NEBNext® Microbiome DNA Enrichment Kit

NEB #E2612S/L

6/24 reactions

Version 7.0_1/20

Table of Contents

The NEBNext Microbiome DNA Enrichment Kit Includes

Each kit contains sufficient reagents for the effective separation of CpG methylated DNA from a mixed pool containing microbial or viral DNA. If starting with 1 µg of input DNA per experiment, the volumes provided are sufficient for preparation of up to 6 reactions (NEB #E2612S) and 24 reactions (NEB #E2612L). All reagents in Box 1 should be stored at –20°C.

Box 1: Store at –20°C.

NEBNext MBD2-Fc Protein

NEBNext Bind/wash Buffer (5X)

16s rRNA Universal Gene Bacterial Control Primers (20 µM each, supplied as a mix)

RPL30 Human DNA Control Primers (20 µM each, supplied as a mix)

Box 2: Store at 4°C. Do not freeze.

NEBNext Protein A Magnetic Beads

Required Materials Not Included

- λ DNA-HindIII Digest (NEB #N3012)
- 6-Tube Magnetic Separation Rack (NEB #S1506)
- Gel Loading Dye Blue (6X) (NEB #B7021)
- Nuclease-free water
- 0.8% Agarose Gel
- 1X TE Buffer, pH 7.5
- Agencourt® AMPure® XP Beads (Beckman Coulter, Inc. #A63881) or Beckman SPRIselect Reagent Kit (Beckman Coulter, Inc. #B23317)
- 100% Ethanol for Alcohol Precipitation, 80% Ethanol for AMPure XP or SPRIselect
- DNA Low Bind Microcentrifuge Tubes
- Rotating Mixer
- Gel running equipment

- Heat block or Thermomixer
- Microcentrifuge
- Proteinase K, Molecular Biology Grade (optional; required for eluting captured host DNA) (NEB #P8107)
- Optional: Luna® Universal qPCR Master Mix (NEB #M3003)

Introduction

The NEBNext Microbiome DNA Enrichment Kit facilitates enrichment of microbial DNA from samples containing methylated host DNA (including human), by selective binding and removal of the CpG-methylated host DNA. Importantly, microbial diversity remains intact after enrichment.

In human DNA, 4–6% of cytosines are methylated, and 60–90% of these methylated cytosines are at CpG sites (1,2). In contrast, methylation at CpG sites in microbial species is rare. The NEBNext Microbiome DNA Enrichment Kit uses a simple and fast magnetic bead-based method to selectively bind and remove CpG-methylated host DNA. The method uses the MBD2-Fc protein, which is composed of the methylated CpG-specific binding protein MBD2, fused to the Fc fragment of human IgG. The Fc fragment binds readily to Protein A, enabling effective attachment to Protein A-bound magnetic beads. The MBD2 domain of this protein binds specifically and tightly to CpG methylated DNA. Application of a magnetic field then pulls out the CpG-methylated (eukaryotic) DNA, leaving the non-CpG-methylated (microbial) DNA in the supernatant (3). If desired, the host DNA captured in the magnetic bead pellet can be eluted, and a protocol is provided for this.

The NEBNext Microbiome DNA Enrichment Kit is suitable for a wide range of sample types, including samples with high levels of contaminating host DNA, (3,4) and is also effective for separation of organelle DNA (e.g., mitochondria, chloroplast) from eukaryote nuclear DNA (5). The kit is compatible with downstream applications including next generation sequencing on all platforms, qPCR and end-point PCR.

References

1. Lister, R. et al. (2009) *Nature*, 462, 315–322.
2. Tucker, K. L. (2001) *Neuron*, 30, 649–652.
3. Feehery, G. R. et al. (2013) *PLoS One* 8(10):e76096.
4. Zheng, Z. (2014), *Genome Announcements* 2(2):e00273–14.
5. Yigit, E. et al. (2014). *Appl. Plant Sci.* 2014 2(11): 1400064.

Method Overview

Step I—Prepare Genomic DNA

DNA should be free of proteins, proteinase, SDS and organic solvents; size should be ≥ 15 kb for optimal performance.

Step II—Combine MBD2-Fc and Magnetic Beads in 1X Bind/wash Buffer.

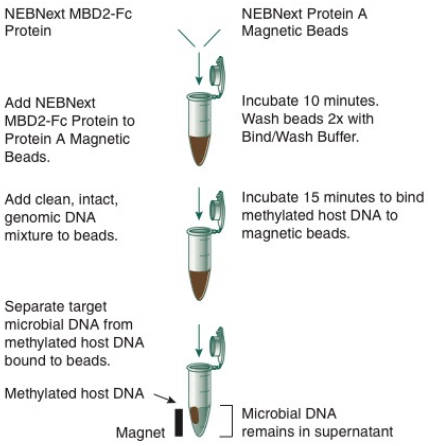
Incubate the reaction for 10 minutes at room temperature. Wash beads two times in Bind/wash Reaction Buffer.

Step III—Add DNA to MBD2-Fc Magnetic Beads.

Incubate the reaction for 15 minutes at room temperature with gentle mixing.

Step IV—Collect Supernatant Fraction containing enriched microbial DNA and Bead Fraction-Containing Host DNA.

Figure 1. Separation workflow.



Protocols

Note: It is important to use sterile technique to avoid environmental DNA contamination.

DNA Preparation and Quantitation

Any method for the purification of protein-free genomic DNA can be used, including Proteinase K treatment followed by phenol/chloroform extraction and ethanol precipitation, lysozyme digestion, Qiagen® column preparation, NEB Monarch column preparation (for genomic DNA) or other methods. Sonication, nebulization, chaotropic salts, enzymatic fragmentation, rough handling, multiple freeze-thaws, or any other procedure that would cause DNA to shear, should be avoided. Samples should be in DNase-free TE buffer (pH 7.5) and be at least **15 kb or greater in size, and free of small molecular weight fragments.** If the DNA fragment size is < 15 kb, enrichment will be less efficient. Determine DNA quality and quantity by agarose gel electrophoresis of the sample alongside a DNA marker (e.g., Lambda DNA-HindIII Digest, NEB #N3012). **It is also important to quantitate the amount of DNA in the experimental sample by A_{260} measurement using a spectrophotometer, such as a Nanodrop® instrument or Qubit® Fluorometer.**

Prebind MBD2-Fc Protein to Magnetic Beads

Before proceeding, you need to determine the volume of prepared beads required for enriching microbial DNA from your sample.

- For every 6.25 ng of input DNA you will need 1 µl of MBD2-Fc-bound magnetic beads for enrichment; therefore, if your total input DNA is 1 µg, you will need 160 µl of MBD2-Fc-bound magnetic beads.
- The amount of MBD2-Fc-bound magnetic beads required (Y) can be calculated using the following equation:

$$Y = \frac{\text{amount of MBD2-Fc-bound magnetic beads (µl)}}{} = \frac{\text{Input DNA (ng)}}{\text{6.25 ng/µl}}$$

For example:

$$\frac{\text{1000 (ng)}}{\text{6.25 ng/µl}} = \text{160 µl}$$

The following protocol will yield 160 µl MBD2-Fc-bound magnetic beads for a 1 µg input sample. For other input sample amounts, scale accordingly.

1. Resuspend NEBNext Protein A Magnetic Beads by gently pipetting the slurry up and down until the suspension is homogeneous. Alternatively, rotate the tube on a rotating mixer gently for 15 minutes at 4°C. **Do not vortex.**
2. Prepare 1X Bind/wash Buffer on ice by diluting 1 part NEBNext Bind/wash Buffer (5X) with 4 parts DNase-free water. One individual reaction, from start to finish, will require 4 ml of 1X Bind/wash Buffer.
3. In one tube, add 16 µl of MBD2-Fc protein and 160 µl of Protein A Magnetic Beads. For input amounts other than 1 µg, add (0.1 x Y) µl of MBD2-Fc protein and (Y) µl of Protein A magnetic beads. Mix by pipetting up and down until the beads are completely homogeneous, at least 5-10 times.

   Note: Y is calculated on page 3.
4. Mix the bead-protein mixture by placing the tube in a rotating mixer for 10 minutes at room temperature.
5. Briefly spin the tube and place on the magnetic rack for 2–5 minutes or until the beads have collected to the wall of the tube and the solution is clear.
6. Carefully remove the supernatant with a pipette without disturbing the beads.
7. Add 1 ml of 1X Bind/wash Buffer (kept on ice) to the tube to wash the beads. Pipette up and down until the beads are completely homogeneous, at least 5-10 times.
8. Mix the beads on a rotating mixer for 3 minutes at room temperature.
9. Briefly spin the tube and place on the magnetic rack for 2–5 minutes or until the beads have collected to the wall of the tube and the solution is clear.
10. Carefully remove the supernatant with a pipette without disturbing the beads.
11. Repeat steps 7–10.
12. Remove the tube from the rack and add 160 µl of 1X Bind/wash Buffer (kept on ice) to resuspend the beads. For input amounts other than 1 µg, add (Y) µl of 1X Bind/wash Buffer to resuspend the beads. Mix by pipetting up and down a few times.

    Note: Y is calculated on page 3.
13. The MBD2-Fc-bound magnetic beads are stable for up to 7 days at 4°C.

Capture Methylated Host DNA

1. Add 1 µg in up to 200 µl input DNA to the tube containing the 160 µl of MBD2-Fc-bound magnetic beads. For other DNA input amounts, add DNA to (Y) µl of MBD2-Fc bound magnetic beads.

   Note: Y is calculated on page 3.

2. Add undiluted Bind/wash Buffer (5X) for a final concentration of 1X. (For example add 10 µl of Bind/wash Buffer (5X) if the DNA input sample was 40 µl, add 4 µl of Bind/wash Buffer (5X) if the DNA sample was 16 µl). Pipette the sample up and down until the beads are completely homogenous, at least 5-10 times.

   Volume of 5X Bind/wash Buffer to add (µl) = Vol input DNA (µl)/4

3. Agitate the tube on a rotating mixer for 15 minutes at room temperature with rotation.

Collect Enriched Microbial DNA

1. Briefly spin the tube and place on the magnetic rack for 5 minutes until the beads have collected to the wall of the tube and the solution is clear.
2. Carefully remove the supernatant with a pipette, without disturbing the beads and transfer it to a clean microcentrifuge tube. This supernatant contains the target microbial DNA. Store this sample at –20°C or proceed directly to purification:

   Purify the samples by either AMPure XP bead cleanup (page 4) or ethanol precipitation (page 5).

   To elute captured host DNA, see page 5.

Option A: Agencourt AMPure XP/SPRIselect Bead Cleanup

1. Vortex AMPure XP/SPRIselect Beads to resuspend.
2. If your enriched sample volume x 2.8 from the previous step exceeds your tube volume, split into two tubes.
3. Add 1.8X volume of resuspended AMPure XP or SPRIselect beads to the sample. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix.
4. Incubate samples for at least 5 minutes at room temperature.
5. Place the tube on the appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.
6. After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
7. Add 400 µl of freshly prepared 80% ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
8. Repeat Step 7 once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnetic stand and remove traces of ethanol with a p10 pipette tip.
9. Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

   Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn brown and start to crack, they are too dry.

10. Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 50 µl of 1X TE (use 25 µl for each tube if sample was split in two).
11. Mix well by pipetting up and down 10 times. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.
12. Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer eluate to a new microcentrifuge tube (combine the eluates if sample was split in two).
13. The sample can now be used for NGS library construction or other downstream analysis (see page 5 for quantitation by agarose gel or qPCR).

Option B: Ethanol Precipitation

1. Add 2.5 volumes of 100% ethanol*, incubate for 10 minutes on ice, then centrifuge the sample for 30 minutes at 13,000 rpm (16,000 rcf). Remove the ethanol, allow the pellet to air dry, and then resuspend the pellet in a small quantity (50 µl) of TE buffer.
2. If the ethanol pellet contained any residual beads, the resuspended sample can be placed on the magnetic rack 5 minutes to concentrate the beads on the inner wall of the tube, and the supernatant can be transferred to a fresh microcentrifuge tube.
3. The sample can now be used for NGS library construction or other downstream analysis (see page 5 for quantitation by agarose gel or qPCR).

   * For ethanol precipitation of the proteinase 1 < digested host DNA also add 0.3 M (final) Na-Acetate

NGS Library Construction

Enriched samples are compatible with NEBNext DNA Library Prep reagents for the following platforms:

- Illumina® (NEB #E6040, E7370, E7645, E7103, E7805, E6177)
- Ion Torrent™ (NEB #E6270, #E6285)

Optional Protocol for Eluting Captured Host DNA

This step elutes the captured host DNA from the MBD2-Fc-bound magnetic beads.

1. While the tube with the beads (page 4, section "Collect Enriched Microbial DNA", Step 2) is still in the magnetic rack, add 1 ml of 1X Bind/wash Buffer (kept on ice) to wash the beads.
2. Carefully remove the wash buffer with a pipette without disturbing the beads.
3. Add 150 µl of 1X TE and 15 µl of Proteinase K to the sample pellet. Mix beads by gentle vortexing (1,200 rpm) or by flicking the tube. (Note: The pellet may be difficult to resuspend initially due to the high concentration of genomic DNA bound to the beads.) Incubate the slurry in a heat block or thermomixer set at 65°C for 20 minutes, with occasional mixing (3–5 times).
4. Briefly centrifuge the sample at 13,000 rpm in a microcentrifuge.
5. Place the tube on the magnetic rack for 2–5 minutes or until the beads have collected to the wall of the tube and the solution is clear.
6. Carefully remove the supernatant and transfer to a fresh microcentrifuge tube.
7. This eluted sample contains the methylated host DNA. Store this sample at –20°C, or proceed directly to purification via using AMPure XP bead cleanup (see page 4) or ethanol precipitation (see page 5).

Downstream Analysis

Quantitation of DNA by agarose gel electrophoresis

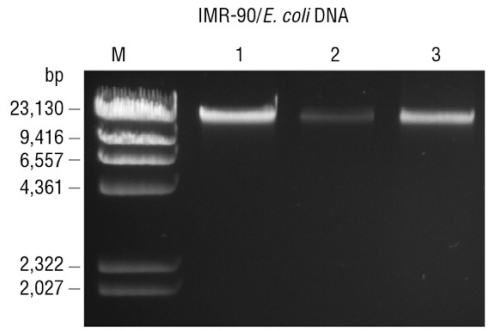
1. Prepare the unenriched sample by diluting 0.1 µg of unenriched input DNA in TE Buffer for a final volume of 20 µl.
2. Aliquot 20 µl of the unenriched DNA, supernatant fraction containing the enriched mircobial DNA, and eluted fraction containing the eukaryotic host DNA into separate tubes. Add 5 µl of Gel Loading Dye, Blue (6X) (NEB #B7021) or other loading buffer to each sample.
3. Load 20 µl of each sample alongside an appropriate DNA marker (λ DNA-HindIII Digest, NEB #N3012) on a 0.8% agarose gel. Since the supernatant fraction containing enriched microbial DNA will be depleted of host DNA, the total amount of DNA seen on the gel will be reduced compared to the input control. (See example in Figure 2, page 6)

Validation of enrichment by qPCR

1. Prepare the unenriched sample by diluting the same quantity as previously used for enrichment in TE Buffer in a final volume of 50 µl.
2. For each sample (input, enriched and purified microbial DNA, aliquoted and purified host (human DNA), aliquot the volume appropriate for your qPCR reaction in triplicate. Do this for all primer pairs used (16s rRNA gene and RPL30 gene or other primers for host DNA).
3. Add the appropriate volume of qPCR primers for your qPCR kit. Dilute as needed. Primers are supplied as 20 µM each.
4. Perform qPCR and analyze results as appropriate for your qPCR reagents and instrument.
5. In the microbial fraction, little to no change in the Cq value for enriched universal bacterial control primers should be observed between the input fractions. A significant Cq shift should be observed between the input and enriched fraction with RPL30 or other

host primers. In the host fraction, a small shift of Cq should be observed with the RPL30 or other host primers. A significant shift should be observed for the 16SrRNA primers.

Figure 2. Agarose gel containing unenriched, enriched and captured host DNA.



Lane M, Lambda DNA-Hind III Digest, NEB #N3012; Lane 1, Unenriched input DNA;
Lane 2, Supernatant fraction containing enriched microbial DNA;
Lane 3, Eluted DNA from magnetic bead pellet containing host DNA.

Control Primers

PRODUCT	INDEX PRIMER SEQUENCE
16s rRNA Gene Universal Bacteria Control Primers (20 µM)	5´-CCATGAAGTCGGAATCGCTAG forward 5´-GCTTGACGGGCGGTGT reverse Amplicon- CCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTC CCGGGCCTTGTACACACCGCCCGTCAAGC 85 bp
RPL30 Human DNA Control Primers (20 µM)	5´-GCCCGTTCAGTCTCTTCGATT forward 5´-CAAGGCAAAGCGAAATTGGT reverse Amplicon- GCCCGTTCAGTCTCTTCGATTACCTCAAAGCTGGGCAGTTGTTAGCGAGAAT GACCAATTTCGCTTTGCCTTG 73 bp

Kit Components

NEB #E2612S Table of Components

NEB #	PRODUCT	VOLUME
E2614A	NEBNext MBD2-Fc Protein	0.1 ml
E2615A	NEBNext Protein A Magnetic Beads	1.0 ml
E2616A	NEBNext Bind/Wash Buffer	12 ml
E2618A	16s rRNA Gene Universal Bacteria Control Primers	0.012 ml
E2619A	RPL30 Human DNA Control Primers	0.012 ml

NEB #E2612L Table of Components

NEB #	PRODUCT	VOLUME
E2614AA	NEBNext MBD2-Fc Protein	0.4 ml
E2615AA	NEBNext Protein A Magnetic Beads	4.0 ml
E2616AA	NEBNext Bind/Wash Buffer	48 ml
E2618AA	16s rRNA Gene Universal Bacteria Control Primers	0.048 ml
E2619AA	RPL30 Human DNA Control Primers	0.048 ml

Revision History

REVISION #	DESCRIPTION	DATE
3.0	Changed the supplied volume of the primers and included a dilution step of the primers prior to qPCR.	
4.0	Added 24 reaction size.	8/15
5.0	Update required materials not included. Update the Protocol. Create kit component tables to replace individual kit component pages. Create Control Primers Chart.	8/18
6.0	Adjust text for "Validation of enrichment by qPCR". Add optional material not included: NEB #M3003.	12/18
7.0	Updated to new manual format.	1/20

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