IFU Handbook

Version: 1.0 *Ref: HB_RC-ITSMB* *Revision date: 2026-06-03*

EasySeq™

ITS Microbiome Kit

For short-read sequencers

NimaGen.

Innovators in DNA Sequencing Technologies

Contents

Product and Company Information

EasySeq™ ITS Microbiome Kit

REF RC-ITSMB096

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 Instructions For Use

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

Reverse Complement PCR Kit Contents

NimaGen Part# RC-ITSMB096 (store at -20 °C)	Contents
ITS Microbiome Probe Panel (REF: PM-ITSMB)	1x Tube (24 µL)
2x Master Mix HiFi Polymerase (REF: MM096)	1x Tube (1150 µL)
Probe Dilution Buffer Enhanced (REF: PDB-Enh)	1x Tube (216 µL)

Storage

All RC-PCR components of the kit should be stored at a temperature of -20 °C. The expiry date of each individual tube is stated on the label.

The dehydrated index plates should be stored dry, below 25 °C, and are stable for 1 year under these conditions. The expiry date is stated on the box label and plate.

Intended Use

EasySeq™ ITS Microbiome Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention or treatment of a disease. Research Use Only (RUO).

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.

Make sure to place the remaining strip(s) of the index plate(s) back into the provided plastic bag and include the silica gel pouch. Try to leave as little air as possible inside the plastic bag, while sealing it.

Quality Control

In accordance with NimaGen's ISO-certified Quality Management System, each lot of the EasySeq™ ITS Microbiome Kit is tested against predetermined specifications to ensure consistent product quality.

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-U01, IDX96-U02, IDX96-U03, IDX96-U04. **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™ QuantStudio™, MiniAmp™ and SimpliAmp™ Thermal Cyclers, and Bio-Rad C1000 Touch™ 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
Optional: ZymoBIOMICS Microbial Community DNA Standard (D6305/D6306)	Zymo research

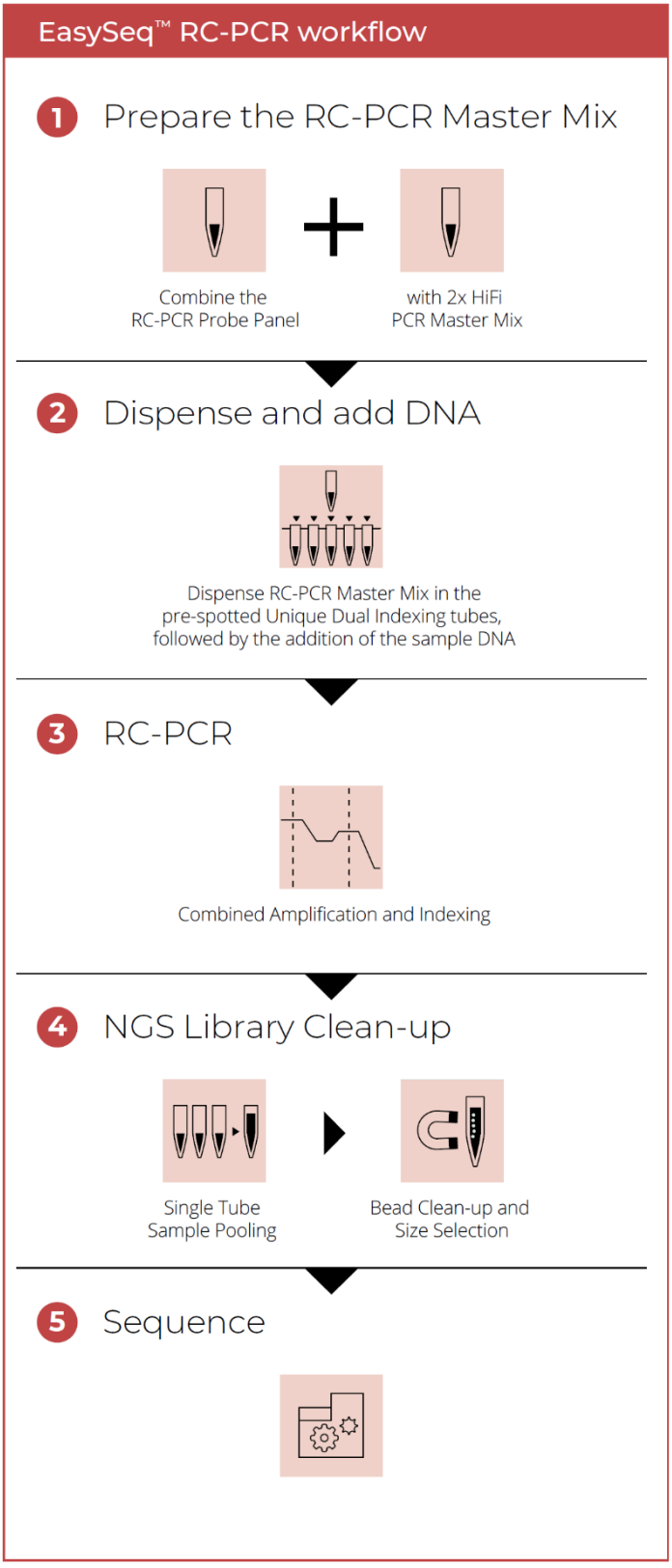
Product Description

The kit is based on the unique and patented Reverse Complement PCR (RC-PCR) technology to create Next-Generation Sequencing (NGS) libraries for sequencing. The RC-PCR technology provides a safe, robust and simple workflow, combining amplification with indexing and adapter attachment in a single reaction, decreasing the risk of PCR contamination and sample swapping.

Next-Generation Sequencing parts of the ribosomal RNA gene is the gold standard to classify and perform compositional profiling of fungal species. The EasySeq™ ITS Microbiome Kit contains all reagents to generate libraries for short-read sequencers. Our method supports an NGS driven analysis of the mycobiome in DNA extracted from different kinds of specimen, like soil, fecal, environmental, and water samples. The kit is developed for highly diverse microbial communities to enable researchers to deconvolute the composition in an unmatched safe and straightforward workflow. The kit is not intended for diagnostic use, but for research use only.

A single fragment is generated comprising the ITS2 region of the rRNA operon, and is optimized to amplify a broad range of fungal taxa.

EasySeq™ Workflow



Important Notes

Sample Collection and Storage

Optimal results are obtained with fresh samples or those that have been immediately frozen and stored at temperatures between -80 °C and -15 °C. Repeated freezing and thawing should be avoided, as this can diminish template integrity and availability. Additionally, using low-quality starting material may result in shorter DNA fragments and lower overall yield.

Input DNA Guidelines

DNA samples should ideally be free of PCR inhibitors and only contain microbial DNA. Additionally, all DNA samples should be normalized to 1 ng/µL for optimal results. Use of a fluorescent quantification method like Qubit® prior normalization is recommended.

Non-ideal Sample Types

Low biomass or high host DNA containing samples are difficult to normalize prior PCR. The maximum DNA input is 100 ng total DNA, including host DNA. However, diluting these samples 10 or 100 times can improve microbial DNA amplification.

Processing of Samples

The standard protocol of this kit has been optimized for sample types containing high levels of microbial DNA but the kit can also be used for non-ideal samples. Please refer to the table below to determine the appropriate protocol. The samples in an experiment should be grouped and handled per sample category for optimal results. An extended protocol is provided for users who require full normalization control but this significantly increases hands-on-time.

	Ideal samples	Non-ideal samples	
Sample category	High microbial DNA	Low microbial DNA / high host DNA	Low microbial DNA & high host DNA / PCR inhibitors in DNA sample
Example sample type	Fecal, soil, culture	Water samples, intestinal tissue	Skin swabs, biopsies
Normalization method	Prior RC-PCR	After RC-PCR	After RC-PCR
Recommended protocol	Standard	Non-ideal	Non-ideal with additional cleanup

The following protocols are included in this handbook:

- Standard for ideal samples that can be normalized prior PCR
- Non-ideal for samples that can't be normalized prior PCR
- Non-ideal for challenging samples that can't be normalized prior PCR
- Extended for the most control of normalization

Standard Protocol

This protocol is designed for sample types containing high levels of fungal DNA.

RC-PCR Thermal Cycling

This thermal cycling program is specifically designed for the EasySeq™ ITS Microbiome workflow and has been tested on Bio-Rad C1000™, Applied Biosystems SimpliAmp™, MiniAmp™, Quantstudio™ thermal cyclers at NimaGen. Using a different thermal cycler might require other settings.

This protocol takes approximately **5.5 hours** to complete, but may vary per thermal cycler used. When running this protocol for the first time, start the cycling program without samples, to check the predicted duration of 5.5 hours.

Temp	Duration	Ramp Rate (from previous step)	Cycles
98 °C	2 minutes	4 °C/sec*	1 x
80 °C	1 second	4 °C/sec	
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	
58 °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	20 x
80 °C	1 second	4 °C/sec	
58 °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 but not exceeding 4 °C/sec.

Sample Normalization

DNA samples should be free of PCR inhibitors and only contain microbial DNA. Use a fluorescent quantification method (Qubit®) to quantify each sample prior normalization. Dilute all DNA samples to equal concentration, 1 ng/µL is recommended.

Reverse Complement PCR Reaction Set-up

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.

1. Thaw on ice:
   - RC-PCR Probe Panel (Green cap)
   - Probe Dilution Buffer (Blue cap)
   - HiFi Master Mix (Purple 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.

   Note: Always ensure all components are fully thawed and thoroughly mixed before use.

2. Take the IDX PCR plate and break off the number of strips needed.

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

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

3. Prepare in a fresh 1.5 mL tube the RC-PCR mix by combining and mixing:
   - 0.2 µL RC-PCR Probe Panel per reaction (Green cap)
   - 1.8 µL Probe Dilution Buffer per reaction (Blue cap)
   - 10 µL HiFi Master Mix per reaction (Purple 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.

4. Remove the seal from the PCR plate or strip(s).
5. Dispense 12 µL of the RC-PCR mix (from step 3) to each well of the plate/strip(s).
6. Add to each well 8 µL of DNA sample.

   Optional: Add ZymoBIOMICS Microbial Community DNA standard as a positive control (at least 100 pg total).

7. Close the tube strips **firmly** with the caps provided. Mix by short vortexing, followed by a quick spin. Verify that the color of the reaction mix is homogeneously pink.
8. 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.

Safe stopping point: The reactions can be stored at 4 °C for up to 48 hours. For longer storage, keep the samples at -20 °C. It is best to continue with the samples within a month.

Purification

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

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

1. Bring the magnetic bead solution (AmpliClean™ or AMPure XP) to **room temperature** for at least one hour.

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

2. Pool 5 µL RC-PCR product from each reaction into a 1.5 mL tube (increase volume to 15 µL when processing less than 24 samples).

3. Mix well and transfer 100 µL of this pool to a new 1.5 mL tube.

4. Beads purification using AmpliClean:

Purification

a. Vortex the beads thoroughly to resuspend.
b. Add 80 µL bead solution to the 100 µL pool (from step 3) 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 1-3 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 1-2 minutes at room temperature. **Do not over-dry.** Immediately continue with the Elution.

5. Elution:

a. On magnet: Add 45 µL Low TE buffer or Tris-HCl pH 8.0 buffer 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 1-3 minutes, or until the solution is fully cleared.
e. On magnet: Carefully bring 40 µL of the clear solution into a new 1.5 mL tube, making sure not to transfer any of the beads.

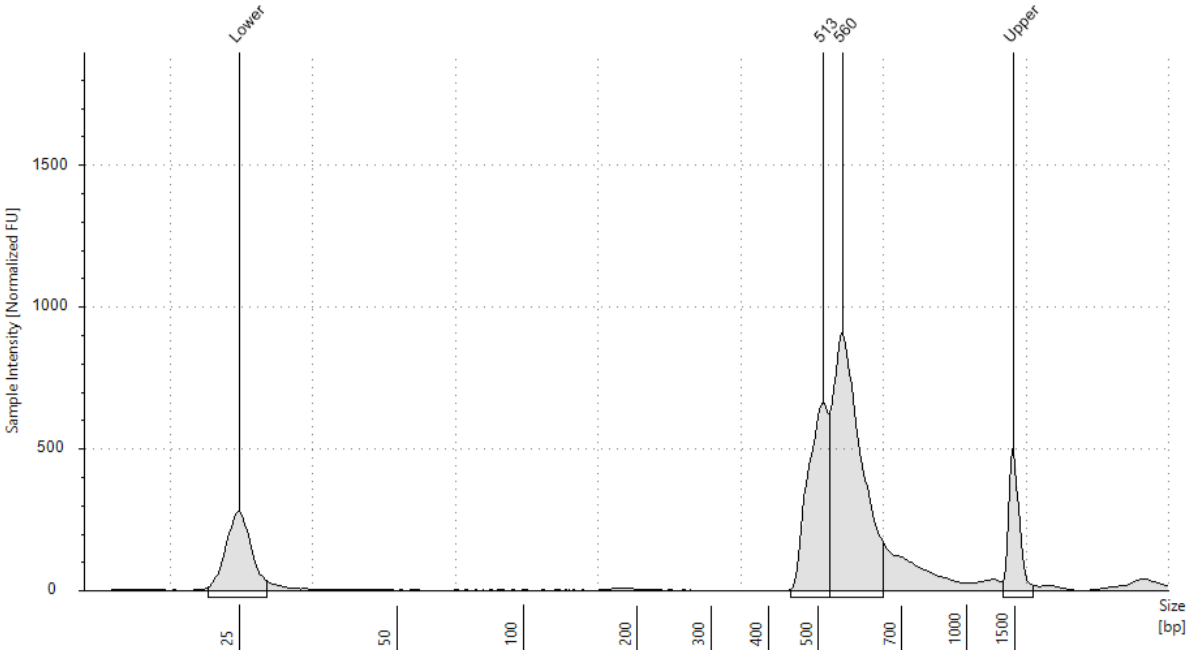
The libraries are now ready for a quantitative and qualitative check, followed by NGS.

Safe stopping point: The libraries can be stored at 4 °C for up to a week after elution. For longer storage, keep the samples at -20 °C. It is best to sequence the library within a month. Perform a new quantification after storing the library for longer than a day at either temperature.

Library Quantitative and Qualitative Check

Quantify the pooled and cleaned library with a Qubit® HS measurement. Verify the quality of the library using a TapeStation, Bioanalyzer or equivalent according to manufacturer's protocol.

Example of a clean library on TapeStation:



Downstream Processing using an Illumina® sequencer

1. Perform sequencing according to the manufacturer's instructions.
2. We recommend using the average length based on library QC like TapeStation calculating the library molarity, but a length of 560 bp could be used if no QC was performed.
3. We advise a read depth of 30 000 reads per sample for complex microbiome samples.
4. A spike-in of 5% PhiX is recommended for QC purposes.
5. We advise to start with a lower loading concentration for their initial sequence run and adjust in subsequent runs if needed. This avoids overclustering and potentially failure of the run. See the table below for sequencing guidelines.

Table Illumina sequencer and sample multiplexing guidelines

Sequencer	Reagent kit	Run setup	Number of samples*	Paired-end reads	Library concentration
MiSeq	V2 Nano 500 cycles	251-10-10-251	31	1 million	7 pM
MiSeq	V2 500 cycles	251-10-10-251	475	15 million	7 pM
MiSeq	V3 600 cycles	301-10-10-301	768	25 million	8 pM
NextSeq 1000/2000	P1 600 cycles	301-10-10-301	768	100 million	1000 pM**
NextSeq 1000/2000	P2 600 cycles	301-10-10-301	768	300 million	1000 pM**
MiSeq i100	5 M Reagent kit (600 cycles)	301-10-10-301	158	5 million	80 pM
MiSeq i100	25 M Reagent kit (600 cycles)	301-10-10-301	768	25 million	80 pM
MiSeq i100	50 M Reagent kit (600 cycles)	301-10-10-301	768	50 million	80 pM

*Theoretical maximum with 30 000 paired-end reads per sample or maximum number of indices available (n=768).
**Based on Illumina recommendations and assuming onboard denature and dilution.

Data Analysis

After sequencing on a short-read sequencer and demultiplexing, the data could be processed by *in-house* or open-source pipelines like DADA2 and QIIME2. Commercial options for ITS analysis are compatible with our library prep kit, examples are 1928, GenomeDetective, and BugSeq.

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This protocol is designed for sample types containing low levels of fungal DNA or low levels of fungal DNA and high host DNA background.

RC-PCR Thermal Cycling

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This protocol takes approximately **6 hours** to complete, but may vary per thermal cycler used. When running this protocol for the first time, start the cycling program without samples, to check the predicted duration of 6 hours.

Temp	Duration	Ramp Rate (from previous step)	Cycles
98 °C	2 minutes	4 °C/sec*	1 x
80 °C	1 second	4 °C/sec	
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	
58 °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	25-30 x
80 °C	1 second	4 °C/sec	
58 °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 but not exceeding 4 °C/sec.

Reverse Complement PCR Reaction Set-up

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.

1. Thaw on ice:
   - RC-PCR Probe Panel (Green cap)
   - Probe Dilution Buffer (Blue cap)
   - HiFi Master Mix (Purple 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.

   Note: Always ensure all components are fully thawed and thoroughly mixed before use.

2. Take the IDX PCR plate and break off the number of strips needed.

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

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

3. Prepare in a fresh 1.5 mL tube the RC-PCR mix by combining and mixing:
   - 0.2 µL RC-PCR Probe Panel per reaction (Green cap)
   - 1.8 µL Probe Dilution Buffer per reaction (Blue cap)
   - 10 µL HiFi Master Mix per reaction (Purple 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.

4. Remove the seal from the PCR plate or strip(s).
5. Dispense 12 µL of the RC-PCR mix (from step 3) to each well of the plate/strip(s).
6. Add to each well 8 µL of DNA sample.

   Optional: Add ZymoBIOMICS Microbial Community DNA standard as a positive control (at least 100 pg total).

7. Close the tube strips **firmly** with the caps provided. Mix by short vortexing, followed by a quick spin. Verify that the color of the reaction mix is homogeneously pink.
8. 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.

Safe stopping point: The reactions can be stored at 4 °C for up to 48 hours. For longer storage, keep the samples at -20 °C. It is best to continue with the samples within a month.

Quantification and Normalization

To optimize the read depth distribution across samples with variable or unknown input, we recommend performing a DNA quantification step after RC-PCR and prior to pooling. Quantification using the Qubit™ High Sensitivity (HS) assay, followed by equimolar pooling, improves read distribution within the EasySeq™ ITS Microbiome Library Prep workflow.

Normalization procedure

Determine the concentration of RC-PCR product using a 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
c. Measure according to manufacturer's instructions
d. Perform equimolar pooling ("EasySeq Pooling Calculator.xlsx" can be used [downloadable from the NimaGen website]).
e. Proceed with the purification using AmpliClean™ or AMPure XP

In case equal volume pooling is preferred, pool 5 µL RC-PCR product from each reaction into a 1.5 mL tube (increase volume to 15 µL when processing less than 24 samples). Transfer 100 µL to a new tube and proceed with the purification using AmpliClean™ or AMPure XP.

Purification

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

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

Bring the magnetic bead solution (AmpliClean™ or AMPure XP) to **room temperature** for at least one hour.

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Purification

a. Vortex the beads thoroughly to resuspend.
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c. Incubate for 5 minutes.

On magnet:
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e. Remove and discard the liquid carefully, without disturbing the beads.
f. Add 300 µL (freshly prepared) 75% ethanol, without disturbing the beads.
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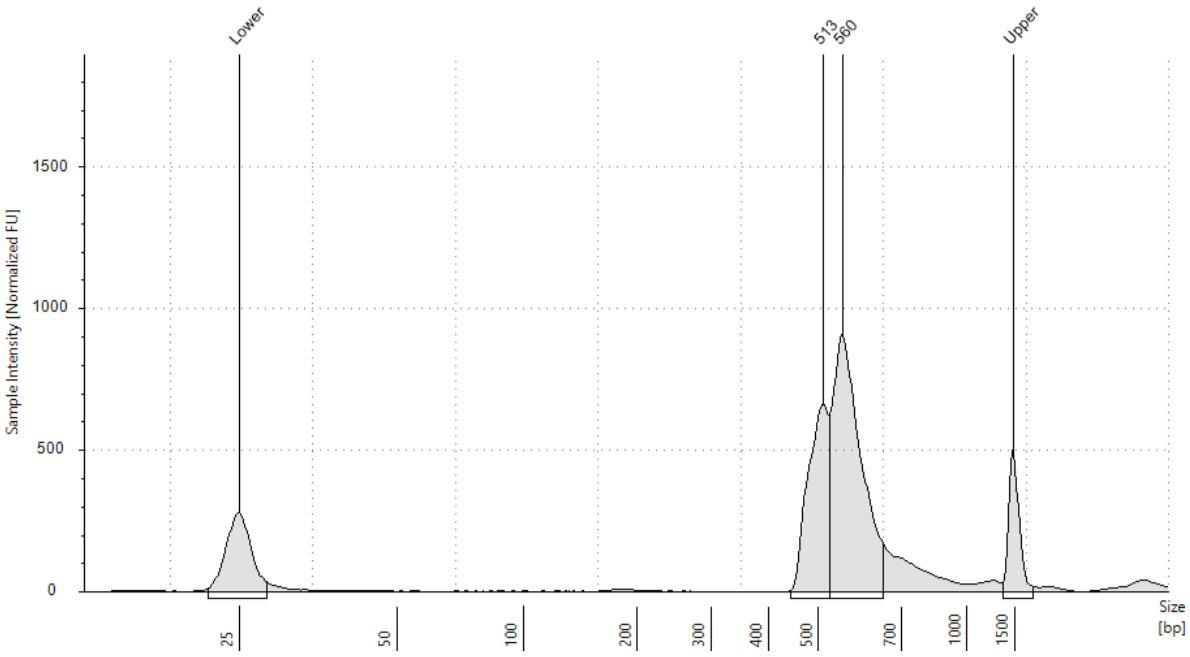

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*It is recommended to allow for a 10% excess when preparing the RC-PCR mix to correct for any pipetting loss. The kit contains extra reagent to facilitate this.

4. Remove the seal from the PCR plate or strip(s).
5. Dispense 12 µL of the RC-PCR mix (from step 3) to each well of the plate/strip(s).
6. Add to each well 8 µL of DNA sample.

   Optional: Add ZymoBIOMICS Microbial Community DNA standard as a positive control (at least 100 pg total).

7. Close the tube strips **firmly** with the caps provided. Mix by short vortexing, followed by a quick spin. Verify that the color of the reaction mix is homogeneously pink.
8. 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.

Safe stopping point: The reactions can be stored at 4 °C for up to 48 hours. For longer storage, keep the samples at -20 °C. It is best to continue with the samples within a month.

Quantification and Normalization

To optimize the read depth distribution across samples with variable or unknown input, we recommend performing a DNA quantification step after RC-PCR and prior to pooling. Quantification using the Qubit™ High Sensitivity (HS) assay, followed by equimolar pooling, improves read distribution within the EasySeq™ ITS Microbiome Library Prep workflow.

Normalization procedure

Determine the concentration of RC-PCR product using a 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
c. Measure according to manufacturer's instructions
d. Perform equimolar pooling ("EasySeq Pooling Calculator.xlsx" can be used [downloadable from the NimaGen website]).
e. Proceed with the purification using AmpliClean™ or AMPure XP

In case equal volume pooling is preferred, pool 5 µL RC-PCR product from each reaction into a 1.5 mL tube (increase volume to 15 µL when processing less than 24 samples). Transfer 100 µL to a new tube and proceed with the purification using AmpliClean™ or AMPure XP.

Purification

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

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

Bring the magnetic bead solution (AmpliClean™ or AMPure XP) to **room temperature** for at least one hour.

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

Purification #1

a. Vortex the beads thoroughly to resuspend.
b. Add 80 µL bead solution to the 100 µL pool 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 1-3 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 1-2 minutes at room temperature. **Do not over-dry.**
k. Add 105 µL Low TE buffer.

Off magnet:
l. Resuspend the beads by pipetting up and down, by flicking or by short vortexing.
m. Incubate for 2 minutes.

On magnet:
n. Wait for 1-3 minutes, or until the solution is fully cleared.
o. Carefully bring 100 µL of the clear solution into a new 1.5 mL tube, ensuring not to transfer any of the beads.

Purification #2

Off magnet:
p. Vortex the beads thoroughly to resuspend.
q. Add 80 µL bead solution to the 100 µL pool (from step **o.**) and mix well immediately by pipetting up and down at least 5 times or by short vortexing.
r. Incubate for 5 minutes.

On magnet:
s. Place the tube for 1-3 minutes on the magnet, or until the solution is fully cleared.

t. Remove and discard the liquid carefully, without disturbing the beads.
u. Add 300 µL (freshly prepared) 75% ethanol, without disturbing the beads.
v. Wait for 1 minute.

w. Repeat steps **t.**, **u.** and **v.** for a second ethanol wash step.
x. 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).
y. Dry with open cap for 1-2 minutes at room temperature. **Do not over-dry**. Immediately continue with the Elution.

Elution

a. On magnet: Add 45 µL Low TE buffer or Tris-HCl pH 8.0 buffer 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 1-3 minutes, or until the solution is fully cleared.
e. On magnet: Carefully bring 40 µL of the clear solution into a new 1.5 mL tube, making sure not to transfer any of the beads.

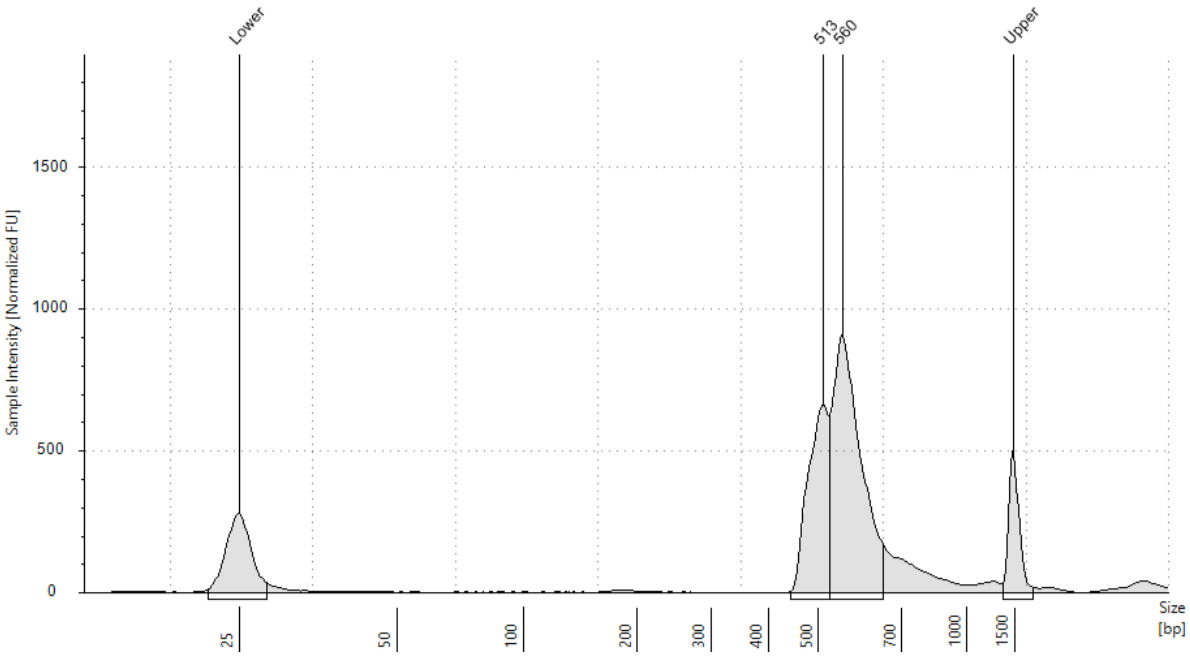
The libraries are now ready for a quantitative and qualitative check, followed by NGS.

Safe stopping point: The libraries can be stored at 4 °C for up to a week after elution. For longer storage, keep the samples at -20 °C. It is best to sequence the library within a month. Perform a new quantification after storing the library for longer than a day at either temperature.

Library Quantitative and Qualitative Check

Quantify the pooled and cleaned library with a Qubit® HS measurement. Verify the quality of the library using a TapeStation, Bioanalyzer or equivalent according to manufacturer's protocol.

Example of a clean library on TapeStation:



Downstream Processing using an Illumina® sequencer

1. Perform sequencing according to the manufacturer's instructions.
2. We recommend using the average length based on library QC like TapeStation calculating the library molarity, but a length of 560 bp could be used if no QC was performed.
3. We advise a read depth of 30 000 reads per sample for complex microbiome samples.
4. A spike-in of 5% PhiX is recommended for QC purposes.
5. We advise to start with a lower loading concentration for their initial sequence run and adjust in subsequent runs if needed. This avoids overclustering and potentially failure of the run. See the table below for sequencing guidelines.

Table Illumina sequencer and sample multiplexing guidelines

Sequencer	Reagent kit	Run setup	Number of samples*	Paired-end reads	Library concentration
MiSeq	V2 Nano 500 cycles	251-10-10-251	31	1 million	7 pM
MiSeq	V2 500 cycles	251-10-10-251	475	15 million	7 pM
MiSeq	V3 600 cycles	301-10-10-301	768	25 million	8 pM
NextSeq 1000/2000	P1 600 cycles	301-10-10-301	768	100 million	1000 pM**
NextSeq 1000/2000	P2 600 cycles	301-10-10-301	768	300 million	1000 pM**
MiSeq i100	5 M Reagent kit (600 cycles)	301-10-10-301	158	5 million	80 pM
MiSeq i100	25 M Reagent kit (600 cycles)	301-10-10-301	768	25 million	80 pM
MiSeq i100	50 M Reagent kit (600 cycles)	301-10-10-301	768	50 million	80 pM

*Theoretical maximum with 30 000 paired-end reads per sample or maximum number of indices available (n=768).
**Based on Illumina recommendations and assuming onboard denature and dilution.

Data Analysis

After sequencing on a short-read sequencer and demultiplexing, the data could be processed by *in-house* or open-source pipelines like DADA2 and QIIME2. Commercial options for ITS analysis are compatible with our library prep kit, examples are 1928, GenomeDetective, and BugSeq.

Extended Protocol

This protocol is designed for users who require full normalization control. Instead of a pooled cleanup, individual cleanups will be performed.

RC-PCR Thermal Cycling

This thermal cycling program is specifically designed for the EasySeq™ ITS Microbiome workflow and has been tested on Bio-Rad C1000™, Applied Biosystems SimpliAmp™, MiniAmp™, Quantstudio™ thermal cyclers at NimaGen. Using a different thermal cycler might require other settings.

This protocol takes approximately **6 hours** to complete, but may vary per thermal cycler used. When running this protocol for the first time, start the cycling program without samples, to check the predicted duration of 6 hours.

Temp	Duration	Ramp Rate (from previous step)	Cycles
98 °C	2 minutes	4 °C/sec*	1 x
80 °C	1 second	4 °C/sec	
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	
58 °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	25-30 x
80 °C	1 second	4 °C/sec	
58 °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 but not exceeding 4 °C/sec.

Reverse Complement PCR Reaction Set-up

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.

1. Thaw on ice:
   - RC-PCR Probe Panel (Green cap)
   - Probe Dilution Buffer (Blue cap)
   - HiFi Master Mix (Purple 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.

   Note: Always ensure all components are fully thawed and thoroughly mixed before use.

2. Take the IDX PCR plate and break off the number of strips needed.

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

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

3. Prepare in a fresh 1.5 mL tube the RC-PCR mix by combining and mixing:
   - 0.2 µL RC-PCR Probe Panel per reaction (Green cap)
   - 1.8 µL Probe Dilution Buffer per reaction (Blue cap)
   - 10 µL HiFi Master Mix per reaction (Purple 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.

4. Remove the seal from the PCR plate or strip(s).
5. Dispense 12 µL of the RC-PCR mix (from step 3) to each well of the plate/strip(s).
6. Add to each well 8 µL of DNA sample.

   Optional: Add ZymoBIOMICS Microbial Community DNA standard as a positive control (at least 100 pg total).

7. Close the tube strips **firmly** with the caps provided. Mix by short vortexing, followed by a quick spin. Verify that the color of the reaction mix is homogeneously pink.
8. 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 barcodes. From this point, RC-PCR product purification is performed using a magnetic bead based purification to remove primers, dimers and salts.

Safe stopping point: The reactions can be stored at 4 °C for up to 48 hours. For longer storage, keep the samples at -20 °C. It is best to continue with the samples within a month.

Purification

The purification involves one-sided size selection using magnetic beads, minimizing the number of reads lost to residual primers and dimers. The procedure will be performed per individual sample instead of a pooled purification.

Bring the magnetic bead solution (AmpliClean™ or AMPure XP) to **room temperature** for at least one hour.

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

Purification #1

a. Vortex the beads thoroughly to resuspend.
b. Add 16 µL bead solution to each sample 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 1-3 minutes on the magnet, or until the solution is fully cleared.

e. Remove and discard the liquid carefully, without disturbing the beads.
f. Add 80 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 1-2 minutes at room temperature. **Do not over-dry.**
k. Add 22 µL Low TE buffer.

Off magnet:
l. Resuspend the beads by pipetting up and down, by flicking or by short vortexing.
m. Incubate for 2 minutes.

On magnet:
n. Wait for 1-3 minutes, or until the solution is fully cleared.
o. Carefully bring 20 µL of the clear solution into a new PCR plate, ensuring not to transfer any of the beads.

Purification #2

Off magnet:
p. Vortex the beads thoroughly to resuspend.
q. Add 16 µL bead solution to each sample and mix well immediately by pipetting up and down at least 5 times or by short vortexing.
r. Incubate for 5 minutes.

On magnet:
s. Place the tube for 1-3 minutes on the magnet, or until the solution is fully cleared.

t. Remove and discard the liquid carefully, without disturbing the beads.
u. Add 80 µL (freshly prepared) 75% ethanol, without disturbing the beads.

v. Wait for 1 minute.

w. Repeat steps **t.**, **u.** and **v.** for a second ethanol wash step.
x. 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).
y. Dry with open cap for 1-2 minutes at room temperature. **Do not over-dry**. Immediately continue with the Elution.

Elution

a. On magnet: Add 12 µL Low TE buffer or Tris-HCl pH 8.0 buffer 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 1-3 minutes, or until the solution is fully cleared.
e. On magnet: Carefully bring 10 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.

Safe stopping point: The libraries can be stored at 4 °C for up to a week after elution. For longer storage, keep the samples at -20 °C. It is best to sequence the library within a month. Perform a new quantification after storing the library for longer than a day at either temperature.

Quantification and Pooling

Determine the concentration of the purified samples using a 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
c. Measure according to manufacturer's instructions
d. Perform equimolar pooling (“EasySeq Pooling Calculator.xlsx” can be used [downloadable from the NimaGen website]).

Library Quantitative and Qualitative Check

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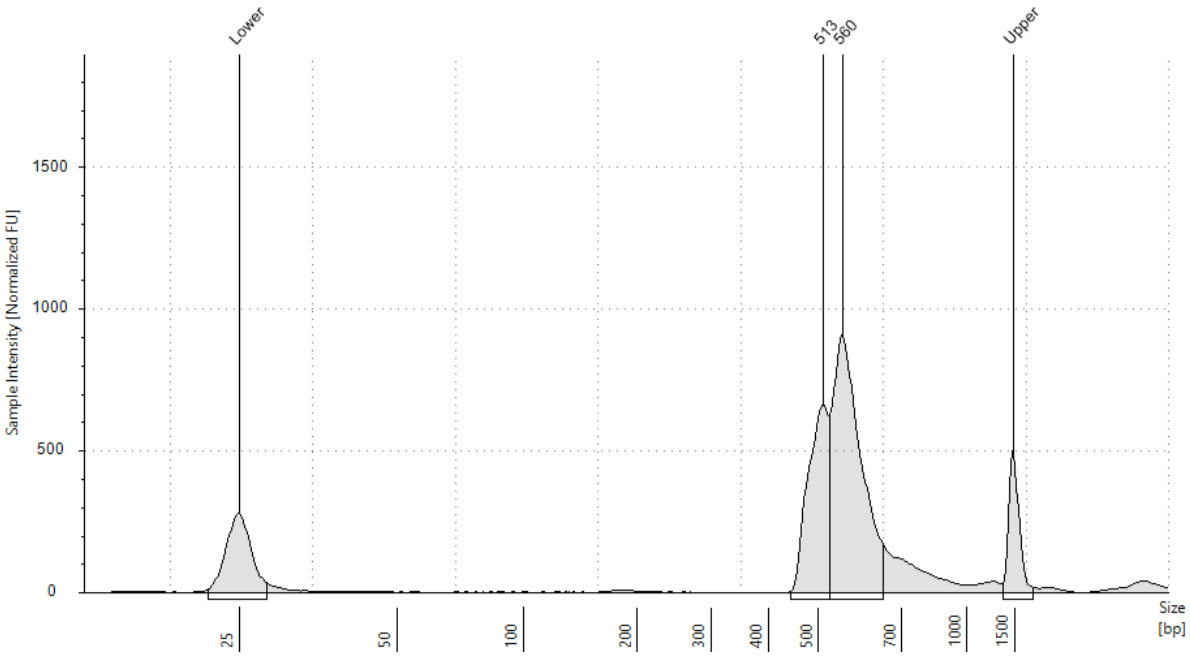

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Data Analysis

After sequencing on a short-read sequencer and demultiplexing, the data could be processed by *in-house* or open-source pipelines like DADA2 and QIIME2. Commercial options for ITS analysis are compatible with our library prep kit, examples are 1928, GenomeDetective, and BugSeq.

Troubleshooting

This guide could be useful to solve potential issues that might occur. If problems persist, please contact our technical support team at techsupport@nimagen.com**.** If possible, provide details of the experiment, QC, and add data of the positive control.

Low PCR yield

DNA contains PCR inhibitors	Use an appropriate DNA isolation kit or method to remove PCR inhibitors. Check the DNA quality with a spectrophotometer like the Nanodrop.
Insufficient input DNA	Concentrate the sample or repeat DNA isolation with more input material. Alternatively, 35-40 PCR cycles could be used but will result in a higher percentage of background ITS reads.

Cleanup

Low yield	Increase pool volume from 100 µL to 200 µL and elute in a lower volume of lowTE or Tris-HCl pH 8.0.

Primer-dimers peak after cleanup

Insufficient input DNA	Because of very low input DNA or negative samples, primer-dimers may be formed.
Improper washing of beads	Ensure that the whole bead pellet is covered with fresh 75% EtOH. Furthermore, resuspending the beads during washing could aid in primer-dimer removal.
Excess ethanol not removed	After the second wash, spin down the beads, return the tube on the magnet, and remove any residual liquid.

Low sequence output

Over or under loaded flow cell	Most likely this is caused by incorrect quantification. Make sure that the quantification method is calibrated. Also, insufficient removal of primer-dimers and adapters could result in low sequencing output. Furthermore, over or under loading of a flow cell could have detrimental effects on the sequencing run.

High percentage of chimeric reads

Too many PCR cycles	PCR artifacts like chimeric reads cannot be avoided but can be minimized by using the lowest number of cycles possible, depending on the DNA input. Though 25 cycles is a good starting point, lower number of cycles should be used when high percentage of chimeric reads are observed.
High DNA input	Chimeric reads are also linked to amount of input DNA. Dilute the sample or decrease PCR cycles.

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	2026-06-03

Appendix

Appendix A: Primer Sequences

ITS2 forward primer:
GCATCGATGAAGAACGCAGC

ITS2 reverse primer:
TCCTCCGCTTATTGATATGC

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