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This manual is for Reference Purposes Only. DO NOT use this protocol to run your assays. Periodically, optimizations and revisions are made to the kit and protocol, so it is important to always use the protocol included with the kit.

NEXTflex™ Metagenomics Application for Sciclone NGS Workstation

(Compatible with NEXTflex™ 16S V1-V3 Amplicon-Seq Kit, NEXTflex™ 16S V4 Amplicon-Seq Kit 2.0, NEXTflex™ 16S V5-V6 Amplicon-Seq Kit and NEXTflex™ 18S ITS Amplicon-Seq Kit)



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NEXTflex™ Metagenomics Amplicon-Seq Automation Guide

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Product Overview

The NEXTflex™ Metagenomics Amplicon-Seq Application is designed to prepare multiplexed amplicon libraries. The NEXTflex 16S Amplicon-Seq Kits enable analysis of the entire microbial community within a sample, without the bias introduced by the culturing required for other, non-NGS based 16S rRNA analysis methods. 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 NEXTflex amplicon processing: an initial PCR amplification using customized PCR primers that target the desired domain, 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. Using this kit, library preparation may be automated using the Maestro-based NEXTflex Metagenomics Amplicon-Seq Application on the PerkinElmer Sciclone NGS Workstation.

The NEXTflex Metagenomics Amplicon-Seq Workflow on the Sciclone NGS Workstation allows for preparation of up to 96 libraries at a time from between 1 ng and 50 ng high quality genomic DNA, using up to 384 unique barcoded primers. The user provides the number of samples to process (1 to 12 columns of 8 samples each) and selects either to use pre-arrayed barcoded primers in a hard shell PCR plate, or have the workstation array up to 32 barcoded primers at the start of each run. The workflow typically takes less than 3 hours to complete.

Application Name	NEXTflex Metagenomics Amplicon-Seq Steps
NEXTflex Metagenomics Amplicon-Seq	PCR 1 setup
	PCR 1 cleanup
	PCR 2 setup
	PCR 2 cleanup

Required Hardware

Part	Vendor / Part Number
Sciclone NGS Workstation	Perkin Elmer
Inheco 384-well plate adapter	NGS Sciclone accessory CLS 100853
Inheco 96-well adapters (2)	NGS Sciclone accessory CLS 128372
Inheco 96-well adapter/shaker	NGS Sciclone accessory CLS 100852
Magnet	Agencourt 96 ring magnet CLS128316

Required Software

- Maestro 6.0 software or later
- Maestro-based NEXTflex Metagenomics Amplicon-Seq Application

Required Consumables

Reagents

- NEXTflex™ reagents:
 - ◊ 16S V5-V6 Amplicon-Seq Kit (4205-01--07) or
 - ◊ 16S V4 Amplicon-Seq Kit 2.0 (4203-01--07) or
 - ◊ 16S V1-V3 Amplicon-Seq Kit (4202-01--07) or
 - ◊ 18S ITS Amplicon-Seq Kit (4210-01--07)
 - ◊ See kit manual for components and storage conditions.
- Ethanol 80% (room temperature)
- Agencourt® AMPure® XP beads (Beckman Coulter Genomics, Cat # A63881)

Plates and Reservoirs

PerkinElmer Part No.	Vendor / Part Number	Part	Quantity Needed
CLS127737	BioRad HSP-9631	96 Well PCR Plate, Bio-Rad Hardshell, Full Skirt	6-7 dependent on options selected
CLS111426	PerkinElmer	Pipette Tip, 150 µL, Art, Box, 10-96 Sterile Racks	2-12 dependent on number of samples processed and options selected
CLS133355	Seahorse Bioscience 201379-100	Deepwell-96 POS, Square 2.0 mL well, Polypropylene, Seahorse	1
CLS128469	Seahorse Bioscience 201244-100	Reservoir-Deepwell, 252 mL, Seahorse	1
CLS112785	Seahorse Bioscience 200856-100	946 Lid-Universal, Robotic friendly, Polystyrene	7
6008890	Corning 3672	Microplate-384 well, Round bottom, Polypropylene, pack of 10	1

Miscellaneous Equipment

- Adhesive PCR Plate Seal (BioRad, Cat # MSB1001)
- Thermal cycler
- 2, 10, 20, 200 and 1000 µL pipettes / multichannel pipettes
- Nuclease-free barrier pipette tips
- Microcentrifuge
- 1.5 mL nuclease-free microcentrifuge tubes
- Agilent 2100 Bioanalyzer
- Applied Biosystems 7900HT Fast Real-Time PCR System (or equivalent)

Warnings and Precautions

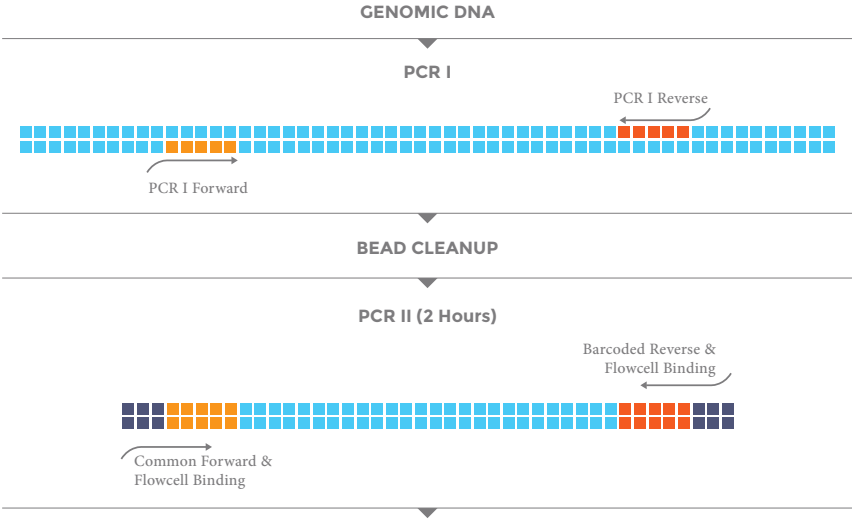
Bioo Scientific strongly recommends that you read the following warnings and precautions. Periodically, optimizations and revisions are made to the components and manual. Therefore, it is important to follow the protocol included with the kit. If you need further assistance, you may contact your local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

- Follow all safety precautions as recommended by PerkinElmer for the Sciclone NGS Workstation.
- 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.
- Allow AMPure XP beads to come to room temperature for at least 30 minutes prior to use

NEXTflex METAGENOMICS AMPLICON-SEQ AUTOMATION PROTOCOL

Metagenomics Amplicon-Seq Workflow on the Sciclone NGS Workstation

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



Starting Material

Up to 96 samples of 1 ng - 50 ng high quality DNA in 33 µL nuclease-free water.

NEXTflex Metagenomics Amplicon-Seq Library Preparation

This application begins with the 33 µL DNA samples in a hard-shell, 96-well PCR plate and proceeds with PCR I setup and cleanup, followed by PCR II setup and cleanup. PCR is performed off the deck.

Materials for Amplicon-Seq Library Preparation

Bio Scientific Supplied

NEXTflex Metagenomics Amplicon-Seq Components

GREEN CAP - NEXTflex™ PCR Master Mix

ORANGE CAP - NEXTflex™ PCR I Primer Mix*

YELLOW CAP or PCR PLATE - NEXTflex™ PCR II Barcoded Primer Mix

CLEAR CAP BOTTLE - Resuspension Buffer

* Use the appropriate Primer 1 mix for your application

User Supplied

Ethanol, 80%, freshly prepared, 100 mL

Agencourt AMPure XP Magnetic Beads

NEXTflex Metagenomics Amplicon-Seq Library Preparation Method

The library preparation method allows for NEXTflex Barcode Primer Mixes that are pre-aliquoted in a hard-shell PCR plate (where sample in sample well A1 would receive the barcoded primer mix from well A1 of the barcode plate, etc...), or for up to 32 NEXTflex Barcoded Primer Mixes to be arrayed by the machine, according to the Barcode Indexing tab in the NEXTflex Metagenomics Amplicon-Seq Workbook.

1. Power on the Sciclone NGS Workstation and the Inheco unit, and then start the PC controller.

Date	Random of Columns	Purification of Bead	Total Number of Samples	PCR I Mix	PCR II Mix	PCR II Barcoded Primer Mix	PCR II Barcoded Primer Mix	PCR II Barcoded Primer Mix	PCR II Barcoded Primer Mix	PCR II Barcoded Primer Mix	PCR II Barcoded Primer Mix
1/11/2016	5	5	5	41	41	41	41	41	41	41	41

PCR I Mixture	1 Sample	W Sample
NEXTflex Amplicon-Seq Master Mix	50	200
PCR I Primer Mix	2	200
Total	52	300

PCR II Mixture	1 Sample	W Sample
NEXTflex Amplicon-Seq Master Mix	50	200
PCR II Primer Mix	2	200
Total	52	300

Figure 2: Setup guide for the NEXTflex Metagenomics Amplicon-Seq. Enter the number of columns to process (1-12) in cell 'D2'

- Specify the number of samples and, if necessary, the appropriate NEXTflex Barcoded Primer Mixes, in the Maestro-based NEXTflex Metagenomics Amplicon-Seq Application Workbook:

- The path of the workbook is: [C:\ProgramData\Caliperls\Maestro\Workbooks\NEXTflex Metagenomics Amplicon-Seq Workbook.xls](#)
- Only columns of 8 samples can be processed at a time.
- Enter the number of columns to process in cell 'D2' of the library preparation tab.
- If Barcoded Primer Mixes are to be arrayed by the Sciclone, respective barcoded adapters are set by well ID in the Indexing tab of the workbook. Up to 32 NEXTflex Barcoded Primer Mixes for sample multiplexing can be used in one run. Alternatively, the NEXTflex Barcoded Primer Mixes can be placed in a hard-shell, 96-well PCR plate, allowing for up to 96 samples to be multiplexed.

Enter Barcoded Primer index Well # below			Enter Name of barcoded primer Mix in the Index Name column			
Sample Well	Index Well	Index Name	Index Well	Index Name	# Samples	Vol Needed
A1	1	ID001	1	ID001	2	10
B1	2	ID002	2	ID002	2	10
C1	3	ID003	3	ID003	2	10
D1	4	ID004	4	ID004	3	14
E1	5	ID005	5	ID005	2	10
F1	6	ID006	6	ID006	2	10
G1	7	ID007	7	ID007	2	10
H1	8	ID008	8	ID008	2	10
A2	9	ID009	9	ID009	2	10
B2	10	ID010	10	ID010	2	10
C2	11	ID011	11	ID011	2	10
D2	12	ID012	12	ID012	2	10
E2	1	ID001	13	ID013	1	6
F2	2	ID002	14	ID014	1	6
G2	3	ID003	15	ID015	1	6
H2	4	ID004	16	ID016	1	6
A3	4	ID004	17	ID017	1	6
B3	5	ID005	18	ID018	0	0
C3	6	ID006	19	ID019	0	0
D3	7	ID007	20	ID020	0	0
E3	8	ID008	21	ID021	0	0
F3	9	ID009	22	ID022	0	0
G3	10	ID010	23	ID023	0	0
H3	11	ID011	24	ID024	0	0
A4	12	ID012	25	ID025	0	0
B4	13	ID013	26	ID026	0	0
C4	14	ID014	27	ID027	0	0
D4	15	ID015	28	ID028	0	0
E4	16	ID016	29	ID029	0	0
F4	17	ID017	30	ID030	0	0
G4		#N/A	31	ID031	0	0
H4		#N/A	32	ID032	0	0
A5		#N/A				

Figure 3: The Adapter Indexing worksheet in the NEXTflex Metagenomics Amplicon-Seq Workbook. The Sciclone can array up to 32 NEXTflex Barcoded Primer Mixes according to the user input in the adapter indexing worksheet. Alternatively, the NEXTflex barcoded primers can be pre-arrayed in a hard shell PCR plate.

- Save the worksheet after all edits have been made. **DO NOT change the name or path of the workbook.**
- The NEXTflex Metagenomics Amplicon-Seq Workbook contains reagent and plate setup maps for processing the number of columns specified in step 2 above. Refer to the Library Prep tab for the library preparation method.
 - Master mix calculations, where necessary, are found in the workbook to the right of the plate setup maps

This application requires one master mix to be prepared by the user. The master mix must be combined prior to starting the Maestro-based NEXTflex Metagenomics Amplicon-Seq Library Preparation application.

The PCR I Master Mix is composed of the following components*:

15 μ L	NEXTflex™ PCR Master Mix
2 μ L	NEXTflex™ PCR I Primer Mix
<hr/>	
17 μ L	TOTAL VOLUME

*See workbook for preparation of master mix with appropriate overages for automation.

- Aliquot the prepared reaction mixes into hard-shell PCR plates as determined by the spreadsheet.
 - Keep sample plate and master mix plate on ice until ready to run the application.
 - Ensure no wells used have trapped air bubbles.
 - Prepare the remaining plates as depicted in the workbook.
 - Into a Seahorse deep well reservoir, add 100 mL of freshly prepared 80% EtOH solution. Cover the reservoir with a lid and store at room temperature.
 - In addition to the reagents and plates listed in the workbook, 1-2 clean hard-shell PCR plates are needed depending on the options selected for the run. Also, a Seahorse deep well plate is needed for waste collection.
4. Run the Maestro software. Open the NEXTflex Metagenomics Amplicon-Seq Library Preparation application.
 5. Start the run by pressing the play button. If operating under the Editor Mode, make sure to start the Main Method.

At the start of the run, the user will be prompted with a pop-up window. The user must select the NEXTflex Barcoded Primer Mix format. NEXTflex Barcoded Primer Mixes can be pre-arrayed in a Hard Shell PCR plate or placed in the Master Mix plate (arranged according to the NEXTflex Metagenomics Amplicon-Seq Workbook) and arrayed by the workstation. Select the correct format for your application.

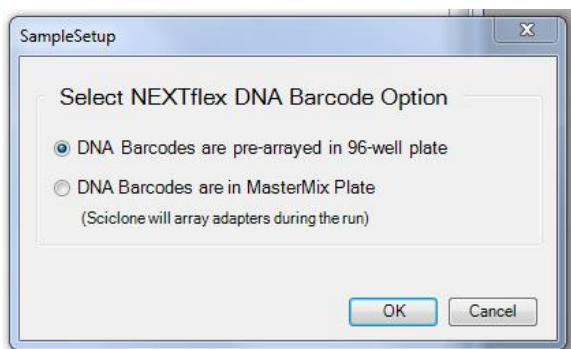


Figure 4: Barcoded Primer Mix options

6. The user will be prompted to verify the number of sample columns to be processed. Ensure the software shows the correct number of sample columns being processed. If not, the Excel workbook from Step 2 will need to be corrected, and the application restarted from the Main Method.

7. The NEXTflex Metagenomics Amplicon-Seq Library Preparation application requires the user to verify that the following Inheco adapters and magnets are on the deck:

Position A3: 96-well

Position A4: 384-well

Position D2: 96-well

Position D4: 96-well shaker

Position B4: Magnet

8. Set up the Sciclone NGS Workstation deck as indicated by the application. This involves placement of reagent and sample plates, as well as consumables such as pipette tip boxes.
9. The Setup window will show a graphic of the completed deck setup. Ensure the positioning of all plates and boxes is correct, and then click "Finished" to begin the automated library preparation method. A blinking green light on top of the machine indicates the Maestro-based NEXTflex Metagenomics Amplicon-Seq Application is running.
10. The Sciclone NGS Workstation will proceed through the automated reaction steps and their respective cleanups: PCR I setup, PCR I cleanup, PCR II setup, and PCR II cleanup.
11. Once PCR setup is completed, the application will pause. Seal the PCR plate and place it on a thermocycler. Use the appropriate cycling conditions found in the kit manual.

Once the final cleanup is completed, the final library plate can be safely stored at -20°C or used for immediate validation (qPCR and/or Bioanalyzer).

RELATED PRODUCTS

Illumina Compatible RNA NGS Kits and Adapters

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

NEXTflex™ Small RNA Barcode Primers

NEXTflex™ Poly(A) Beads

Illumina Compatible DNA NGS Kits and Adapters

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

NOTES

NOTES



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We can't wait to hear from you!



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