



Instructions For Use

Version: 1.0 Ref: IFU-MitoHVR

Revision date: 2023-12-22

Mitochondrial DNA Hypervariable Region Sequencing

For MPS Library Prep by Reverse Complement PCR



NimaGen.

Innovators in
DNA Sequencing
Technologies

Product and Company Information

IDseek® Mitochondrial DNA Hypervariable Region Sequencing Kit



IDS-HVR96

Research Use Only



NimaGen B.V.
Hogelandseweg 88
6545 AB Nijmegen
The Netherlands
Tel: +31 (0)24 820 02 41
Email: info@nimagen.com

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
	Matrix code containing the reference number, lot number and use-by date

Product Description

Forensic DNA profiling utilizes mitochondrial DNA for analysis of hypervariable regions (mutation hotspots) in very low quantities of (highly degraded) nuclear mtDNA that does not provide a full STR profile. For laboratories in those countries where full genome sequencing in forensics is restricted by guidelines or regulations.

IDseek® Mitochondrial DNA Hypervariable Region Sequencing kit provides multiplex amplicon-based MPS library preparation for sequencing the hypervariable region of the mitochondrial genome. This Reverse Complement Polymerase Chain Reaction (RC-PCR) based library prep kit contains all reagents to generate Illumina compatible libraries in a simple, sensitive, robust and safe method for cost-effective and high-quality mitochondrial DNA analysis.

Mitochondrial HVR region (16010 – 591)

Reverse Complement PCR Kit Contents

NimaGen Part# IDS-HVR96 (store at -20 °C)	Contents
IDseek® RC-PCR Probe Panel A (REF: PM-IDS-MHVR-A)	1x Tube (24 µL) ●
IDseek® RC-PCR Probe Panel B (REF: PM-IDS-MHVR-B)	1x Tube (24 µL) ●
2x PCR Master Mix (Hot Start HiFi) (REF: MMHS096)	2x Tube (1150 µL) ○
IDS Probe Dilution Buffer (REF: PDB-IDS-2x)	1x Tube (432 µL) ●

Required Materials, Not Included

Description	Vendor
<p>Index Primer Plate, dehydrated. Choose one of the 4 available EasySeq™ Unique Dual Index plates for Illumina.</p> <p>Available REF: IDX96-U01D, IDX96-U02D, IDX96-U03D, IDX96-U04D.</p> <p>Note: The index sequences are available from the download section of the NimaGen website.</p>	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
Water, PCR Grade	Multiple Vendors
Qubit Fluorometer incl. High Sensitivity consumables	Thermo Fisher Scientific
<p>Thermocycler with heated lid, (0.2 mL standard PCR tubes), compatible with semi-skirted ABI style PCR plates and option for ramp rate programming.</p> <p>Note: Kit is validated for Applied Biosystems™ Veriti™, MiniAmp™ and SimpliAmp™ Thermal Cyclers.</p>	Multiple Vendors
NaOH Solution (2 N), MPS 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 MPS Sequencing Instrument (MiSeq®/MiSeq FGx®)	illumina®/ Verogen®
<p>Illumina MiSeq® Reagent kit v2 (300 or 500-cycle)</p> <p>Or Illumina MiSeq® Reagent kit v3 (600-cycle)</p> <p>Or Verogen MiSeq FGx® Reagent Kit</p> <p>Or Verogen MiSeq FGx® Reagent Micro Kit</p>	illumina®/ Verogen®

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.

Protocol

1. Thermocycling Program

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

Heated lid at 105 °C.

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 (Green 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 (Green cap)
- 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 (Green cap)
- 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
- 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 plate or strip(s).

2.6. Dispense 12 µL of the RC-PCR mix Panel A (from step 2.3) to each well of the plate/strip(s) A.

2.7. Dispense 12 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: 8 µL of DNA solution (optimal: 50 pg total DNA input).

2.9. Close the tube strips **carefully** with the caps provided, there should be an audible click. Mix by short vortexing, followed by a quick spin. Verify that the colour of the reaction mix is homogenously pink.

2.10. Place the samples in the thermal cyclers 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, minimizing the number of reads lost to residual primers and primer-dimers. The input quality and quantity of your samples will impact the PCR yield. Samples can be pooled based on the total input quantity of the PCR to ensure low-input samples have appropriate read depth.

- 3.1. Bring the magnetic bead solution (AmpliClean™ or AMPure XP) to room temperature.
- 3.2. Perform steps 3.3 to 3.7 for both Panel A and Panel B individually.
- 3.3 Create three separate pools based on the input quantity of each reaction. Pool 10 µL RC-PCR product from each reaction in the same input range into a 1.5 mL tube.

- Pool 1: ± 50 – 12.5 pg
- Pool 2: ± 12.5 – 3 pg
- Pool 3: < 3 pg and NTC

Note: Perform all subsequent steps for each pool individually.

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

- 3.4 Mix well and transfer 40 µL of this pool into a new 1.5 mL tube.
- 3.5 Add 60 µL Low TE buffer or molecular grade H₂O to the tube and mix well (total volume is now 100 µL).
- 3.6 Bead purification:

Purification #1

- a. Vortex the beads thoroughly to resuspend.
 - b. Add 80 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 the liquid carefully, without disturbing the beads.
 - f. Add 300 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. (Optional: 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**. Immediately continue with the Elution.

3.7. Elution:

- a. On magnet:** Add 40 μ L Low TE buffer to the tube and close 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 3-5 minutes, or until the solution is fully cleared.
- e. On magnet:** Carefully bring 30 μ L of the clear solution into a new 1.5 mL tube, making sure not to transfer any of the beads.

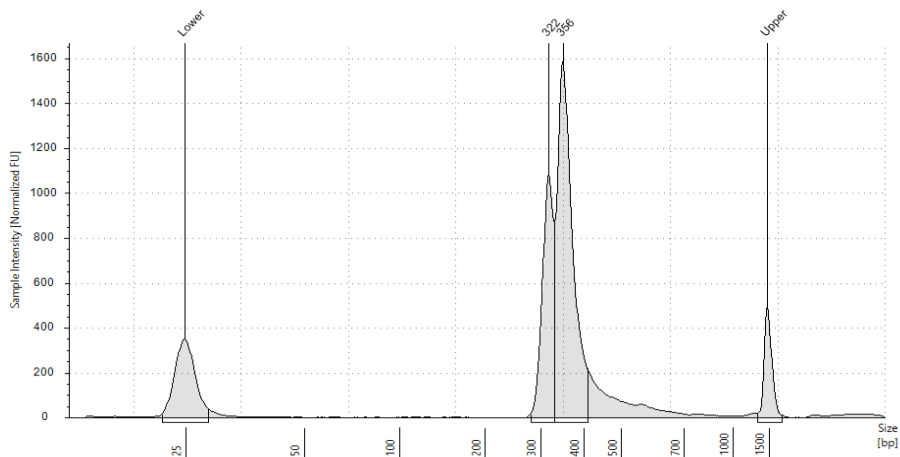
The libraries are now ready for a quantitative and qualitative check, followed by NGS.

4. Sequencing

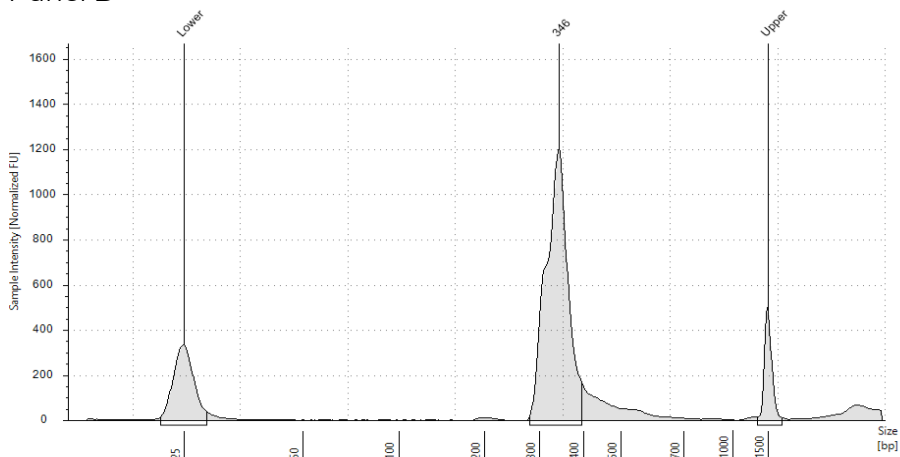
- 4.1. Determine the final concentration of the libraries by a double Qubit (HS) measurement:
- Bring the Qubit reagents to room temperature.
 - Label the Qubit tubes on the lid according to the number of samples to be used plus 2 standards.
 - Dilute the Qubit dsDNA HS Reagent 1:200 in Qubit dsDNA HS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

Example of clean libraries on TapeStation:

Panel A



Panel B



4.3. Perform sequencing on an Illumina® platform or MiSeq™ FGx in research mode, according to the manufacturer's manual.

- a. We recommend a length of 340 bp for calculating the library molarity for panel A and panel B.
- b. Combine equimolar solutions of panel A and B in a ratio of 1:1 prior to denaturing.
- c. We advise to maintain a minimal read depth of 12.000 reads per sample for reference samples or a minimum of 120.000 reads per sample for traces, mixed samples or samples with low RC-PCR input (<250 pg).
- 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 the MiSeq a concentration of 8 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**
			Reference	challenging		
MiSeq	V2 Nano 300 cycles	151-10-10-151	79	7	1 million	8 pM
MiSeq	V2 Nano 500 cycles	251-10-10-251	79	7	1 million	8 pM
MiSeq	V2 Micro 300 cycles	151-10-10-151	316	31	4 million	8 pM
MiSeq	V2 300 cycles	151-10-10-151	1187*	118	15 million	8 pM
MiSeq	V2 500 cycles	251-10-10-251	1187*	118	15 million	8 pM
MiSeq	V3 600 cycles	301-10-10-301	1979*	197	25 million	8 pM
MiSeq	MiSeq FGx® Reagent Kit	301-10-10-301	1979*	197	25 million	8 pM
MiSeq	MiSeq FGx® Reagent Micro Kit	301-10-10-301	633*	63	8 million	8 pM

* Maximum number of indices available (n=384).

** Based on Illumina recommendations and assuming onboard denature and dilution.

Data Analysis

The IDseek® Mitochondrial DNA Hypervariable Region Sequencing Kit does not come with predetermined data analysis software, instead the user is given full freedom to implement the analysis tools which best suits their needs. The bed file is available for data analysis.

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	2023-12-22

Legal Notice

IDseek, MitoHVR, EasySeq and AmpliClean are trademarks of NimaGen B.V.. All other product names and trademarks are the property of their respective owners. Reverse Complement PCR (RC-PCR) is IP protected. Exclusive license granted to NimaGen B.V., Nijmegen, The Netherlands by SALISBURY NHS FOUNDATION TRUST (SALISBURY, WILTSHIRE, GB). It is strictly prohibited to produce, distribute, import, or sell RC-PCR based methods or kits without the patent owner's or licensee's written consent.

Disclaimer

Although the information in this document is presented in good faith and believed to be correct at the time of printing, NimaGen makes no representations or warranties as to its completeness or accuracy. NimaGen has no liability for any errors or omissions in this document, including your use of it.

Published by

NimaGen B.V.
Hogelandseweg 88
6545 AB Nijmegen
The Netherlands
www.nimagen.com

© 2023 NimaGen
All rights reserved.