

NEXTFLEX® Combo-Seq™ mRNA/miRNA Automation Kit

NOVA-5139-53

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Product Overview

The NEXTFLEX® Combo-Seq™ mRNA/miRNA Automation Kit enables the user to generate up to 96 combined mRNA and microRNA libraries from 5 ng – 100 ng of total RNA, without the need for rRNA depletion or poly(A) selection on the PerkinElmer® Sciclone® G3 NGSx workstation. The workflow relies on a novel technique in which poly(A)-tailed RNA species are selectively reverse transcribed then fragmented by RNase H into fragments of a useful length. The mRNA fragments are subsequently processed along with the small RNAs present in the sample. Libraries prepared with this kit are directional (strand-specific) and compatible with Illumina® sequencers.

NEXTFLEX® Combo-Seq™ mRNA/miRNA sequencing libraries contain Unique Dual Indices (UDIs), which are designed to specifically address the index-hopping phenomenon associated with Illumina® platforms utilizing a patterned flow cell. These UDIs prevent mis-assigned reads from appearing in final datasets, allowing for the highest assurance of data integrity.

Materials and Reagents

Provided Reagents

Contents, Storage and Shelf Life

The NEXTFLEX® Combo-Seq™ mRNA/miRNA Automation Kit contains enough material to prepare 96 libraries using the PerkinElmer® Sciclone® G3 NGSx workstation for Illumina® sequencing. The volumes provided support a single, 96-samples run or two, 48-sample runs. The shelf life of all reagents is at least 6 months when stored properly. All components can safely be stored at -20°C, except: NEXTFLEX® Adapter Depletion Solution, Resuspension Buffer, and Nuclease-free Water, which can be stored at room temperature, and NEXTFLEX® Cleanup Beads, which should be stored at 4°C.

Kit Contents	Cap Color	Amount	Storage Temp.
NEXTFLEX® Anchored Oligo(dT) Primer	PINK CAP	227 µL	-20°C
RT/RNase H Annealing Buffer	PINK CAP	227 µL	-20°C
RT/RNase H Reaction Buffer	PINK CAP	578 µL	-20°C
NEXTFLEX® Combo-Seq™ Reverse Transcriptase	PINK CAP	378 µL	-20°C
NEXTFLEX® Thermostable RNase H	PINK CAP	608 µL	-20°C
NEXTFLEX® PAP Buffer	RED CAP	578 µL	-20°C
NEXTFLEX® Poly(A)	RED CAP	145 µL	-20°C
NEXTFLEX® tRNA/YRNA Blocker	RED CAP	131 µL	-20°C
Adapter Depletion Solution	WHITE CAP	5 mL	Room Temp.
NEXTFLEX® Combo-Seq™ 5' Ligation Buffer	LIGHT PURPLE CAP	854 µL	-20°C
NEXTFLEX® Combo-Seq™ 5' 4N Adapter	LIGHT PURPLE CAP	197 µL	-20°C
NEXTFLEX® 5' Ligation Enzyme Mix	LIGHT PURPLE CAP	263 µL	-20°C
NEXTFLEX® Combo-Seq™ RT Buffer	BLUE CAP	(2) 757 µL	-20°C
NEXTFLEX® UDI Barcoded Primer Mix*	GREEN CAP	6 µL each*	-20°C
NEXTFLEX® PCR Master Mix	GREEN CAP	832 µL	-20°C
Resuspension Buffer	YELLOW CAP	(2) 1.5 mL	Room Temp.
Nuclease-free Water	WHITE CAP	10 mL	Room Temp.
NEXTFLEX® Cleanup Beads	WHITE CAP	16 mL	4°C

* Pre-plated, see figure below

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

Index Primer Option

Please enter the number of columns to process (1-12)

Please enter the starting column for the index primer plate:

For Example, if columns 1-3 were used prior, enter column 4 to continue to work down the plate.
Please ensure the number of eligible index columns are equal to or less than number of columns to process.

Pre-plated NEXTFLEX® UDI Barcoded PCR Primer Plate

(left) Barcode ID for the provided plate. Sequences can be found at the bottom of this document. Barcodes will be added column-wise to the PCR reaction. Any unused columns can be used in a subsequent run by selecting the starting column from an interface (right) in the Maestro software.

Sciclone® NGS Hardware, Software, Applications and Consumables

Required Hardware

Part	Vendor/Part Number
Sciclone® NGS Workstation	PerkinElmer®
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
Agencourt® 96 ring magnet	Agencourt® CLS128316
Spacer Assembly for Agencourt® 96 ring magnet	Agencourt® CLS128316

Required Software

Maestro 6.3 software or later

Required Consumables

Part No.	Vendor / Part Number	Part	Quantity Needed for 96 samples
6008870	PerkinElmer®	Bio-Rad® Hard-Shell® 96 Well PCR Plate, Full Skirt	15
111426	PerkinElmer®	Pipette Tip, 150 µL, Art, Box, 10-96 Sterile Racks	33
6008880	PerkinElmer®	Deepwell-96 POS, Square 2.0 mL well, Polypropylene, Seahorse	2
6008700	PerkinElmer®	Reservoir-Deepwell, 12 column, 21mL	3
6000030	PerkinElmer®	946 Lid-Universal, Robotic friendly, Polystyrene	9
6008890	PerkinElmer®	Microplate-384 well, Round bottom, Polypropylene, pack of 10	1

Required Reagents Not Provided

- **Vapor-Lock – Qiagen; Cat No./ID: 981611**
- 5 ng – 100 ng total RNA in up to 11 µL Nuclease-free Water
- Human Universal RNA (Biochain # R4234565) or other total RNA, as a control (optional)
- Isopropanol
- 80% Ethanol, freshly prepared
- Adhesive PCR Plate Seal (Bio-Rad, Cat # MSB1001)
- Thermal cycler compatible with Bio-Rad® Hard Shell Plates (see Bio-Rad website for recommendations)
- Vortex

Provided Maestro Applications

Application Name	NEXTFLEX® Combo-Seq Steps	Approximate Run Time (Including offline Incubations and PCR)
Library Prep 1	Step A – Reverse Transcription and RNase H Digestion	1 hour, 40 minutes
	Step B - Polyadenylation	1 hour, 45 minutes
	Step C – 5' 4N Adapter Ligation	1 hour
	Step D – First Strand Synthesis	1 hour, 30 minutes
Library Prep 2	Step E – Barcoded PCR Setup	20-45 minutes (depending on # of cycles)
Post-PCR Size Selection Cleanup	Step F – Size Selection & Cleanup	1 hour

Warnings and Precautions

- Ensure that all pipette tips, microcentrifuge tubes, and other consumables are RNase-free.
- Take extreme care to keep an RNase-free workspace. It is recommended that an RNase decontamination solution such as RNase-Zap® (SigmaAldrich) be used on gloves and workspace surfaces before making mastermixes.
- DTT in buffers may precipitate after freezing. If precipitate is seen, vortex buffer for 1 - 2 minutes or until the precipitate is in solution. The performance of the buffer is not affected once the precipitate is in solution.
- Vortex and centrifuge each component prior to use, in order to ensure material has not lodged in the cap or side of the tube.
- Do not remove enzymes from -20°C until immediately before use; return to -20°C immediately after use.
- Do not freeze NEXTFLEX® Cleanup Beads. NEXTFLEX® Cleanup Beads should be stored at 4°C and brought to room temperature before use. Vortex and ensure homogenous appearance before use.
- Except where specified, thermal cycling should be performed with a heated lid.

Starting Material

The NEXTFLEX® Combo-Seq™ mRNA/miRNA Automation Kit is designed for use with 5 ng – 100 ng of total RNA. Upstream depletion of ribosomal RNA is neither required nor recommended. RNA should be free of DNA, divalent cations, salts such as guanidinium salts, organics such as phenol, reducing agents such as DTT, and chelators such as EDTA. Best results are obtained using high-quality starting material with a RIN \geq 8. The use of degraded RNA will result in a low proportion of mRNA-mapping reads in the sequencing library.

Some total RNA extraction and purification methods may not efficiently isolate small RNAs. Users should verify that their extraction and purification method isolates total RNA including small RNAs such as miRNA.

The NEXTFLEX® Combo-Seq™ mRNA/miRNA Kit includes an optional reagent designed to deplete tRNA fragments and Y RNA fragments which are abundant in many types of input RNA. Users who wish to deplete these RNA fragments should add 1 μ L of NEXTFLEX® tRNA/YRNA Blocker prior to 5' ligation, as indicated in Steps B-11 and B-14.

If the user is performing the procedure for the first time, we recommend including a positive control such as 20 ng of total RNA (Biochain R4234565 or similar).

Using Qiagen® Vapor-Lock™ barrier

Qiagen® Vapor-Lock™ barrier must be used during the Library Prep 1 application. Qiagen® Vapor-Lock™ barrier should be manually applied directly to the top of the samples by pipetting prior to beginning the run. Qiagen® Vapor-Lock™ barrier will be removed during the bead cleanup at the end of Step A and will not inhibit any downstream applications.

Offline Steps

Two steps are done offline in a thermal cycler: Step B – Polyadenylation and Step E – Library Amplification.

Step ID	Condition								
Step B – Polyadenylation	15 minutes – 37°C 20 minutes – 90°C < 5 minutes – 4°C								
Step E – PCR	<table border="0"> <tr> <td>30 sec – 98°C</td> <td rowspan="5">} cycle as per chart below</td> </tr> <tr> <td>10 sec – 98°C</td> </tr> <tr> <td>20 sec – 65°C</td> </tr> <tr> <td>15 sec – 72°C</td> </tr> <tr> <td>2 min – 72°C</td> </tr> <tr> <td>Hold – 4°C</td> <td></td> </tr> </table>	30 sec – 98°C	} cycle as per chart below	10 sec – 98°C	20 sec – 65°C	15 sec – 72°C	2 min – 72°C	Hold – 4°C	
30 sec – 98°C	} cycle as per chart below								
10 sec – 98°C									
20 sec – 65°C									
15 sec – 72°C									
2 min – 72°C									
Hold – 4°C									
Input RNA Amount	# of PCR Cycles								
100 ng	13								
20 ng	16								
10 ng	17								
5 ng	18								

Appendix: PCR Primer Sequences

NEXTFLEX®	Sequence
NEXTFLEX® Anchored Oligo(dT) Primer	TTTTTTTTTTTTTTTTTTVN
NEXTFLEX® Combo-Seq™ 5' 4N Adapter	3'ddNVTTTTTTTTT5'5'rGrUrUrCrArGrArGrUrUrCrArGrArCrGrArCrGrArUrCrNrNrNrN3'
RT primer (included in NEXTFLEX® Combo-Seq™ RT Buffer)	TCCTTGGCACCCGAGAATTCCA(7) ₁₀ VN where (7) ₁₀ contains modified bases
UDI primer 1 – P7 (included in NEXTFLEX® UDI Barcoded Primer Mix)	CAAGCAGAAGACGGCATAACGAGATXXXXXXXXX ¹ GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
UDI primer 2 – P5 (included in NEXTFLEX® UDI Barcoded Primer Mix)	AATGATACGGCGACCACCGAGATCTACACXXXXXXXXX ² ACACGTTACAGTTCACAGTCCGA

XXXXXXXXX¹ denotes the P7 index region of the primer. The index sequences that are added to each barcoded library via PCR are listed below. Note: UDI primer 1 contains the reverse complement of the sequence listed under P7 Index. The final library, however, will contain the P7 Index listed below, and the sequencer will read the index as listed below.

XXXXXXXXX² denotes the P5 index region of the primer. The index sequences that are added to each barcoded library via PCR are listed below.

NEXTFLEX® Combo-seq mRNA/miRNA UDI primers

NEXTFLEX®	i7 sequences	i5 sequences	NEXTFLEX®	i7 sequences	i5 sequences	NEXTFLEX®	i7 sequences	i5 sequences	NEXTFLEX®	i7 sequences	i5 sequences
Index 1	AAGATCAT	AATAATAG	Index 25	AAGAGTTG	AATATTGA	Index 49	AAGACTGT	AATATCTG	Index 73	AAGTACAG	AATAGCAA
Index 2	TGCTATTC	TTAGTAGC	Index 26	TTATACAA	TTCTCAAT	Index 50	TTCGGAAC	TTAGCTAA	Index 74	TGAATGGA	TTCGCTTG
Index 3	GACGTGTC	TGCGTGGC	Index 27	GATTAGGA	GTGGAACG	Index 51	GAAGATCG	GCTTCTTG	Index 75	TCGAAGCT	GCCACGCG
Index 4	CGGTAGTC	ACCAATTG	Index 28	CTCTGGCG	AGTTACGG	Index 52	CGGAGGCT	CGTTATAT	Index 76	CTCATAAT	GCGCTGAC
Index 5	GACAGCAG	CTACTGGT	Index 29	GATACCTA	GCGAGATC	Index 53	GAAGAAGC	TGCGCGAT	Index 77	TCTTAGGC	TACACAAC
Index 6	CTTGTAACA	AGCAGAGT	Index 30	CGCGTATC	AGCGTACG	Index 54	CTGCGAAG	CGACATGG	Index 78	CTCTACAC	GTATGGTC
Index 7	GACAAGTG	CATTATCG	Index 31	GATACGAT	CTTGGTAC	Index 55	GAATCTGA	GAAGCCTC	Index 79	TCTAGGTT	AGTCACAA
Index 8	CTCGCCTT	GTGCAGTC	Index 32	CTGGAGCT	CGCAGCTG	Index 56	CTGCGGTA	GATTGGAG	Index 80	CGTTCTGC	CCGCTAAT
Index 9	GAGCGTCA	GTTACAC	Index 33	GAACGATA	ACGGCACA	Index 57	GTCTTGCT	GTCTATCG	Index 81	TCCTACAG	CCTAGTGG
Index 10	CGCGCTCG	CCGCATAC	Index 34	CGGTCCAT	GCGGTGTG	Index 58	CTGACAGT	AATACAGG	Index 82	CTTCACGA	GCTAGAGT
Index 11	GAGCTCTA	GTGGCGAA	Index 35	GAAGTGGC	CGATACTA	Index 59	GAATTGAA	TACTCTGT	Index 83	TCAGTGAG	CGTGCAGG
Index 12	CTATAGGA	GTGGATAC	Index 36	CGGTGAGA	CAGCTACA	Index 60	CGTCGTCA	TGTTGGTA	Index 84	CTTCTTAA	CGTAGTTC
Index 13	AAGAGAGC	AATATAAC	Index 37	AAGACATA	AATATGCT	Index 61	AAGTAAGT	AATAGATT	Index 85	AAGTTATC	AATTAATG
Index 14	TTATAGCG	TTCTAGGT	Index 38	TTATGTAT	TTCTCCGC	Index 62	TGACGGAA	TTCTCTCA	Index 86	TGACCGCG	TTAATTC
Index 15	GAGCTAAG	AGATTGTG	Index 39	GAACAGAT	CTATATTG	Index 63	GAATACGG	GCACACAA	Index 87	TCAGACTT	TGTGCAAC
Index 16	CGAAGCCA	CAATCCGT	Index 40	CTTGGCCT	CAGGAAGG	Index 64	CTTATCAG	TGTGAGTC	Index 88	CTGCATCA	CGATCGTT
Index 17	TTGTGGCT	TTACTTAC	Index 41	GAACATTC	GCCGTGCA	Index 65	TCCGCGTC	GTGCGAGG	Index 89	TCATGCGT	CCGACGAC
Index 18	CGCGAGAC	TTGTCGAC	Index 42	CTGATATA	GAGTTGCG	Index 66	CTGAGTGC	GCATCACA	Index 90	CTTGCGGA	CTCCGCGC
Index 19	GAGATCGG	AAGGAGCG	Index 43	GAAGCGAG	AACCTCAC	Index 67	TCCTAACG	TCCAACCT	Index 91	TCATCTCT	GCCACCTC
Index 20	CGCACTTA	CTCGAAGC	Index 44	CGGCCTCT	AACCATGG	Index 68	CGAGATAA	TGGTACAC	Index 92	CCGGCTGA	CCTCTGGA
Index 21	GATGTCAG	GTTAGAAC	Index 45	GAAGTCGA	TACTGTTC	Index 69	TCGCGATT	CCAAGCAC	Index 93	TGCCCGCG	GACACAGA
Index 22	CTACTTCG	CGAACTGT	Index 46	CGGCCATG	GACTGAGC	Index 70	CTAGTTAT	CCTTGATG	Index 94	CAGAGCTC	GCAATTCG
Index 23	GATTACTC	CATGTCTC	Index 47	GAAGTTAC	GTCCAAGT	Index 71	TCGCACTC	GATGGTAA	Index 95	TGCGATAG	CACACATG
Index 24	ACTCAGAC	CGACTATA	Index 48	CTTAGAGA	CCGATGCG	Index 72	CTATTATC	CCTCCGAG	Index 96	CCATTGAC	CAGATTCT

Revision History

Version	Date	Description
V0.1	June 2019	Initial release
V20.04	April 2020	Change in RT primer formulation

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For more information, visit applied-genomics.com

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