

FOR REFERENCE PURPOSES

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™ Diabetes-4 Amplicon Panel
(Illumina Compatible)
Catalog #4238-01 (8 reactions)



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NEXTflex™ Diabetes-4 Amplicon Panel - 4238-01

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Product Overview

The NEXTflex™ Diabetes-4 Amplicon Panel produces barcoded amplicon libraries compatible with Illumina platforms. Libraries are constructed using genomic DNA extracted from blood or cell samples. FFPE or cfDNA samples are not compatible with this kit. This panel contains a total of 38 primer pairs in two pools that allow for the amplification and sequencing of all coding exons of the *PAX4*, *INS*, and *BLK* loci. Amplicon regions of interest range in size between 134 - 234 bp. The regions of interest plus primer pad sites, which comprise the read portion of the libraries, range between 200 - 298 bp. These target regions are amplified in PCR I, which is followed by adapter ligation. PCR II then enriches for the product of interest, as well as introducing unique barcodes and sequences necessary for downstream sequencing (Fig. 1). NEXTflex™ Cleanup Beads are included, and have been validated with amplicon library preparation. NEXTflex™ Diabetes-4 Amplicon Primer Mixes are optimized to achieve high coverage uniformity and reduce off-target reads.

The NEXTflex Diabetes-4 Amplicon Panel covers 6.2 kilobases comprising 26 coding exons. Libraries have 100% uniformity at $\geq 0.2x$ mean coverage and 100% on-target reads. Up to 1000 samples can be multiplexed with at least 100x coverage on a single Illumina 2x150 MiSeq lane for detection of germline mutations. Standard Illumina sequencing primers may be used with this kit.

Contents, Storage and Shelf Life

The NEXTflex™ Diabetes-4 Amplicon Panel contains enough material to prepare 8 sample libraries. The shelf life of all reagents is 12 months when stored properly. All components should be stored at -20°C, except the Nuclease-free Water and Resuspension Buffer, which can be safely stored at room temperature, and NEXTflex™ Cleanup Beads and NEXTflex™ PCR Enhancers, which should be stored at 4°C.

| Kit Contents | Amount |
|--|-------------|
| PINK CAP | |
| NEXTflex™ Diabetes-4 Amplicon Primer Mix 1 | 32 μ L |
| BLUE CAP | |
| NEXTflex™ Diabetes-4 Amplicon Primer Mix 2 | 32 μ L |
| ORANGE CAP | |
| NEXTflex™ PCR Enhancer 1 | 16 μ L |
| RED CAP | |
| NEXTflex™ PCR Enhancer 2 | 48 μ L |
| CLEAR CAP | |
| NEXTflex™ Hot Start PCR I Master Mix (A) | 192 μ L |

| | |
|--------------------------------------|-----------|
| LIGHT PURPLE CAP | |
| NEXTflex™ Ligation Mix | 336 µL |
| NEXTflex™ Amplicon DNA Adapter | 20 µL |
| YELLOW CAP | |
| NEXTflex™ PCR II Barcoded Primer Mix | 4 µL each |
| GREEN CAP | |
| NEXTflex™ PCR II Master Mix | 80 µL |
| WHITE CAP | |
| Nuclease-free Water | 1.5 mL |
| Resuspension Buffer | 1.5 mL |
| BROWN CAP | |
| NEXTflex™ Cleanup Beads | (2) 1 mL |

Required Materials not Provided

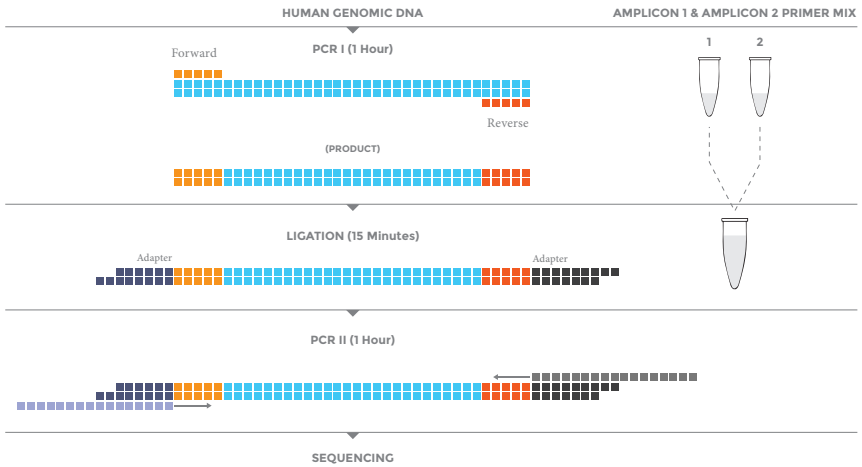
- 20 - 100 ng of extracted genomic DNA (two 10 - 50 ng aliquots in up to 30 µL nuclease-free water each)
- Ethanol 80% (room temperature)
- 96 well PCR Plate Non-skirted (Phenix Research, Cat # MPS-499) or similar
- 96 well Library Storage and Pooling Plate (Fisher Scientific, Cat # AB-0765) or similar
- Adhesive PCR Plate Seal (BioRad, Cat # MSB1001)
- 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
- Microcentrifuge
- 1.5 mL nuclease-free microcentrifuge tubes
- Vortex

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.
- Do not heat NEXTflex™ Adapters above room temperature.
- Try to maintain a laboratory temperature of 20°–25°C (68°–77°F).
- DNA sample quality may vary between preparations. It is the user's responsibility to utilize high quality DNA. 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.
- NEXTflex™ Diabetes-4 Amplicon Primer Mixes are required for PCR I amplification.

NEXTflex™ Diabetes-4 Amplicon Panel Preparation Flow



Chart

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

Starting Material

The NEXTflex™ Diabetes-4 Amplicon Panel has been optimized and validated using 10 - 50 ng of high quality genomic DNA for each PCR I primer pool.

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. DTT in buffers may precipitate after freezing. If precipitate is seen in any mix, vortex for 1 minute or until the precipitate is in solution. The performance of the mix is not affected once precipitate is in solution.
3. Before every use, allow NEXTflex™ Cleanup Beads to come to room temperature and vortex until liquid appears homogenous.

STEP A: PCR I - Targeted Diabetes-4 Amplification

Materials

Bioo Scientific Supplied

PINK CAP - NEXTflex™ Diabetes-4 Amplicon Primer Mix 1

BLUE CAP - NEXTflex™ Diabetes-4 Amplicon Primer Mix 2

ORANGE CAP - NEXTflex™ PCR Enhancer 1

RED CAP - NEXTflex™ PCR Enhancer 2

CLEAR CAP - NEXTflex™ Hot Start PCR I Master Mix (A)

WHITE CAP - Nuclease-Free Water

User Supplied

Thermocycler

96 Well PCR Plate

Adhesive PCR Plate Seal

For each reaction, 10 - 50 ng of genomic DNA in up to 30 µL

1. For each sample, prepare two separate reactions using Diabetes-4 Primer Mix 1 and 2 by combining the following reagents in adjacent wells in a PCR plate. **Note: It is recommended to combine these reagents as a master mix if processing multiple samples.**

Reaction 1

| | |
|-------|--|
| _ µL | Genomic DNA (10 - 50 ng in up to 30 µL) |
| _ µL | Nuclease-free Water |
| 4 µL | NEXTflex™ Diabetes-4 Amplicon Primer Mix 1 |
| 1 µL | NEXTflex™ PCR Enhancer 1 |
| 3 µL | NEXTflex™ PCR Enhancer 2 |
| 12 µL | NEXTflex™ Hot Start PCR I Master Mix (A) |
| <hr/> | |
| 50 µL | TOTAL |

Reaction 2

| | |
|-------|--|
| _ µL | Genomic DNA (10 - 50 ng in up to 30 µL) |
| _ µL | Nuclease-free Water |
| 4 µL | NEXTflex™ Diabetes-4 Amplicon Primer Mix 2 |
| 1 µL | NEXTflex™ PCR Enhancer 1 |
| 3 µL | NEXTflex™ PCR Enhancer 2 |
| 12 µL | NEXTflex™ Hot Start PCR I Master Mix (A) |
| <hr/> | |
| 50 µL | TOTAL |

2. Mix thoroughly by pipette.

3. Apply adhesive PCR plate seal and place in thermocycler for the following PCR cycles:

| | | |
|--------|------|------------|
| 2 min | 98°C | |
| 20 sec | 98°C | } 4 cycles |
| 4 min | 68°C | |
| 20 sec | 98°C | } 4 cycles |
| 4 min | 66°C | |
| 20 sec | 98°C | } 4 cycles |
| 4 min | 64°C | |
| 20 sec | 98°C | } 4 cycles |
| 4 min | 62°C | |
| Hold | 4°C | |

4. Proceed immediately to Step B: PCR I Cleanup.

STEP B: PCR I Cleanup

Materials

Bioo Scientific Supplied

WHITE CAP - Resuspension Buffer

BROWN CAP - NEXTflex™ Cleanup Beads (room temperature)

User Supplied

80% Ethanol, freshly prepared (room temperature)

Magnetic Stand

50 μ L PCR I Reactions 1 & 2 (from Step A)

1. Add 30 μ L of NEXTflex™ Cleanup Beads to each reaction. Mix thoroughly until homogenized.
2. Incubate at room temperature for 5 minutes.
3. Place the 96 well PCR Plate on the magnetic stand at room temperature for 5 minutes, or until the supernatant appears completely clear.
4. Do not discard the supernatant in this step. Transfer the clear supernatant to a new well. Be careful not to disrupt the magnetic bead pellet or transfer any magnetic beads with the supernatant.
5. Add 30 μ L of NEXTflex™ Cleanup Beads to supernatant. Mix thoroughly until homogenized.
6. Incubate at room temperature for 5 minutes.
7. Place the 96 well PCR plate on the magnetic stand at room temperature for 5 minutes, or until the supernatant appears completely clear.
8. Remove and discard the supernatant. Do not disturb beads. Some liquid may remain in wells.
9. With plate on stand, gently add 200 μ L of freshly prepared 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully remove ethanol by pipette.
10. Repeat previous step, for a total of 2 ethanol washes. Ensure all ethanol has been removed.
11. Remove the plate from the magnetic stand and let dry at room temperature for 5 minutes, or until bead pellet is visibly dry.
12. Resuspend dried beads with 16 μ L of Resuspension Buffer. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the well.
13. Incubate resuspended beads at room temperature for 3 minutes.
14. Place the 96 well PCR plate on the magnetic stand at room temperature for 5 minutes, or until sample appears clear.
15. Transfer 14 μ L of clear supernatant (purified PCR I Reaction) to new well.
16. Proceed immediately to Step C: Adapter Ligation.

STEP C: Adapter Ligation

Materials

Bioo Scientific Supplied

LIGHT PURPLE CAP - NEXTflex™ Amplicon DNA Adapter, NEXTflex™ Ligation Mix

User Supplied

Thermocycler

Adhesive PCR Plate Seal

Ice

14 µL Purified PCR I Reactions 1 & 2 (from Step B)

1. For each sample, combine the following reagents on ice in a nuclease-free 96 well PCR Plate:

| | |
|---------|--------------------------------|
| 14 µL | Purified PCR I Reaction 1 |
| 14 µL | Purified PCR I Reaction 2 |
| 2.5 µL | NEXTflex™ Amplicon DNA Adapter |
| 42 µL | NEXTflex™ Ligation Mix |
| <hr/> | |
| 72.5 µL | TOTAL |
2. Mix thoroughly by pipette.
3. Apply adhesive PCR plate seal and incubate in a thermocycler for 15 minutes at 22°C.
4. Proceed immediately to Step D: Cleanup.

STEP D: Cleanup

Materials

Bioo Scientific Supplied

WHITE CAP - Resuspension Buffer

BROWN CAP - NEXTflex™ Cleanup Beads (room temperature)

User Supplied

80% Ethanol, freshly prepared (room temperature)

Magnetic Stand

72.5 µL Adapter Ligated DNA (from Step C)

1. Add 58 µL of NEXTflex™ Cleanup Beads to each sample. Mix thoroughly until homogenized.
2. Incubate at room temperature for 5 minutes.
3. Place the 96 well PCR Plate on the magnetic stand at room temperature for 5 minutes, or 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, gently add 200 µL of freshly prepared 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully 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 5 minutes or until bead pellet is visibly dry.
8. Resuspend dried beads with 40 µL of 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 3 minutes.
10. Place the 96 well PCR plate on the magnetic stand at room temperature for 5 minutes, or until sample appears clear.
11. Gently transfer 38 µL of clear sample to new well.
12. Proceed immediately to Step E: PCR II Amplification.

STOPPING POINT: Alternatively, the procedure may be stopped at this point with samples stored at -20°C. To restart, thaw frozen samples on ice before proceeding to Step E: PCR II Amplification.

STEP E: PCR II Amplification

Materials

Bioo Scientific Supplied

YELLOW CAP - NEXTflex™ PCR II Barcoded Primer Mix

GREEN CAP - NEXTflex™ PCR II Master Mix

WHITE CAP - Resuspension Buffer

BROWN CAP - NEXTflex™ Cleanup Beads (room temperature)

User Supplied

Thermocycler

96 Well PCR Plate

Adhesive PCR Plate Seal

80% Ethanol, freshly prepared (room temperature)

Magnetic Stand

38 µL Purified Adapter Ligated DNA (from Step D)

1. For each sample, combine the following reagents on ice in a nuclease-free 96 well PCR plate:

| | |
|-------|--|
| 38 µL | Purified Adapter Ligated DNA (from Step D) |
| 2 µL | NEXTflex™ PCR II Barcoded Primer Mix |
| 10 µL | NEXTflex™ PCR II Master Mix |
| <hr/> | |
| 50 µL | TOTAL |

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

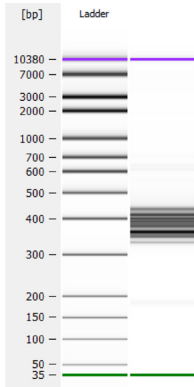
| | | |
|--------|------|-------------|
| 20 min | 65°C | } 12 cycles |
| 2 min | 98°C | |
| <hr/> | | |
| 30 sec | 98°C | |
| 30 sec | 65°C | |
| 60 sec | 72°C | |
| <hr/> | | |
| 4 min | 72°C | |

4. Remove PCR plate from the thermocycler. Add 40 µL of NEXTflex™ Cleanup Beads to each sample and mix thoroughly until homogenized.
5. Incubate at room temperature for 5 minutes.
6. Place the 96 well PCR plate on the magnetic stand at room temperature for 5 minutes, or until the supernatant appears completely clear.
7. Remove and discard the supernatant. Do not disturb beads. Some liquid may remain in wells.

8. With plate on stand, gently add 200 μ L of freshly prepared 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully remove ethanol by pipette.
9. Repeat previous step, for a total of 2 ethanol washes. Ensure all ethanol has been removed.
10. Remove the plate from the magnetic stand and let dry at room temperature for 5 minutes or until bead pellet is visibly dry.
11. Resuspend dried beads with 22 μ L of Resuspension Buffer. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the well.
12. Incubate resuspended beads at room temperature for 3 minutes.
13. Place the 96 well PCR plate on the magnetic stand at room temperature for 5 minutes, or until sample appears clear.
14. Gently transfer 20 μ L of clear sample to a new well and proceed to library analysis or seal plate with adhesive PCR plate seal and store at -20°C . Qubit (Life Technologies) and Bioanalyzer (Agilent) are recommended to quantify and analyze quality of the library.

LIBRARY VALIDATION

A



B

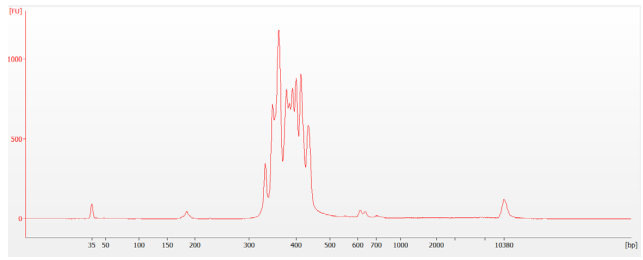


Figure 2. High Sensitivity DNA Chip Output:

- A) NEXTflex Diabetes-4 Amplicon Panel Library - 20 ng input (Bioanalyzer gel image)
- B) NEXTflex Diabetes-4 Amplicon Panel Library - 20 ng input (electropherogram)

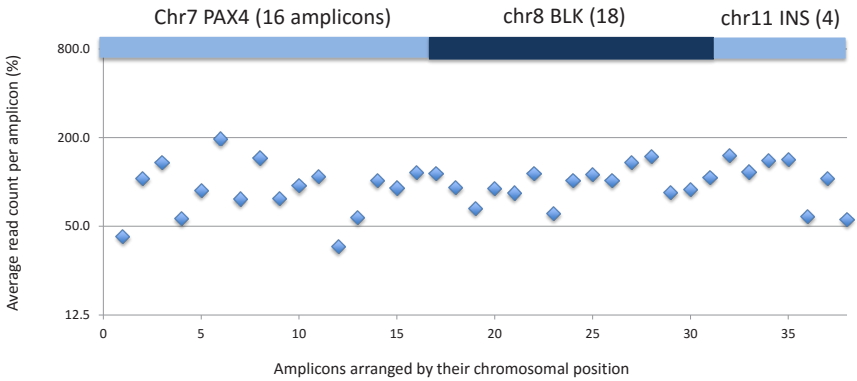


Figure 3. Performance of 38 amplicons from NEXTflex™ Diabetes-4 Amplicon Panel on an Illumina Sequencing Platform. Numbers in parentheses indicate number of amplicons.

Oligonucleotide Sequences

| NEXTflex™ PCR II Barcoded Primer Mix | |
|--------------------------------------|---|
| NEXTflex™ | Sequence 5' → 3' |
| PCR II Forward Primer | AATGATACGCGACCACCGAGATCTACTCTTTCCTACACGACGCTCTTCCGATCT |
| PCR II Reverse Barcoded Primer | CAAGCAGAAGACGGCATAACGAGATXXXXXXXXXXXXX'GTGACTGGAGTT CAGACGTGTGCTCTTCCGATCT |

'XXXXXXXXXXXXX' denotes the index region of the primer. The index sequences and the respective reverse complement sequences contained in each primer are listed below. The reverse complement is the sequence reported in the index read.

Reverse Primer Index Sequences and Reverse Complements

| Barcoded Primer | Sequence 5' → 3' | Reverse Complement |
|-----------------|------------------|--------------------|
| 1 | GGCCGGCTAGAT | ATCTAGCCGGCC |
| 2 | AAGGAAGAGATA | TATCTTTCCTT |
| 3 | GGACGGCATCTA | TAGATGCCGTCC |
| 4 | AAGGAAGGAGCG | CGCTCCTTCCTT |
| 5 | GGACGGCGCTCG | CGAGCGCCGTCC |
| 6 | CCGACTCTCGA | TCGAGAGTCCGG |
| 7 | GGCCGGCCGAGC | GCTCGGCCGGCC |
| 8 | CCGACTGAGCT | AGCTCAGTCCGG |
| 9 | GGACGGGCAGT | ACTGCCGCGTCC |
| 10 | CCGGAGAAGTAA | TTACTTCTCCGG |
| 11 | GGCCGCGGTCA | TGACGCGCGGCC |
| 12 | CCGGAGATCATT | AATGATCTCCGG |
| 13 | GGACGTACGCTT | AAGCGTACGTCC |
| 14 | AAGGACTGATAA | TTATCAGTCTT |
| 15 | GGACGCGATGAC | GTCATCGCGTCC |
| 16 | CCGGAGAGACGG | CCGTCTCTCCGG |
| 17 | GGACGTAGCGAA | TTCGCTACGTCC |
| 18 | CCGGAAGAGCGT | ACGCTCTTCCGG |
| 19 | GGCCGCTACTG | CAGTACGCGGCC |
| 20 | AAGGATCAGTAC | GTAAGTATCTT |
| 21 | GGCCGTATATCC | GGATATACGGCC |
| 22 | CCGGAAGCTATG | CATAGCTTCCGG |
| 23 | GGCCGATGCCTC | GAGGCATCGGCC |
| 24 | CCGGATCCTTAT | ATAAGGATCCGG |

| | | |
|----|---------------|--------------|
| 25 | GGACGATCGGAG | CTCCGATCGTCC |
| 26 | CCGGATCGAATA | TATTCGATCCGG |
| 27 | GGACGATTAAGA | TCTTAATCGTCC |
| 28 | CCGGATCAGGCG | CGCCTGATCCGG |
| 29 | GGACGATATTCT | AGAATATCGTCC |
| 30 | CCGGATCTCCGC | GCGGAGATCCGG |
| 31 | GGACCGGCCATG | CATGGCCGGTCC |
| 32 | AAGGTACGTGAC | GTCACGTACCTT |
| 33 | GGACCGGTTGCA | TGCAACCGGTCC |
| 34 | CCGGTCAACAGG | CCTGTTGACCGG |
| 35 | GGACCTGGGCT | AGCCCAAGGTCC |
| 36 | CCGGTACCAAGC | GCTTGGTACCGG |
| 37 | GGACCTTCCCGA | TCGGGAAGGTCC |
| 38 | CCGGTACGTTTCG | CGAACGTACCGG |
| 39 | GGCCCTTAAATC | GATTTAAGGGCC |
| 40 | AAGGTCAGTTCT | AGAACTGACCTT |
| 41 | GGACCAAGGCGG | CCGCCTTGGTCC |
| 42 | CCGGTTGCATCA | TGATGCAACCGG |
| 43 | GGCCCAACCGCC | GGCGGTTGGGCC |
| 44 | CCGGTTGGTAGT | ACTACCAACCGG |
| 45 | GGACCAATTATT | AATAATTGGTCC |
| 46 | CCGGTTGACGAC | GTCGTCAACCGG |
| 47 | GGCCTGAGATTT | AAATCTCAGGCC |
| 48 | CCGGCCGCGCAC | GTGCGGGCCGG |
| 49 | GGACTGACTAAA | TTAGTCAGTCC |
| 50 | CCGGCCGGCGTG | CACGCCGGCCGG |
| 51 | GGACTGATCGGG | CCCATCAGTCC |
| 52 | CCGGCCGATACA | TGTATCGGCCGG |
| 53 | GGACTCTGAAAG | CTTTCAGAGTCC |
| 54 | CCGGCCGCGGTA | TACCGGCGCCGG |
| 55 | GGACTCTCTTTC | GAAAGAGAGTCC |
| 56 | AAGGCTAGCCAG | CTGGCTAGCCTT |
| 57 | GGCCTCTCCCT | AGGGAAGAGGCC |
| 58 | AAGGCTACGGTC | GACCGTAGCCTT |
| 59 | GGACTTAGGGA | TCCCTAGAGTCC |
| 60 | AAGGCTATAACT | AGTTATAGCCTT |
| 61 | GGACTTCGAGGC | GCCTCGAAGTCC |
| 62 | AAGGCCGCGACG | CGTCGCGGCCTT |
| 63 | GGCCTTCCTCCG | CGGAGGAAGGCC |
| 64 | AAGGCCGGCTGC | GCAGCCGGCCTT |
| 65 | GGACTTCTCTTA | TAAGAGAAGTCC |

| | | |
|-----|---------------|---------------|
| 66 | AAGGCCGATCAT | ATGATCGGCCTT |
| 67 | GGACTTCAGAAT | ATTCTGAAGTCC |
| 68 | AAGGCCGTAGTA | TACTACGGCCTT |
| 69 | GGACTAGGACCA | TGGTCCTAGTCC |
| 70 | CCGGCTAATGTT | AACATTAGCCGG |
| 71 | GGACTAGCTGGT | ACCAGCTAGTCC |
| 72 | CCGGCTATACAA | TTGTATAGCCGG |
| 73 | GGACTAGTCAAC | GTTGACTAGTCC |
| 74 | CCGGCTACGTGG | CCACGTAGCCGG |
| 75 | GGACTAGAGTTG | CAACTCTAGTCC |
| 76 | AAGGCCGCGCACA | TGTGCGCGCCTT |
| 77 | GGCCACAGTACC | GGTACTGTGGCC |
| 78 | AAGGGTTAATTT | AAATTAACCCCTT |
| 79 | GGCCACATGCAA | TTGCATGTGGCC |
| 80 | AAGGGTTCGGG | CCCGGAACCCCTT |
| 81 | GGACACAACGTT | AACGTTGTGTCC |
| 82 | AAGGGTTGGCCC | GGGCCAACCCCTT |
| 83 | GGACATGGTGTG | CACACCATGTCC |
| 84 | CCGGGAACCAAA | TTTGGTTCCCGG |
| 85 | GGACATGCACAC | GTGTGCATGTCC |
| 86 | CCGGGAATTGGG | CCCAATTCCCGG |
| 87 | GGACATGACACA | TGTGTGCATGTCC |
| 88 | CCGGGAAGGTTT | AAACCTTCCCGG |
| 89 | GGACAACGTCAT | ATGACGTTGTCC |
| 90 | CCGGGTAAAGGA | TCCTTAACCCGG |
| 91 | GGACAACGACG | CGTCAGTTGTCC |
| 92 | CCGGGTTCCTTC | GAAGGAACCCGG |
| 93 | GGCCAACACTGC | GCAGTGTGGCC |
| 94 | CCGGGTGGAAG | CTTCCAACCCGG |
| 95 | GGCTGGTCATAC | GTATGACCAGCC |
| 96 | CCGAACCTTAGG | CCTAAGGTTCCGG |
| 97 | GGATGGTACGCA | TGCGTACCATCC |
| 98 | CCGAACCGGCTT | AAGCCGTTCCGG |
| 99 | GGATGCAGTTAT | ATAACTGCATCC |
| 100 | CCGAAGGCCCTC | GAGGGCCTTCGG |
| 101 | GGCTGCACAATA | TATTGTGCAGCC |
| 102 | CCGAAGGTTTCT | AGAAACCTTCGG |
| 103 | GGATGCATGGCG | CGCCATGCATCC |
| 104 | CCGAAGGAAAGA | TCTTTCCTTCGG |
| 105 | GGATGCAACCGC | GCGGTTGCATCC |
| 106 | AAGAATTGGGAT | ATCCAATTCTT |

| | | |
|-----|--------------|--------------|
| 107 | GGCTGTGGTCGA | TCGACCACAGCC |
| 108 | AAGAACCAAGAG | CTCTTGGTTCTT |
| 109 | GGCTGTGCAGCT | AGCTGCACAGCC |
| 110 | AAGAACCGGAGA | TCTCCGTTCTT |
| 111 | GGCTGTGACTAG | CTAGTCACAGCC |
| 112 | AAGAACCTTCTC | GAGAAGGTTCTT |
| 113 | GGATGACCACGG | CCGTGGTCATCC |
| 114 | CCGAATTGGTCA | TGACCAATTCGG |
| 115 | GGATGACTGTAA | TTACAGTCATCC |
| 116 | CCGAATTAACTG | CAGTTAATTCGG |
| 117 | GGCTGACACATT | AATGTGTCAGCC |
| 118 | AAGAAGGTTGAA | TTCAACCTTCTT |
| 119 | GGATCGAGAAGC | GCTTCTCGATCC |
| 120 | AAGATATATTAT | ATAATATATCTT |
| 121 | GGATCGACTTCG | CGAAGTCGATCC |
| 122 | CCGATCGGCCGA | TCGGCCGATCGG |
| 123 | GGATCGATCCTA | TAGGATCGATCC |
| 124 | CCGATCGATTAG | CTAATCGATCGG |
| 125 | GGATCGAAGGAT | ATCCTTCGATCC |
| 126 | CCGATCGTAATC | GATTACGATCGG |
| 127 | GGCTCCTGATCA | TGATCAGGAGCC |
| 128 | CCGATGCCGCGG | CCGCGGCATCGG |
| 129 | GGATCCTCTAGT | ACTAGAGGATCC |
| 130 | AAGATTATATAC | GTATATAATCTT |
| 131 | GGTCCTTCGAC | GTCGAAGGAGCC |
| 132 | CCGATGCATATT | AATATGCATCGG |
| 133 | GGATCCTAGCTG | CAGCTAGGATCC |
| 134 | AAGATTAGCGCA | TGCGCTAATCTT |
| 135 | GGCTCCTCTGAA | TTCAGGAGAGCC |
| 136 | CCGATATTACGT | ACGTAATATCGG |
| 137 | GGATCTCTCAGG | CCTGAGAGATCC |
| 138 | AAGATCGCGTAA | TTACGCGATCTT |
| 139 | GGATCTCAGTCC | GGACTGAGATCC |
| 140 | CCGATATGCATG | CATGCATATCGG |
| 141 | GGATCAGGAGAG | CTCTCCTGATCC |
| 142 | AAGATGCCGATC | GATCGGCATCTT |
| 143 | GGCTCAGCTCTC | GAGAGCTGAGCC |
| 144 | CCGATTAGCTAT | ATAGCTAATCGG |
| 145 | GGATCAGTCTCT | AGAGACTGATCC |
| 146 | AAGATGCATCGA | TCGATGCATCTT |
| 147 | GGCTCAGAGAGA | TCTCTCTGAGCC |

| | | |
|-----|----------------|--------------|
| 148 | CCGATTATAGCG | CGCTATAATCGG |
| 149 | GGCTTGGCCTGA | TCAGGCCAAGCC |
| 150 | CCGACCAGTCCG | CGGACTGGTCCG |
| 151 | GGATTGGTTTACAG | CTGAACCAATCC |
| 152 | CCGACCACAGGC | GCCTGTGGTCCG |
| 153 | GGCTTGGAAGTC | GACTTCCAAGCC |
| 154 | AAGACTACTGAAG | CTTCAGTGTCTT |
| 155 | GGATTCCGGTGG | CCACCGGAATCC |
| 156 | CCGACGTCACCA | TGGTGACGTCCG |
| 157 | GGCTTCCTTGTT | AACAAGGAAGCC |
| 158 | CCGACGTACAAC | GTTGTACGTCCG |
| 159 | GGATTCCAACAA | TTGTTGGAATCC |
| 160 | AAGACTGTGTTT | AAACACAGTCTT |
| 161 | GGATTAACCCAT | ATGGGTTAATCC |
| 162 | CCGACTGGTTTC | GAAACCAGTCCG |
| 163 | GGGTTAATTGTC | GCAAATTAACCC |
| 164 | CCCACTGCAAAG | CTTTGCAGTGGG |
| 165 | GGATAGCGCAA | TTTGCCTATCC |
| 166 | AAGAGAGAGTGG | CCACTCTCTCTT |
| 167 | GGATAGCCGTTT | AAACGGCTATCC |
| 168 | CCGAGCTTCA | TGTGAAGCTCCG |
| 169 | GGATAGCTACCC | GGGTAGCTATCC |
| 170 | AAGAGAGCTGTT | AACAGCTCTCTT |
| 171 | GGCTAGCATGGG | CCCATGCTAGCC |
| 172 | AAGAGAGGACAA | TTGTCCTCTCTT |
| 173 | GGATACGGCTTC | GAAGCCGTATCC |
| 174 | AAGAGTCCTCAG | CTGAGGACTCTT |
| 175 | GGCTACGCGAAG | CTTCGCGTAGCC |
| 176 | AAGAGTCGAGTC | GACTCGACTCTT |
| 177 | GGATACGTAGGA | TCCTACGTATCC |
| 178 | AAGAGTCAGACT | AGTCTGACTCTT |
| 179 | CCAGCGGCCAT | ATGGCGCGCTGG |
| 180 | TTGCTAGAGGGC | GCCCTTAGCAA |
| 181 | CCCGCGCTAACG | CGTTAGCGCGGG |
| 182 | TTGCTAGCTTTA | TAAAGCTAGCAA |
| 183 | CCAGCGCATTGC | GCAATGCGCTGG |
| 184 | TTGCTAGGAAAT | ATTTCTAGCAA |
| 185 | CCAGCTAGCACC | GGTGCTAGCTGG |
| 186 | TTGCTCTCTGGG | CCCAGAGAGCAA |
| 187 | CCAGCATGCTGA | TCAGCATGCTGG |
| 188 | TTGCTGACTCCT | AGGAGTCAGCAA |

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| 189 | CCAGCATCGACT | AGTCGATGCTGG |
| 190 | TTGCTGATCTTC | GAAGATCAGCAA |
| 191 | CCAGCATTAGTC | ACTAATGCTGG |
| 192 | TTGCTGAAGAAG | CTTCTCAGCAA |
| 193 | CCAGCATATCAG | CTGATATGCTGG |
| 194 | TTGCTGAGAGGA | TCCTCTCAGCAA |
| 195 | CCCGTGTGTCTC | GAGACACACGGG |
| 196 | TTGCCAACCTAG | CTAGGTTGGCAA |
| 197 | CCAGTGCAGAG | CTCTGACACTGG |
| 198 | TTGCCAATTCGA | TCGAATTGGCAA |
| 199 | CCAGTGTACTCT | AGAGTACACTGG |
| 200 | TTGCCAAGGATC | GATCCTTGGCAA |
| 201 | CCCGTCAAGTAA | TTCCTGACGGG |
| 202 | TTGCCCTAACGG | CCGTTAAGGCAA |
| 203 | CCAGTCACACTT | AAGTGTGACTGG |
| 204 | TTGCCTTGGTAA | TTACCAAGGCAA |
| 205 | CCCGTACTGGAT | ATCCAGTACGGG |
| 206 | TTGCCGGAATA | TATTTCCGGCAA |
| 207 | CCGGTACACCTA | TAGGTGTACCGG |
| 208 | TTACCGGTTTAT | ATAAACCGGTAA |
| 209 | CCGGACTTCTAG | CTAGAAGTCCGG |
| 210 | TTACGTAATCTC | GAGATTACGTAA |
| 211 | CCGGACTAGATC | GATCTAGTCCGG |
| 212 | TTCCGTAGCTCT | AGAGCTACGGAA |
| 213 | CCACATGGTCAA | TTGACCATGTGG |
| 214 | TTGGGCCAAGGG | CCCTTGGCCCAA |
| 215 | CCACAGTCATGC | GCATGACTGTGG |
| 216 | TTGGGAATTAAT | ATTAATTCCCAA |
| 217 | CCACAGTGTACG | CGTACACTGTGG |
| 218 | TTGGGAACCGGC | GCCGGTCCCAA |
| 219 | CCACTAGAGAAA | TTTCTCTAGTGG |
| 220 | TTGGCGCGCTGG | CCAGCGCGCCAA |
| 221 | CCACTAGTCTTT | AAAGACTAGTGG |
| 222 | TTGGCGCATCAA | TTGATGCGCCAA |
| 223 | CCACTCAGTTC | GAAGTGAAGTGG |
| 224 | TTGGCCGGCACT | AGTGCCGGCCAA |
| 225 | CCACTTCTCAAG | CTTGAGAAGTGG |
| 226 | TTGGCCGCGTGA | TCACGCGGCCAA |
| 227 | CCACTTCTGGA | TCCAGGAAGTGG |
| 228 | TTGGCCGTACAG | CTGTACGGCCAA |
| 229 | CCACTTCGACCT | AGGTCGAAGTGG |

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|-----|---------------|---------------|
| 230 | TTGGCCGATGTC | GACATCGGCCAA |
| 231 | CCACTCTAGCCG | CGGCTAGAGTGG |
| 232 | TTGGCTAGCGTA | TACGCTAGCCAA |
| 233 | CCACTCTCGGC | GCCGAAGAGTGG |
| 234 | TTGGCTAATACG | CGTATTAGCCAA |
| 235 | CCACTCTCTAAT | ATTAGAGAGTGG |
| 236 | TTGGCTATATGC | GCATATAGCCAA |
| 237 | CCACTCTGATTA | TAATCAGAGTGG |
| 238 | TTGGCTACGCAT | ATGCGTAGCCAA |
| 239 | CCACTGAAGGGT | ACCCTTCAGTGG |
| 240 | TTGGCATGCCAC | GTGGCATGCCAA |
| 241 | CCACTGATCCCA | TGGGATCAGTGG |
| 242 | TTGGCATCGGTG | CACCGATGCCAA |
| 243 | CCACTGACTTTG | CAAAGTCAGTGG |
| 244 | TTGGCATTAAACA | TGTTAATGCCAA |
| 245 | CCACTGAGAAAC | GTTTCTCAGTGG |
| 246 | TTGGCATATTGT | ACAATATGCCAA |
| 247 | CCACCAATTTAC | GTAAATTTGGTGG |
| 248 | TTGGTGTCAAGT | ACTTGACACCAA |
| 249 | CCACCAACCCGT | ACGGGTTGGTGG |
| 250 | TTGGTGTGTTC | TGAACACACCAA |
| 251 | CCACCAAGGGCA | TGCCCTTGGTGG |
| 252 | TTGGTGTACCTG | CAGGTACACCAA |
| 253 | CCACCTTAATAT | ATATTAAGGTGG |
| 254 | TTGGTCAGTAGC | GCTACTGACCAA |
| 255 | CCACCTTCCGCG | CGCGGAAGGTGG |
| 256 | TTGGTCATGCTA | TAGCATGACCAA |
| 257 | CCACCTTGGCGC | GCGCCAAGGTGG |
| 258 | TTGGTCACATCG | CGATGTGACCAA |
| 259 | CCACCGGAAGCC | GGCTTCCGGTGG |
| 260 | TTGGTACTGAGG | CCTCAGTACCAA |
| 261 | CCACCGCCTAA | TTAGGCCGGTGG |
| 262 | TTGGTACGTCTT | AAGACGTACCAA |
| 263 | CCACGATATAGC | GCTATATCGTGG |
| 264 | TTGGAGAGATAT | ATATCTCTCCAA |
| 265 | CCACGATATCG | CGATAATCGTGG |
| 266 | TTGGAGACTATA | TATAGTCTCCAA |
| 267 | CCACGATCGCTA | TAGCGATCGTGG |
| 268 | TTGGAGATCGCG | CGCGATCTCCAA |
| 269 | CCACGATGCGAT | ATCGCATCGTGG |
| 270 | TTGGAGAAGCGC | GCGCTTCTCCAA |

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|-----|--------------|--------------|
| 271 | CCGCGTAATTCA | TGAATTACGCGG |
| 272 | TTAGACTGAATG | CATTCAGTCTAA |
| 273 | CCGTCTCTTCC | GGAAGAGGACGG |
| 274 | GGAATGCGCCGT | ACGGCGCATTCC |
| 275 | CCCTCTGAAGG | CCTTCAGGAGGG |
| 276 | GGGATGCATTAC | GTAATGCATCCC |
| 277 | CCGTCGAAGCTC | GAGCTTCGACGG |
| 278 | TTAATATTATAG | CTATAATATTA |
| 279 | CCGTCGATCGAG | CTCGATCGACGG |
| 280 | GGCATCGATATA | TATATCGATGCC |
| 281 | CCATCGACTAGA | TCTAGTCGATGG |
| 282 | GGGATCGGCGCG | CGCGCCGATCCC |
| 283 | CCGTCGAGATCT | AGATCTCGACGG |
| 284 | GGCATCGCGCGC | GCGCGCGATGCC |
| 285 | CCATGACACTAC | GTAGTGTCATGG |
| 286 | GGGAATTGGAGG | CCTCCAATCCC |
| 287 | CCCTGACTGATG | CATCAGTCAGGG |
| 288 | TTGAAGGAAGAC | GTCTTCCTTCAA |
| 289 | CCGTGACGTCGT | ACGACGTCACGG |
| 290 | GGCAATTAAGAA | TTCTTAATTGCC |
| 291 | CCATGTGACATA | TATGTCACATGG |
| 292 | TTGAACCTTGAT | ATCAAGGTTCAA |
| 293 | CCATGTGTGTAT | ATACACACATGG |
| 294 | TTGAACCAACTA | TAGTTGGTTCAA |
| 295 | CCGTGCAACGCT | AGCGTTGCACGG |
| 296 | TTCAATTGGCTC | GAGCCAATTGAA |
| 297 | CCATGCATGCGA | TCGCATGCATGG |
| 298 | TTGAATTCGGAG | CTCGGAATTCAA |
| 299 | CCGTGCACATAG | CTATGTGCACGG |
| 300 | GGAAAGGTTAGC | GCTAACCTTTCC |
| 301 | CCGTGCAGTATC | GATACTGCACGG |
| 302 | GGAAAGGCCGAT | ATCGGCCTTTCC |
| 303 | CCATGGTACCGG | CCGGTACCATGG |
| 304 | GGGAACCGGGAC | GTCCCGGTTCCC |
| 305 | CCGTGGTCAATT | AATTGACCACGG |
| 306 | GGAAACCTTTCA | TGAAAGGTTTCC |
| 307 | CCGCATGACTGG | CCAGTCATGCGG |
| 308 | TTCGGCCGAAA | TTTCCGGCCGAA |
| 309 | TTGGGAAGGCCG | CGGCCTTCCCAA |
| 310 | AAACATGACGTC | GACGTCATGTTT |
| 311 | GGACATGTGTGT | ACACACATGTCC |

| | | |
|-----|---------------|---------------|
| 312 | AAGGGCCAACCA | TGGTTGGCCCTT |
| 313 | TTCCAGATTAGC | GCTAATCTGGAA |
| 314 | AATGGCGCATAG | CTATGCGCCATT |
| 315 | TTTCAGAAATCG | CGATTTCTGAAA |
| 316 | CCCGGATTGCGC | GCGCAATCCGGG |
| 317 | TTCCACTCCCTG | CAGGGAGTGGAA |
| 318 | CCTGGTATGGCA | TGCCATACCAGG |
| 319 | TTCCATCTTCTT | AAGAAGATGGAA |
| 320 | AATGGATACTAC | GTAGTATCCATT |
| 321 | TTTCATCAAGAA | TTCTTGATGAAA |
| 322 | AACGGATGTCGT | ACGACATCCGTT |
| 323 | TTTCAAGGTCT | AGACCCTTGAAA |
| 324 | AACGGTAACATA | TATGTTACCGTT |
| 325 | TTTCAAGCCAGA | TCTGGCTTGAAA |
| 326 | AACGGTAGTGCG | CGCACTACCGTT |
| 327 | TTCCAAGAACTC | GAGTTCTTGGAA |
| 328 | AATGGTATGTAT | ATACATAACCATT |
| 329 | TTCTGCTGGCCT | AGGCCAGCAGAA |
| 330 | AATAAGCACGTA | TACGTGCTTATT |
| 331 | TTCTGCTCCGGA | TCCGGAGCAGAA |
| 332 | AATAAGCTGCAT | ATGCAGCTTATT |
| 333 | TTCTGCTAATTC | GAATTAGCAGAA |
| 334 | AATAAGCGTAGC | CGTACGCTTATT |
| 335 | TTATGTCCGGTTA | TAACCGACATAA |
| 336 | AATAAATACACT | AGTGTATTTATT |
| 337 | TTCTGTCCCAAT | ATTGGGACAGAA |
| 338 | AATAAATTGTGA | TCACAATTTATT |
| 339 | TTCTGTCTTGGC | GCCAAGACAGAA |
| 340 | AATAAATCACAG | CTGTGATTTATT |
| 341 | TTTCACCGCAAT | ATTGCGGTGAAA |
| 342 | AAAGGGTCTGTC | GACAGACCCTTT |
| 343 | AAATTAGATATC | GATATCTAATTT |
| 344 | TTTACTATCGAT | ATCGATAGTAAA |
| 345 | AATTAGTCCTCA | TGAGGACTAATT |
| 346 | TTAAGCCTGATT | AATCAGGCTTAA |
| 347 | AAATAGTTTCTG | CAGAACTATTT |
| 348 | GGTAGAACAGCA | TGCTGTCTACC |
| 349 | AAATAGTAAGAC | GTCTTACTATTT |
| 350 | GGTAGAATGATG | CATCATCTACC |
| 351 | AAATACAGGTCG | CGACCTGTATTT |
| 352 | GGGAGTTCACGC | GCGTGAACCCC |

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|-----|---------------|---------------|
| 353 | AAATACACCAGC | GCTGGTGTATTT |
| 354 | TTTAGGGTGTAG | CTACACCCTAAA |
| 355 | AAATACATTGAT | ATCAATGTATTT |
| 356 | GGTAGTTACATA | TATGTAACCTACC |
| 357 | AAATACAAACTA | TAGTTTGTATTT |
| 358 | GGGAGTTTGTAT | ATACAAACTCCC |
| 359 | AATTATGGGCTC | GAGCCCATAAAT |
| 360 | TTAAGAAAACGCG | CGCGTTTCTTAA |
| 361 | GGACGTAATAGG | CCTATTACGTCC |
| 362 | AAGGACTTCGCC | GGCGAAGTCCTT |
| 363 | GGACCGGAACGT | ACGTTCCGGTCC |
| 364 | CCGGTCATGTCC | GGACATGACCGG |
| 365 | GGCCTGAAGCCC | GGGCTTCAGGCC |
| 366 | CCGGCCGTATGT | ACATACGGCCCG |
| 367 | GGATGGTGTATG | CATACACCATCC |
| 368 | CCGAACCAATCC | GGATTGGTTCGG |
| 369 | GGCTGACGTGCC | GGCACGTCAGCC |
| 370 | CCGAATTCCAGT | ACTGGAATTCCG |
| 371 | GGCTCTCGACTT | AAGTCGAGAGCC |
| 372 | AAGATCGATGCC | GGCATCGATCTT |
| 373 | CCAGAGACTGCC | GGCAGTCTCTGG |
| 374 | TTGCGATGCAGG | CCTGCATCGCAA |
| 375 | CCACTAGCTCCC | GGGAGCTAGTGG |
| 376 | TTGGCGCTAGTT | AACTAGCGCCAA |
| 377 | CCACTAGGAGGG | CCCTCCTAGTGG |
| 378 | TTGGCGCCGACC | GGTCGGCGCCAA |
| 379 | CCACCGGTTCCGG | CCGAACCGGTGG |
| 380 | TTGGTACACTCC | GGAGGTGACCAA |
| 381 | CCGTGGTTGGCC | GGCCAACCACGG |
| 382 | GGCAACCAAAGT | ACTTTGGTTGCC |
| 383 | CCGCATGCAGTT | AACTGCATGCGG |
| 384 | TTAGGCCTTCCC | GGGAAGGCCTAA |

Low Level Multiplexing

Every combination of sequential odd and even numbered barcodes is fully color balanced at all positions of the index. For example, barcodes 5 and 6 offer opposite colors at every position, but barcodes 6 and 7 do not. Larger pools can be made by combining multiple sets of color balanced pairs. For pools of odd numbers of samples, any barcode can be added to a balanced pool. For example, for a pool of 3 samples, pooling barcodes 5, 6, and any other barcode is acceptable.

A BED file of the covered regions is available for download on [our webpage](#).

To receive a complete electronic list of the BED and FASTA files for this kit, please follow the instructions on the label inside the kit box.

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



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