Bioo Scientific
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NEXTflex® 18S ITS Amplicon-Seq Kit - 4

(For Illumina® Platforms)

Catalog #4210-01 (Kit contains 8 reactions)

This product is for research use only.
Not for use in diagnostic procedures.

NEXTflex® 18S ITS Amplicon-Seq Kit - 4 - 4210-01

Product Overview

The NEXTflex® 18S ITS Amplicon-Seq Kit is designed to prepare multiplexed amplicon libraries that span the hypervariable Internal Transcribed Spacer (ITS) region of eukaryotic 18S ribosomal RNA (rRNA) genes. These libraries are compatible with paired-end sequencing on the Illumina® MiSeq® platform.

There are two main steps involved in 18S ITS amplicon processing: an initial PCR amplification using PCR primers that target the ITS domains, and a subsequent PCR amplification that integrates relevant flow cell binding domains and unique 12 base pair sample indices. A limited number of cleanup steps ensures maximum recovery of amplicons for downstream sequencing. It is highly recommended that sequencing of 18S ITS libraries be performed using the Illumina® MiSeq® V3 reagent kit (2X300), which requires the addition of PhiX. The pooled 18S ITS Libraries should contain a final composition of ~5% PhiX control.

Contents, Storage and Shelf Life

The NEXTflex® 18S ITS Amplicon-Seq Kit contains enough material to prepare eight 18S ITS samples from genomic DNA for sequencing on Illumina® platforms. The shelf life of all reagents is 12 months when stored properly. All components can be safely stored at -20°C.

Kit Contents	Amount
GREEN CAP	
NEXTflex® PCR Master Mix	192 µL
ORANGE CAP	
NEXTflex® 18S ITS PCR I Primer Mix	16 µL
YELLOW CAP	
NEXTflex® PCR II Barcoded Primer Mix 1 – 4	4 µL
WHITE CAP	
Resuspension Buffer	1 mL
Nuclease-free Water	1.5 mL

Required Materials not Provided

- 1 ng - 50 ng high-quality genomic DNA in up to 36 µL nuclease-free water for each library
- 96 well PCR Plate Non-skirted (Phenix Research™, Cat # MPS-499) or similar
- Adhesive PCR Plate Seal (Bio-Rad®, Cat # MSB1001)
- Agencourt® AMPure® XP 5 mL (Beckman Coulter® Genomics, Cat # A63880)
- Magnetic Stand -96 (Thermo Fisher Scientific®, Cat # AM10027) or similar
- Thermocycler
- 2, 10, 20, 200 and 1000 µL pipettes / multichannel pipettes
- Nuclease-free barrier pipette tips
- Vortex
- 80% Ethanol, freshly prepared (room temperature)

Revision History

Version	Date	Description
V14.10	October 2014	Initial Product Launch
V15.04	April 2015	The NEXTflex® PCR Master Mix has been reformulated for optimal performance.
V17.04	April 2017	PCR reactions have been optimized.

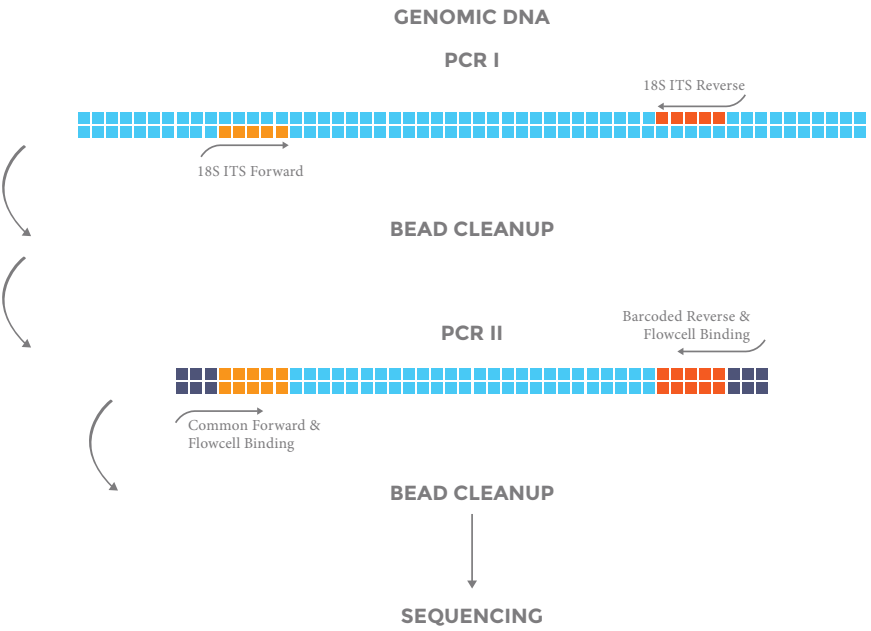
Warnings and Precautions

Bioo Scientific strongly recommends that you read the following warnings and precautions. Periodically, optimizations and revisions are made to the components and manual. Therefore, it is important to follow the protocol included with the kit. If you need further assistance, you may contact your local distributor or Bioo Scientific at nextgen@biooscientific.com.

- Do not use the kit past the expiration date.
- Ensure pipettes are properly calibrated as library preparations are highly sensitive to pipetting error.
- Try to maintain a laboratory temperature of 20°–25°C (68°–77°F).
- Genomic DNA sample quality may vary between preparations. It is the user's responsibility to utilize high quality genomic DNA. Genomic DNA that is heavily nicked or damaged may cause library preparation failure. Absorbance measurements at 260 nm are commonly used to quantify DNA and 260 nm / 280 nm ratios of 1.8 - 2.0 usually indicate relatively pure DNA. Other quantification methods using fluorescent dyes may also be used. The user should be aware that contaminating RNA, nucleotides and single-stranded DNA may affect the amount of usable DNA in a sample preparation.
- It is required that NEXTflex® 18S ITS PCR I & PCR II Primer Mixes are used during PCR amplification steps.

NEXTflex® 18S ITS Amplicon Sample Preparation Flow Chart

Figure 1: Sample flow chart with approximate times necessary for each step.



Starting Material

The NEXTflex® 18S ITS Amplicon-Seq Kit has been optimized and validated using 1 ng - 50 ng of high-quality genomic DNA.

Reagent Preparation

1. Briefly spin down each component to ensure material has not lodged in the cap or side of tube. Keep on ice and vortex each NEXTflex® Mix just prior to use.
2. Allow Agencourt® AMPure® XP Beads to come to room temperature and vortex the beads until liquid appears homogenous before every use.

STEP A: PCR I Amplification

Materials

Bioo Scientific Supplied

GREEN CAP - NEXTflex® PCR Master Mix
ORANGE CAP - NEXTflex® 18S ITS PCR I Primer Mix
WHITE CAP - Nuclease-Free Water

User Supplied

Thermocycler
96 Well PCR Plate
High-Quality Genomic DNA, 1 ng - 50 ng

1. For each sample, combine the following reagents on ice in the PCR plate.

_ µL	High-Quality Genomic DNA (1 ng - 50 ng in up to 36 µL)
_ µL	Nuclease-free Water
12 µL	NEXTflex® PCR Master Mix
2 µL	18S ITS PCR I Primer Mix
50 µL	TOTAL

2. Mix reaction well by pipetting.
3. Apply adhesive PCR plate seal and place in thermocycler for the following PCR cycles:

4 min	95°	
30 sec	95°	
30 sec	60°	Repeat 8 cycles
30 sec	72°	
4 min	72°	

STEP B: PCR I Cleanup

Materials

Bioo Scientific Supplied

WHITE CAP - Resuspension Buffer

User Supplied

Agencourt® AMPure® XP Magnetic Beads (room temperature)
80% Ethanol, freshly prepared (room temperature)
Magnetic Stand

1. Add 50 µL of AMPure® XP Beads to each clear sample. Mix thoroughly by pipetting.
2. Incubate at room temperature for 5 minutes.
3. Place the 96 well PCR Plate on the magnetic stand at room temperature until the supernatant appears completely clear.
4. Remove and discard the supernatant. Do not disturb beads. Some liquid may remain in wells.
5. With plate on stand, add 200 µL of freshly prepared 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Remove ethanol by pipette.
6. Repeat previous step, for a total of 2 ethanol washes. Ensure all ethanol has been removed.
7. Remove the plate from the magnetic stand and let dry at room temperature for 3 minutes.
8. Resuspend dried beads with 17 µL Resuspension Buffer. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the well.
9. Incubate resuspended beads at room temperature for 2 minutes.
10. Place plate on magnetic stand for 5 minutes until the sample appears clear.
11. Transfer 16 µL of clear supernatant (purified PCR I product) to new well.

STEP C: PCR II Amplification

Materials

Bioo Scientific Supplied

GREEN CAP - NEXTflex® PCR Master Mix
YELLOW CAP - NEXTflex® PCR II Primer Mix
WHITE CAP - Nuclease-Free Water

User Supplied

Thermocycler
96 Well PCR Plate
Purified PCR I product

1. For each sample, combine the following reagents on ice in the PCR plate.

16 µL	Purified PCR I product
20 µL	Nuclease-free Water
12 µL	NEXTflex® PCR Master Mix
2 µL	NEXTflex® PCR II Barcoded Primer Mix
50 µL	TOTAL

2. Mix well by pipetting.
3. Apply adhesive PCR plate seal and place in thermocycler for the following PCR cycles:

4 min	95°
30 sec	95°
30 sec	60°
30 sec	72°
4 min	72°

Repeat cycles as recommended in table below

Input to PCR I (ng)	PCR II Cycles
1	20
5	18
10	16
25	12
50	10

STEP D: PCR II Cleanup

Materials

Bioo Scientific Supplied
WHITE CAP - Resuspension Buffer

User Supplied
Agencourt® AMPure® XP Magnetic Beads (room temperature)
80% Ethanol, freshly prepared (room temperature)
Magnetic Stand

1. Add 50 µL of AMPure® XP Beads to each clear sample. Mix thoroughly by pipetting.
2. Incubate at room temperature for 5 minutes.
3. Place the 96 well PCR Plate on the magnetic stand at room temperature until the supernatant appears completely clear.
4. Remove and discard the supernatant. Do not disturb beads. Some liquid may remain in wells.
5. With plate on stand, add 200 µL of freshly prepared 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Remove ethanol by pipette.
6. Repeat previous step, for a total of 2 ethanol washes. Ensure all ethanol has been removed.
7. Remove the plate from the magnetic stand and let dry at room temperature for 3 minutes.
8. Resuspend dried beads with 17 µL Resuspension Buffer. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the well.
9. Incubate resuspended beads at room temperature for 2 minutes.
10. Place plate on magnetic stand for 5 minutes until the sample appears clear.
11. Transfer 16 µL of clear supernatant to new well.
12. To ensure cluster generation, it is recommended that you quantify your library by gel or Agilent® Bioanalyzer® platform. To quantify by gel, load 2 µL of 6X Gel Loading Dye and 6-10 µL of PCR Product on a 2% low melt agarose gel + EtBr.
13. Quantitate DNA library templates for optimal cluster density.

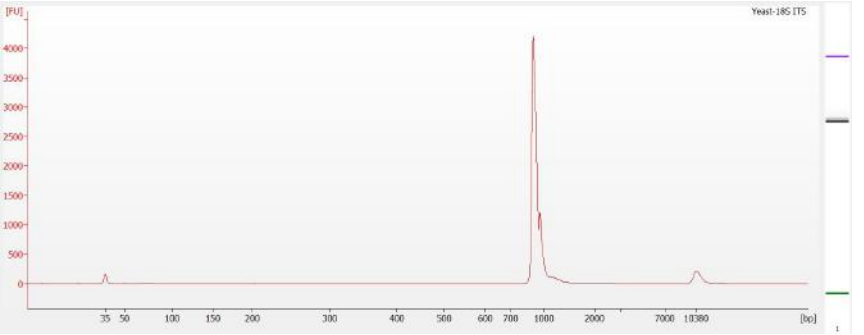


Figure 2. Electropherogram validation of the NEXTflex® 18S ITS PCR product (20 ng S. cerevisiae gDNA; 15 cycles of PCR).

*Important note – Eukaryotic 18S ITS regions vary in base composition and length. For community studies, expect bands that are 450-900 bp.

Oligonucleotide Sequences

NEXTflex® 18S ITS PCR I Primer Mix	
NEXTflex®	**Sequence 5' → 3'**
18S ITS Forward	CTCTTTCCCTACACGACGCTCTTCCGATCTTCCGTAGGTGAACCTGCGG
18S ITS Reverse	CTGGAGTTCAGACGTGTGCTCTTCCGATCTTCCTCCGCTTATTGATATGC

NEXTflex® 18S ITS PCR II Barcoded Primer Mix	
NEXTflex®	**Sequence 5' → 3'**
PCR II Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
PCR II Reverse	CAAGCAGAAGACGGCATACGAGAT[1]XXXXXXXXXXXX[1]GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

[1]XXXXXXXXXXXX denotes the index region of adapter. The index sequences and the respective reverse complement sequences contained in each adapter are listed below.

Reverse Primer Index Sequences and Reverse Complements

Barcoded Primer	Index Sequence (5' → 3')	Reverse Complement
1	GGCCGGCTAGAT	ATCTAGCCGGCC
2	AAGGAAGAGATA	TATCTCTTCCTT
3	GGACGGCATCTA	TAGATGCCGTCC
4	AAGGAAGGAGCG	CGCTCCTTCCTT

For an electronic list of the 18S ITS Primers visit our webpage.

Low Level Multiplexing

Use the following reverse primer combinations for low level multiplexing in this kit:
Pool of 2: (Barcodes 1 & 2) OR (Barcodes 3 & 4)
Pool of 3: (Barcodes 1, 2, & 3), (Barcodes 1, 2, 4), (Barcodes 1, 3, 4), OR (Barcodes 2, 3, 4)

RELATED PRODUCTS

RNA NGS Kits and Adapters for Illumina® Platforms

- NEXTflex® Rapid Directional RNA-Seq Kit
- NEXTflex® RNA-Seq Barcodes
- NEXTflex-96™ RNA-Seq Barcodes

- NEXTflex® Rapid Directional qRNA-Seq™ Kit

- NEXTflex® Small RNA Sequencing Kit v2
- NEXTlfex™ Small RNA Barcode Primers

- NEXTflex® Poly(A) Beads

DNA NGS Kits and Adapters for Illumina® Platforms

- NEXTflex® 16S V4 Amplicon-Seq Kit
- NEXTflex® 16S V4 Amplicon-Seq Kit 2.0
- NEXTflex® 16S V1-V3 Amplicon-Seq Kit
- NEXTflex® 18S ITS Amplicon-Seq Kit

- NEXTflex® Rapid DNA-Seq Kit
- NEXTflex® Cell Free DNA-Seq Kit
- NEXTflex® DNA Barcodes
- NEXTflex-96™ DNA Barcodes
- NEXTflex-HT™ Barcodes
- NEXTflex® Dual-Indexed DNA Barcodes

- NEXTflex® Bisulfite-Seq Kit
- NEXTflex® Bisulfite-Seq Barcodes
- NEXTflex® Methyl-Seq 1 Kit
- NEXTflex® Msp 1

- NEXTflex® ChIP-Seq Kit
- NEXTflex® ChIP-Seq Barcodes
- NEXTflex-96™ ChIP-Seq Barcodes

- NEXTflex® Pre-Capture Combo Kit
- NEXTflex® Rapid Pre-Capture Combo Kit
- NEXTflex® DNA Barcode Blockers

- NEXTflex® PCR-Free DNA Sequencing Kit
- NEXTflex® PCR-Free Barcodes

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THE NGS EXPERTS™

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