Instructions For Use

Version: 1.0 *Ref: IFU-TP53* *Revision date: 2024-09-19*

EasySeq™

TP53 Library Prep Kit

For NGS Library Prep by Reverse Complement PCR

NimaGen.

Innovators in DNA Sequencing Technologies

Product and Company Information

EasySeq™ *TP53* Library Prep Kit

RC-TP53096

Research Use Only

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Symbols Used on Product Labels and in Instruction For Use

Symbol	Description
	Manufacturer
	Use-by date
	Lot number
	Reference number
	Temperature limit for storage
	Contains sufficient for <*n*> tests
	GS1 DataMatrix, containing information about the product

Product Description

The intended purpose of this assay is NGS library preparation for illumina® sequencing of the human TP53 gene coding exons, including a minimum of 20 bases upstream and downstream of each exon.

The assay provides reagents for Multiplex Amplicon-based NGS library preparation and is for research use only, not for diagnostic purposes. The kit is tested and validated for an input DNA amount of 20 ng.

The kit is based on the unique and patented Reverse Complement PCR technology, providing a safe, robust and simple workflow, combining amplification with indexing and adapter addition in a single reaction, decreasing the risk of PCR contamination and sample swapping.

Reverse Complement PCR Kit Contents

NimaGen Part# RC-TP53096 (store at -20 °C)	Contents
TP53 Probe Panel A (REF: PM-TP53-A)	1x Tube (24 µL)
TP53 Probe Panel B (REF: PM-TP53-B)	1x Tube (24 µL)
2x PCR Master Mix (Hot Start HiFi) (REF: MMHS096)	2x Tube (1150 µL)
Probe Dilution Buffer enhanced (REF: PDB-Enh-2x)	1x Tube (432 µL)

Required Materials, Not Included

Description	Vendor
Index Primer Plate, dehydrated. Choose one of the 4 available EasySeq™ Unique Dual Index plates for Illumina. Available REF: IDX96-U01D, IDX96-U02D, IDX96-U03D, IDX96-U04D. **Note:** The index sequences are available from the download section of the NimaGen website. **Note:** If more than 384 indexes are desired, please contact NimaGen for the possibilities.	NimaGen
Adjustable Pipette Set (P10, P20, P100, P200, P1000)	Multiple Vendors
TapeStation, Bioanalyzer Instrument, incl. consumables.	Agilent
Ethanol Absolute, Molecular Biology Grade	Multiple Vendors
AmpliClean™ or AMPure XP Bead Solution	NimaGen / Beckman Coulter
General plasticware, DNAse free (1.5 mL tubes, pipette tips etc.)	Multiple Vendors
Mini Spinner for 1.5 mL tubes and 8-well PCR strips or PCR plates	Multiple Vendors
Magnetic stand for 1.5 mL tubes and/or 96-wells plates	Multiple Vendors / NimaGen
Molecular grade H_2O	Multiple Vendors
Qubit Fluorometer incl. dsDNA High Sensitivity consumables	Thermo Fisher Scientific
Thermal cycler with heated lid, (0.2 mL standard PCR tubes), compatible with semi-skirted ABI style PCR plates and option for ramp rate programming. **Note:** Kit is validated for Applied Biosystems™ Veriti™, MiniAmp™ and SimpliAmp™ Thermal Cyclers.	Multiple Vendors
NaOH Solution (2 N), NGS grade	Multiple Vendors
Tris-HCl (200 mM), pH 7	Multiple Vendors
Low TE (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA)	Multiple Vendors
Illumina NGS Sequencing Instrument **Note:** When running the libraries on a MiSeq System, Exon 11 will not be able to be sequenced bi directionally due to a sequence motif present in that area of the genome which is incompatible with the MiSeq Chemistry.	illumina®
Illumina Reagent kit (300-cycle)	illumina®

General Precautions

Read the Material Safety Data Sheet (MSDS) and follow the handling instructions. Adhere to good laboratory practice when handling both the reagents supplied in this kit and other reagents required.

Use a Pre-PCR environment for setting up the RC-PCR. Sample pooling, purification and library quantification should be performed in a Post-PCR environment.

All reagents need to be thawed and centrifuged before use.
Make sure to mix reagents and reactions properly.

Protocol

1. Thermal Cycling Program

Temp	Duration	Ramp Rate (from previous step)	Cycles
98 °C	2 minutes	N.A.	1 x
98 °C	10 seconds	4 °C/sec*	1 x
58 °C	10 minutes	0.1 °C/sec (or 2% of Max)	
72 °C	1 minute	4 °C/sec	
95 °C	10 seconds	4 °C/sec	2 x
80 °C	1 second	4 °C/sec	
62 °C	90 minutes	0.1 °C/sec (or 2% of Max)	
72 °C	30 seconds	4 °C/sec	
95 °C	10 seconds	4 °C/sec	34 x
80 °C	1 second	4 °C/sec	
62 °C	2 minutes	0.5 °C/sec (or 10% of Max)	
72 °C	30 seconds	4 °C/sec	
16 °C	∞	4 °C/sec	1 x

Heated lid at 105 °C.

***Note:** Use a max ramp rate of 4 °C/sec. If this rate is not an option for your thermal cycler, choose the highest ramp rate possible.
Note: This protocol takes approximately 6-7 hours to complete, but may vary per thermal cycler used. When running this protocol for the first time, start the cycling program as a dummy run, to check the predicted duration of 6-7 hours.

2. Reverse Complement PCR

In a single, closed tube reaction, the target specific RC-probes are working as a template to extend the UDI primers to synthesize functional, tailed and indexed PCR primers. This will be followed by two long hybridization/extension steps of 90 minutes and subsequently a further DNA amplification of the target regions, meanwhile synthesizing more primers.

2.1 Thaw on ice:
- RC-PCR Probe Panel A (Black cap)
- RC-PCR Probe Panel B (Red cap)
- Probe Dilution Buffer (Blue cap)
- HiFi Master Mix (White cap)

Note: The HiFi Master Mix contains iso-stabilizers and may not freeze completely, even when stored at -15 °C to -25 °C. It may contain precipitates when thawed at +2 °C to +8 °C. Always ensure that the Master Mix is fully thawed and thoroughly mixed before use.

2.2. Take two identical IDX PCR plates and break off the number of strips needed. Mark the plates with 'A' and 'B'.

Note: Register the indexes used (IDX set/strip-column number and well position for each sample). Download the index details for setting up the Illumina sample sheet.

Note: For each sample two PCR reactions are needed (Panel A and Panel B). Always use the same well position for the same sample in order to generate identical indexes for each sample in both panels.

Note: Before breaking off 8-well strips, cut the seal at the breaking line with a sharp knife.

2.3. Prepare in a fresh 1.5 mL tube the RC-PCR mix panel A by combining and mixing:
- 0.2 µL RC-PCR Probe Panel A per reaction (Black cap)
- 1.8 µL Probe Dilution Buffer per reaction (Blue cap)
- 4 µL Molecular Grade Water
- 10 µL HiFi Master Mix per reaction (White cap)

2.4. Prepare in a fresh 1.5 mL tube the RC-PCR mix panel B by combining and mixing:
- 0.2 µL RC-PCR Probe Panel B per reaction (Red cap)
- 1.8 µL Probe Dilution Buffer per reaction (Blue cap)
- 4 µL Molecular Grade Water
- 10 µL HiFi Master Mix per reaction (White cap)

Example: 24 samples + 10% extra volume*
- 5.28 µL RC-PCR Probe Panel
- 47.52 µL Probe Dilution Buffer
- 105.6 µL Molecular Grade Water
- 264 µL HiFi Master Mix

*It is recommended to allow for a 10% excess when preparing the RC-PCR mix to correct for any pipetting loss. The kit contains extra reagent to facilitate this.

2.5. Remove the seal from the PCR plates or strip(s).

2.6. Dispense 16 µL of the RC-PCR mix Panel A (from step 2.3) to each well of the plate/strip(s) A.

2.7. Dispense 16 µL of the RC-PCR mix Panel B (from step 2.4) to each well of the plate/strip(s) B

2.8. Add to each well 4 µL of DNA solution (optimal: 20 ng total DNA input).

2.9. Close the tube strips **firmly** with the caps provided. Mix by short vortexing, followed by a quick spin. Verify that the colour of the reaction mix is homogeneously pink.

2.10. Place the samples in the thermal cycler(s) and start the RC-PCR program.

After the RC-PCR, samples have been amplified and tagged with sample-specific indexes and sequencing adapters. From this point, RC-PCR product purification is performed using a magnetic bead based purification to remove primers, dimers and salts.

3. Purification

The purification involves one-sided size selection using magnetic beads, minimising the number of reads lost to residual primers and dimers.

3.1. Bring the magnetic bead solution (AmpliClean™ or AMpure XP) to **room temperature.**

Note: It is important to first let the magnetic bead solution equilibrate to room temperature before continuing with the purification. A colder bead solution will lead to inaccurate size selection.

3.2. Perform steps 3.3 to 3.7 for both Panel A and Panel B individually.

3.3. Pool 5 µL RC-PCR products from each reaction into a 1.5 mL tube.

Note: Before pooling, optionally check the unpurified PCR products on agarose.

3.4. Mix well and transfer 40 µL of this pool to a new 1.5 mL tube.

3.5. Add 60 µL Low TE buffer or molecular grade H_2O to the tube and mix well (total volume is now 100 µL).

3.6. Beads purification:

a. Vortex the beads (AmpliClean™ or AMPure XP) thoroughly to resuspend.
b. Add 90 µL bead solution to the 100 µL pool (from step 3.5) and mix well immediately by pipetting up and down at least 5 times or by short vortexing.
c. Incubate for 5 minutes.

On magnet:

d. Place the tube for 3 minutes on the magnet, or until the solution is fully cleared.

e. Remove and discard all liquid carefully, without disturbing the beads.
f. Add 200 µL (freshly prepared) 75% ethanol, without disturbing the beads.
g. Wait for 1 minute.

h. Repeat steps **e.**, **f.** and **g.** for a second ethanol wash step.
i. Carefully remove all liquid without leaving traces of ethanol. (Optionally: quick spin, then place the tube back on the magnet and remove the last traces of ethanol)
j. Dry with open cap for 2-3 minutes at room temperature. **Do not over-dry.**

3.7. Elution:

a. **On Magnet:** Add 120 µL Low TE buffer to the tube.
b. **Off Magnet:** Resuspend the beads by flicking, or by short vortexing.
c. **Off Magnet:** Incubate for 2 minutes.
d. **On Magnet:** Wait for 1-3 minutes, or until the solution is fully cleared.
e. **On Magnet:** Carefully bring 110 µL of the clear solution into a new 1.5 mL tube, making sure not to transfer any of the beads.

The libraries are now ready for a quantitative and qualitative check, followed by NGS.

4. Sequencing

4.1. Determine the final concentration of the libraries by a double Qubit (HS) measurement:

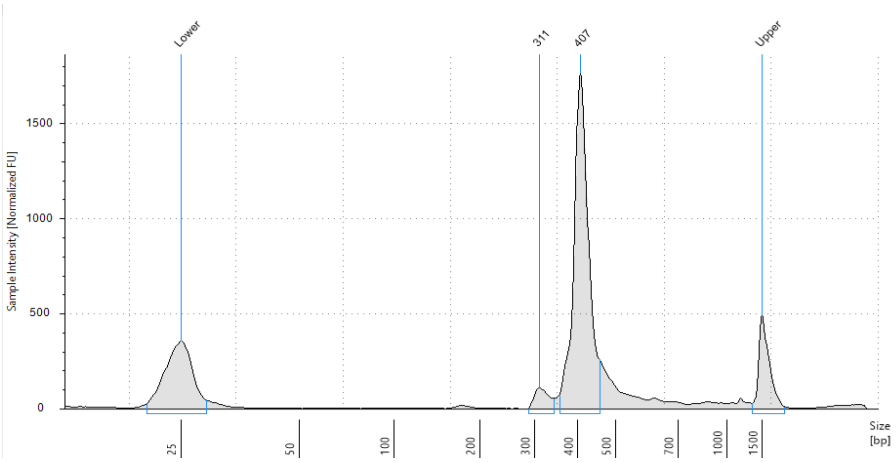
- **a.** Bring the Qubit reagents to room temperature.
- **b.** Label the Qubit tubes on the lid according to the number of samples to be used plus 2 standards.
- **c.** Dilute the Qubit dsDNA HS Reagent 1:200 in Qubit dsDNA HS Buffer for each sample/ standard. It is recommended to allow for >10% excess when preparing the working solution to correct for any pipetting loss.
- **d.** For the standards: mix 190 µL of the working solution with 10 µL of the standard.
- **e.** For the samples: mix 180-199 µL of the working solution with 1-20 µL sample (total 200 µL).
- **f.** Vortex the tubes thoroughly and incubate the tubes for 2 minutes.
- **g.** Measure the standards and the samples using the 'dsDNA High Sensitivity' settings making sure to select the correct sample volume used in step **e.**.

Note: The quantification method may be adapted to an in-house available and/or preferred method, such as qPCR. When quantifying using TapeStation or Bioanalyzer, we recommend to reduce the initial loading concentration of the library on the sequencer to avoid overclustering.

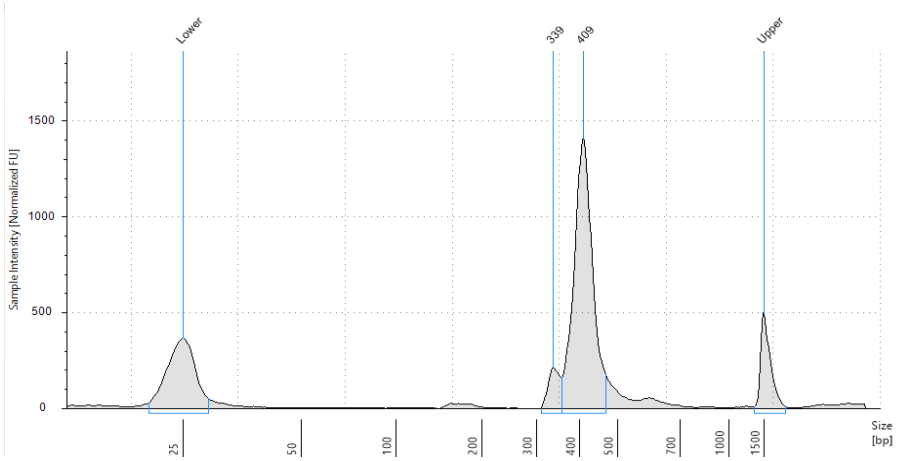
4.2. **Optional but recommended:** Perform a qualitative verification of the libraries on TapeStation or Bioanalyzer, according to the manufacturer's protocol. If needed, dilute the pool. E.g. for TapeStation High Sensitivity kit, dilute to ~2 ng/µL.

Example of clean libraries on TapeStation:

Panel A



Panel B



4.3. Perform sequencing on an illumina® platform, according to the manufacturer's manual.

- **a.** We recommend a length of 410 bp for calculating the library molarity for both panel A and panel B.
- **b.** Combine equimolar solutions of panel A and B in a ratio of 1:1 prior to denaturing.
- **c.** A minimum read depth per sample of 70 000 will result in a lower detection limit of 5% variant allele frequency.
- **d.** A spike-in of 5% PhiX is recommended for QC purposes.
- **e.** We advise to start with a lower loading concentration for the initial sequence run and adjust for subsequent runs if needed. This avoids overclustering and potential failure of the run. For V2 MiSeq a concentration of X pM, V3 MiSeq X pM is recommended. See table 1 for sequencing guidelines.

Table 1 Illumina sequencer and sample multiplexing guidelines

Sequencer	Reagent kit	Run setup	Number of samples	Paired-end reads	Library concentration
Iseq 100	V2 300 cycles	151-10-10-151	54	4 million	50 pM
MiniSeq	Mid output 300 cycles	151-8-8-151	108	8 million	0.8 pM
MiniSeq	High output 300 cycles	151-8-8-151	339	25 million	0.8 pM
MiSeq	V2 nano 300 cycles	151-10-10-151	13	1 million	6-10 pM
MiSeq	V2 Micro 300 cycles		54	4 million	6-10 pM
MiSeq	V2 300 cycles	151-10-10-151	203	15 million	6-10 pM

Customer Support

For technical questions, assistance, or to suggest enhancements, please contact us at techsupport@nimagen.com.

Revision History

Section	Summary of changes	Version	Date
All	New document.	1.0	2024-09-19

Legal Notice

Disclaimer

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