NimaGen.

EasySeq™

Human WES WGS Sample Tracking Kit

NGS library prep by Reverse Complement PCR

Version: RC-HESTv2.5
Revision date: Jun-2022

Quick Reference Guide

Product and Company Information

Product name:	EasySeq™ Human WES WGS Sample Tracking Kit
Product use:	Research Use Only
Company:	NimaGen BV Lagelandseweg 56 6545 CG Nijmegen The Netherlands
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Product Use

The EasySeq™ Human WES WGS Sample Tracking Kit is a single-tube multiplex NGS Library Preparation Kit, for genotyping an optimized panel of 36 exonic, highly variable identification SNPs in Human DNA. This profile can be utilized to extract intrinsic identifiers from human exome or genome sequencing data. The kit provides is intended to create illumina® compatible libraries.

Kit Content: p/n RC-HEST096

Description	Packaging	96 rxn kit
Reverse-Complement PCR HST WES/WGS Probe Panel (black cap)	Tube	24 µL
2x Master Mix HiFi Polymerase (purple cap)	Tube	1.1 mL
Probe Dilution Buffer (blue cap)	Tube	250µL

Choose one of the following options for Indexing and order as a separate item at NimaGen. IDX plates are half-skirted: 4titude FrameStar® Break-A-Way PCR Plate

Kit Content: p/n IDX096-U0x

Description	Packaging	Content
Index Primer Plate with 96 Unique Dual Indexes for Illumina Choice of 8 available sets of 96 indexes: • *IDX96-U01 (UDI 1-96)* • *IDX96-U02 (UDI 97-192)* • *IDX96-U03 (UDI 193-288)* • *IDX96-U04 (UDI 289-384)* • *IDX96-U05 (UDI 385-480)* • *IDX96-U06 (UDI 481-576)* • *IDX96-U07 (UDI 577-672)* • *IDX96-U08 (UDI 673-768)*	96-well plate, sealed, containing 96 different, predispensed, dehydrated and colored primer sets. Ready to use for RC-PCR	1 plate + 12 strips of 8 caps

Compatible with single index reads and unique dual index reads of 8 and 10 bp.

Quick-start Protocol

1. Thermocycling program

Temp:	**Duration:**	**Ramping rate:** **(from previous step)**	Cycles
98°C	1 minute	N/A	1 x
98°C	10 seconds	Max	1 x
80°C	1 second	Max	
58°C	5 minutes	**0.1°C/sec (or 2% of max)**	
72°C	1 minute	Max	
98°C	10 seconds	Max	2 x
80°C	1 second	Max	
58°C	30 minutes	**0.1°C/sec (or 2% of max)**	
72°C	30 seconds	Max	
98°C	10 seconds	Max	34 x
80°C	1 second	Max	
58°C	1 minute	0.5°C/sec (or 10% of max)	
72°C	30 seconds	Max	

Heated lid at 105°C

2. Reverse Complement PCR

In this step, the target specific RC-probes will be transformed into the functional Gene-specific indexing PCR primers. These Gene-specific indexing PCR primers will then be used in a balanced multiplex PCR to simultaneously amplify the targets and add the illumina sequence adaptors and indexes.

2.1 Thaw on ice:

- HST WES/WGS Probe Panel (Black cap)
- Probe Dilution Buffer (Blue cap)
- 2x Master Mix (Purple cap)

2.2. Take an IDX PCR plate and cut off the number of strips needed (8 samples/strip)

Note: Register the indexes used (IDX set/strip-column number and well position for each DNA sample).

2.3. Prepare the Probe-Polymerase master mix, by combining and mixing:

- 0.2 µL HST WES/WGS Probe Panel / sample
- 10 µL 2x Master Mix / sample
- 1.8 µL Probe Dilution Buffer / sample
- 4 µL PCR grade water

Note: There is a 20% extra in the Probe Panel tube to correct for pipetting loss by preparing master mixes. It is recommended to allow for a 10% extra when preparing the master mix.

2.4. Quick-spin the PCR plate or strips, and remove seals carefully.

2.5. Add to each tube: 16 µL of the Probe-Polymerase master mix (from step 2.3).

2.6. Add to each tube: 4 µL (diluted) DNA

2.7. Close tubes, short vortex.

2.8. Perform a short spin.

2.9. Start the RC-PCR program and place samples in the PCR cycler when block is between 60°C and 90°C, close the lid.

The samples have now been amplified and tagged with a sample specific index and sequencing tail. From this point, all PCR products can be pooled together, purified by a single AmpliClean purification to remove primers and salt.

3. Pool, Purify and Sequence

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

3.1. Bring beads solution to Room Temperature.

3.2. Prepare the sequencing pool by combining 5 µL of each PCR reaction (except negative controls) in a new 1.5 mL Eppendorf tube.

Note: If not all samples have the same yield, it is recommended to correct this by adjusting the 5 µL input according to the relative concentration.

3.3. Bring 40 µL of this combination pool to a new, 1.5 mL Eppendorf tube.

3.4. Add 60 µL water (total pool volume is now 100 µL)

3.5. Purify 1:1

a. Vortex beads thoroughly to re-suspend.
b. Add 100 µL beads solution to the 100 µL pool and mix well immediately by pipetting up and down.
c. Incubate for 5 minutes, off magnet.
d. Place on magnet for 3 minutes or for the solution to be fully cleared.
e. Remove and discard 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.
i. Carefully remove all liquid without leaving traces of ethanol.
j. Dry with open cap for 2-3 minutes at Room Temperature. **Do not overdry.**
k. On Magnet: Add 50 µL Low TE.
l. Off Magnet: Re-suspend the beads by flicking or short vortexing.
m. Incubate for 2 minutes, off magnet.
n. Put on magnet and wait for 3-5 minutes or for the solution to be fully cleared.
o. Carefully bring 40 µL of the clear solution to a new 1.5 ml Eppendorf tube making sure not to transfer any of the beads.

3.6 Verify library on TapeStation or Bioanalyzer, according to the manufacturer's protocol. If needed, dilute pool.

a. Example: For TapeStation High Sensitivity kit, dilute 20-30x

3.7 Determine final concentration by a duplo Qubit (HS) measurement according to manufacturer's manual. For molarity calculation, always use a fragment length of 220 bp.

3.8 Perform Sequencing on an illumina NGS platform, according to the manufacturer's manual. Appendix 1 outlines the detailed illumina NGS protocols.

illumina Systems Reference Guides

- **Miseq System Guide**
- **Miseq Denature and Dilute Libraries Guide**
- **MiniSeq System Guide**
- **MiniSeq Denature and Dilute Libraries Guide**
- **Nextseq 550 System Guide**
- **NextSeq System Denature and Dilute Libraries Guide**
- **illumina experiment manager**

APPENDIX 1: iLLUMINA SEQUENCER PROTOCOLS

A: illumina MiSeq protocol

Use illumina MiSeq v2 or v3 kit for 150 cycles (2x75 bp run).
Ref: MiSeq System Denature and Dilute Libraries Guide #15039740 v03.

1. Thaw DNA sample/library, buffer HT1, and MiSeq cartridge.

NOTE: MiSeq cartridge should be thawed by submerging it in (but not covering it completely with) water at room temperature. (thawing takes ~ 1.5hr, do not use warm water as it degrades the enzymes). Store other component of MiSeq kit in 4°C refrigerator until ready to start the MiSeq run.

2. Prepare sample sheet. Workflow: Generate FASTQ. Use the following adapter sequences for trimming in the sample sheet:
   Adapter: GCGAATTTCGACGATCGTTGCATTAACTCGCGAA
   AdapterRead2: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

3. Dilute DNA to 2 nM using 10 mM Tris-HCl, pH 8.5/0.1%Tween20. (illumina EBT Buffer).
4. Prepare <u>fresh</u> 0.2 N NaOH.
5. Mix 10 µL of 2 nM DNA with 10 µL of 0.2N NaOH, vortex, spin down.
6. Incubate for 5 minutes at room temperature.
7. Add 10 µL of 200mM Tris-HCl pH7.0 to hydrolyse the NaOH.
8. Add 970 µL Ice Cold HT1 to DNA/NaOH mix to dilute DNA to 20 pM.
9. Dilute the 20 pM library with Ice Cold HT1 to 9 pM (for v2 kits) or 12 pM (for v3 kits) in a new tube.
   a. Version 2 chemistry.
   b. Dilute 450 of library (20pM) with 550 µL of HT1
      (This is now 1000µL of 9 pM loading concentration)
   c. Invert to mix and then pulse centrifuge.
10. Mix the MiSeq cartridge by inverting 10x, make sure the reagents do not contain ice.
    After mixing, tap the cartridge on the bench 2-3 times to dislodge any air trapped in the bottom of the tubes.
11. Load MiSeq cartridge:
    a. Optional but recommended, use PhiX control: Add a 3% of PhiX control to the library
    b. Load 600 µL of **the library** to the **Load Samples** well.
12. Set up and start MiSeq run: Clean flow cell according to instructions, follow on-screen instructions to load and start instrument.

B: illumina NextSeq protocol

Use illumina Nextseq v2 kit for 150 cycles (2x75 bp run)

1. Thaw DNA sample/library, buffer HT1, and NextSeq cartridge

NOTE: Nextseq cartridge should be thawed by submerging it in (but not covering it completely with) water at room temperature. (thawing takes ~ 1.5hr, do not use warm water as it degrades the enzymes)

2. Prepare sample sheet. Workflow: Generate FASTQ. Use the following adapter sequences for trimming in the sample sheet:

   Adapter: GCGAATTTCGACGATCGTTGCATTAACTCGCGAA
   AdapterRead2: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

3. Dilute DNA to 2 nM using 10 mM Tris-HCl, pH 8.5/0.1%Tween20. (illumina EBT Buffer)
4. Prepare <u>fresh</u> 0.2 N NaOH
5. Mix 10 µL of 2 nM DNA with 10 µL of 0.2N NaOH, vortex, spin down
6. Incubate for 5 minutes at room temperature
7. Add 10 µL of 200mM Tris-HCl pH7.0 to hydrolyse the NaOH
8. Add 970 µL Ice Cold HT1 to DNA/NaOH mix to dilute DNA to 20 pM
9. Dilute the 20 pM library with Ice Cold HT1 to 1 pM in a new tube
   a. Dilute 75 µL of library (20pM) with 1425 µL of HT1
      (This is now 1500µL of 1pM loading concentration)
   b. Invert to mix and then pulse centrifuge.
10. Mix the NextSeq cartridge by inverting 10x, make sure the reagents do not contain ice. After mixing, tap the cartridge on the bench 2-3 times to dislodge any air trapped in the bottom of the tubes.
11. Load NextSeq cartridge:
    a. Optional but recommended, use PhiX control: Add a 3% of PhiX control to the library
    b. Load 1300 µL of **the library** to the **Load Library Here (10)** well
12. Set up and start Nextseq run: Clean flow cell according to instructions, follow on-screen instructions to load and start instrument

C: illumina MiniSeq protocol

Use illumina MiniSeq kit for 150 cycles (2x75 bp run)

1. Thaw DNA sample/library, buffer HT1, and MiniSeq cartridge

NOTE: MiniSeq cartridge should be thawed by submerging it in (but not covering it completely with) water at room temperature. (thawing takes ~ 2,5hr, do not use warm water as it degrades the enzymes)

2. Prepare sample sheet. Workflow: Generate FASTQ. Use the following adapter sequences for trimming in the sample sheet:

   Adapter: GCGAATTTCGACGATCGTTGCATTAACTCGCGAA

   AdapterRead2: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

3. Dilute DNA to 2 nM using 10 mM Tris-HCl, pH 8.5/0.1%Tween20. (Illumina EBT Buffer).
4. Prepare <u>fresh</u> 0.1 N NaOH.
5. Mix 10 µL of 1 nM DNA with 10 µL of 0.1N NaOH, vortex, spin down.
6. Incubate for 5 minutes at room temperature.
7. Add 10 µL of 200mM Tris-HCl pH7.0 to hydrolyse the NaOH.
8. Add 970 µL Ice Cold HT1 to DNA/NaOH mix to dilute DNA to 20pM.
9. Dilute the 20 pM library with Ice Cold HT1 to 1 pM in a new tube.
   a. Dilute 50 µL of library (20pM) with 950 µL of HT1
      (This is now 1000µL of 1 pM loading concentration)
   b. Invert to mix and then pulse centrifuge.
10. Mix the MiniSeq cartridge by inverting 10x, make sure the reagents do not contain ice. After mixing, tap the cartridge on the bench 2-3 times to dislodge any air trapped in the bottom of the tubes.
11. Load MiniSeq cartridge:
    a. Optional but recommended, use PhiX control: Add a 3% of PhiX control to the library
    b. Load 500 µL of **the library** to the **Load Library Here (16)** well

12. Set up and start MiniSeq run: Clean flow cell according to instructions, follow on-screen instructions to load and start instrument

Legal Notice: RC-PCR is patent protected (PCT/GB2016/050558, WO2016146968A1) and exclusively licensed to NimaGen B.V. Nijmegen

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