearby bands will be observed according to the resolution of the system and no bands different from the previous ones will be observed.
- The negative control should not present bands higher than 200 bp, although smaller bands corresponding to the "primer dimer" can be seen, normally around 100-150 bp.
- If any sample has only bands below 150 bp or these are more intense than the 250-300 bp bands, the sample should not be analyzed by NGS, since the PCR products obtained are mainly primer-dimers. These would produce a large number of sequences that are not useful since they only contain primer sequences.

9. TROUBLESHOOTING

- 1) No band is amplified (in any case) or there are only bands corresponding to dimers and primers: verify that all steps of the protocol have been correctly performed and that the DNA extraction has worked correctly.
- 2) There are several reasons why a sample is not amplified:
 - a. The presence of inhibitors or a failure in the extraction of the sample do not allow amplification of the amplicons. To solve it, it is necessary to repeat the extraction, or purify the DNA already obtained to eliminate contaminants, or perform tests with samples that are correctly amplified in other PCR protocols. The kit allows working with samples taken from paraffin and other sources. Paraffin samples are usually extracted without any problem, although they can give more problems than others and specific kits should be used. The OGB-TP53 kit is verified with the "cobas® DNA sample preparation kit" (Roche), the "high purity FFPET DNA isolation kit" (Roche) and the LEV Tissue LEV DNA purification kit from Maxwell 16
 - b. The absence of amplification in one of the tubes and the presence of amplification in other tubes may indicate that a correct extraction was performed. Usually, in this case, the samples that fail are due to the fact that not enough material has been obtained due to a specific error in that sample, that the starting material was degraded or insufficient.
- 3) The negative control has bands in the region of the amplicons obtained in the samples: there has been a contamination in the reaction. It must proceed to decontaminate all the material used in the preparation of the PCR reaction and work areas, etc. and repeat the reaction with one or two samples and a negative control to verify that the problem has been solved.

Any question or problem about the protocols or results can be directed to info@seqplexing.com and we will respond as soon as possible.)

10. BRIEF PROTOCOL FOR EXPERIENCED USERS

10.1. Reaction 1: Region of interest amplification.

- PROCEDURE (24 or 48 reactions). Each sample or control must be amplified separately with Premix A and Premix B.
- 10.1.1. Thaw the tubes with PCR1-Premix A and B. Mix well each tube and centrifuge 5 seconds at maximum speed.
 - 10.1.2. Prepare and label tubes, strips or PCR plates. Two tubes or wells will be used for each sample and control (one for each Premix of PCR1).
 - 10.1.3. Distribute 12 µl of each PCR1 premix in different tubes/wells.
 - 10.1.4. Add 3 µl of genomic DNA (between 5 and 100 ng in total) to each tube/well.
 - 10.1.5. Add 3 µl of water to the negative control tubes (one for each PCR1 Premix).
 - 10.1.6. Close the tubes/wells and centrifuge the reaction mixtures for 10 seconds at maximum speed.
 - 10.1.7. Load in the thermal cycler and use the following PCR program:

STEP	Temp. (°C)	Time (seconds)	Cycles
Activation	98	900	1
Amplification	98	30	25
	55	60	
	60	60	
	65	60	
	70	60	
Final extension	70	1200	1
Stop-conservation	4-10	∞	1

10.2. Labeling reaction with barcodes and the remaining sequences required for NGS reaction of with Illumina® Systems.

THE SECOND PCR REACTION IS ALSO CARRIED OUT IN 2 SEPARATE REACTIONS, SO EACH SAMPLE MUST BE AMPLIFIED IN TWO TUBES USING THE SAME COMBINATION OF BARCODES. See figure 2.

- 10.2.1. Defrost the Mix PCR2-TP53 tube. Mix and centrifuge for 5 seconds.
- 10.2.2. Dispense 12 µl of the Mix of PCR2-TP53 in each tube or well where a sample will be analyzed (1 for reaction A of PCR1 and another for reaction B of each sample and controls).
- 10.2.3. Add 2 µl of the selected barcode mix for each sample (includes 5' and 3' combination) to each tube or well. ATTENTION: THE SAME COMBINATION MUST BE USED FOR ONE SAMPLE IN THE TWO WELLS AND COMBINATION MUST BE DIFFERENT FROM THE OTHER SAMPLES. See recommendation of Barcode Combinations (point 8.3).
- 10.2.4. Add 1 µl of the product of PCR1-Mix A to the well/tube of the PCR2-TP53-Mix A reaction.
- 10.2.5. Add 1 µl of the PCR1-Mix B product to the PCR2-TP53-Mix B reaction well/tube.
- 10.2.6. Add 1 µl of PCR1-Mix water in the negative control reactions.
- 10.2.7. Close the tubes, strips or plates with the corresponding lid.
- 10.2.8. Centrifuge 5 seconds. Check that the entire volume is on the bottom and that there are no bubbles.
- 10.2.9. Place in the thermal cycler and use the following PCR program

STEP	Temp. (°C)	Time (seconds)	Cycles
Activation	95	900	1
Amplification	98	20	38
	60	30	
	72	60	
Final extension	72	600	1
Stop-conservation	15	∞	1

10.2.4. Visualize the results of each sample.

11. RECOMENDATIONS FOR NGS.

The LIBRARIES obtained with our kit must be purified and further processed according to the indications of Illumina®Systems (through a sequencing reaction of 150x2 or higher) using NEXTERA primers. Some general indications are:

- Purify the products obtained with Ampure (AgencourtAMPure XP or BeckmanCoulter).
- It is important not to include in the following steps those samples that do not present correct bands, especially if they have DNA bands of smaller size (possibly the primer-dimer). Samples of these characteristics will produce a reduction in the quality of the results and the number of valid readings.
- It is necessary to use the same amount of amplicons of each sample.
- Quantify the purified product with QuantiFluor™ dsDNA System according to standard procedures.
- Adjust the concentration to 10 nM (1.9 ng / µl) each sample with 0.1% Tris-Tween 20. In the calculation of the readings that will be obtained per sample, it should be considered that 19 amplicons are analyzed.
- Amplified samples can be combined with different OG kits but the proportions must be adjusted according to several parameters. For it, Seqplexing facilitates different spreadsheets so that this adjustment is as simple and reliable as possible.