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**Protocol status:** Working

We use this protocol and it's working

## Microbial DNA enrichment of rectal mucosa tissue samples using NEBNext Microbiome DNA Enrichment Kit

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**ABSTRACT****ABSTRACT**

This protocol outlines the procedure for microbial DNA enrichment of rectal mucosa tissue samples using NEBNext Microbiome DNA Enrichment Kit (NEB #E2612). The same protocol could be used to enrich microbial DNA from any background human DNA by taking advantage of human DNA having CpG methylation sites that are lacking in microbial DNA.

The protocol covers:

1. Microbial DNA enrichment of extracted samples using NEB NEBNext Microbiome DNA Enrichment kit (NEB #E2612).
2. DNA Quantitation of extracted DNA using the Qubit Fluorometer and Agilent Fragment Analyzer.
3. Sample preparation ready for library preparation for microbiome WGS using NEB NEBNext Ultra II FS DNA Library Prep Kit for Illumina (NEB #E7805) and DNA sequencing on the Illumina NextSeq2000.

Previous DNA extraction steps are outlined in protocol

[dx.doi.org/10.17504/protocols.io.eq2lyj15wlx9/v1](https://dx.doi.org/10.17504/protocols.io.eq2lyj15wlx9/v1)

1. DNA extraction from faecal samples using MP Biomedicals FastDNA Spin Kit and FastPrep-24 for bead-beating
2. DNA extraction from rectal mucosa biopsy tissue using a modification of MP Biomedicals FastDNA Spin Kit and FastPrep-24 for bead-beating

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**Keywords:** DNA extraction, rectal mucosa, biopsies, faecal, stool, microbiome, DNA enrichment

## MATERIALS

**Step 1: DNA preparation and quantification**

| Equipment   |       |
|---|-------|
| <b>QubitFlex</b>  | NAME  |
| Fluorometer   | TYPE  |
| Invitrogen  | BRAND |
| Q33326  | SKU   |
|  |       |

| Equipment   |       |
|---|-------|
| <b>Fragment Analyzer</b>  | NAME  |
| capillary based nucleic acid fragment size separation   | TYPE  |
| Agilent   | BRAND |
| M5311AA   | SKU   |
| <a href="https://www.agilent.com/en/product/automated-electrophoresis/fragment-analyzer-systems/fragment-analyzer-systems/5300-fragment-analyzer-system-365721">https://www.agilent.com/en/product/automated-electrophoresis/fragment-analyzer-systems/fragment-analyzer-systems/5300-fragment-analyzer-system-365721</a> | LINK  |

Agilent Genomic DNA Kit (50kb) kit (Agilent #5191-6569)  
 BioRad Gel Doc Go Imaging System (BioRad #730BR10254)

Qubit DNA Broad Range assay kit (Invitrogen #Q32853)  
 Qubit dsDNA HS assay kit (Invitrogen #Q32854)

1M Tris-HCl pH7.5 (Invitrogen #15567-027)  
0.5M EDTA pH8.0 (Invitrogen #15575-038)  
1XTBE Buffer (Thermo-Fisher #B52)

### Step 2: Preparation of MBD beads

| Equipment   |       |
|---|-------|
| Dynal MPC 96 S  | NAME  |
| Plate magnet  | TYPE  |
| Invitrogen  | BRAND |
| 120.27  | SKU   |
|  |       |

LABINCO LD76 Digital Rotary Mixer (#76000)  
NEBNext Microbiome DNA Enrichment kit (NEB #E2612)

### Step 3: Bind MBD2-Fc protein to magnetic beads

NEBNext Microbiome DNA Enrichment kit (NEB #E2612)

### Step 4: Capture methylated host DNA

NEBNext Microbiome DNA Enrichment kit (NEB #E2612)

### Step 5: Collect enriched microbial DNA

No extra materials required

### Step 6: Bead clean-up

Agencourt AMPure XP beads (Beckman #A63881)  
NEBNext Ultra II FS DNA Library Prep Kit for Illumina (NEB #E7805)

### Step 7: Validation of enrichment by qPCR (optional)

Luna Universal qPCR Master Mix (NEBnext #M3003S)

## DNA preparation and quantification

- 1 Samples were extracted using the protocol "DNA extraction from rectal mucosa biopsies and matched faecal samples taken from surgery for microbiome analysis".  
<https://dx.doi.org/10.17504/protocols.io.eq2lyj15wlx9/v1>
- 2 Prepare Tris-EDTA Buffer pH7.5 with  $\text{pH}$  4 mL of 1M Tris-HCl pH7.5 and  $\text{pH}$  800  $\mu\text{L}$  of 0.5M EDTA  $\text{pH}$  8 in 400 mL of distilled water for normalising DNA samples.
- 3 Quantify 2  $\mu\text{L}$  of extracted DNA sample using either Qubit dsDNA Broad Range assay kit or dsDNA High Sensitivity assay kit on the Qubit Flex Fluorometer.
- 4 Determine DNA quality and quantity using an Agilent Fragment Analyzer, use 2ul DNA sample with the Genomic DNA Kit (50kb) kit (#5191-6569). Using the associated PROsize Data Analysis Software, check that DNA fragments are >15kb before processing. This is important as enrichment will be less efficient for fragments smaller than this. (A 0.8% agarose gel can be used as an alternative).
- 5 Normalise samples to 1  $\mu\text{g}$  input DNA with prepared Tris-EDTA Buffer pH7.5. Make up at least 40ul volume. Retain at least 5 ng of DNA for step 36 (Validation of enrichment by qPCR).

## Bind MBD2-Fc protein to magnetic beads

- 6 For every 6.25ng of input DNA, you will need 1  $\mu\text{l}$  of MBD2-Fc-bound magnetic beads for enrichment. The amount of MBD2-Fc-bound magnetic beads required can be calculated using the following equation:  $Y = \text{Input DNA (ng)} / 6.25\text{ng}/\text{ul}$   
i.e. for recommended input of 1000ng/6.25ng/ $\text{ul}$  = 160ul of MBD2-Fc-bound magnetic beads per reaction.
- 7 Prepare batches of MBD beads with a minimum volume of 40  $\mu\text{L}$ , as lower volumes prevents adequate mixing. Remaining unused MBD beads can be stored at  $4^\circ\text{C}$  for up to a week.

- 8 Resuspend the amount of NEBNext Protein A Magnetic Beads required by rotating the tube(s) on a rotating mixer gently for  00:15:00 at  4 °C. Do not vortex. A walk-in fridge is ideal for this step.  
i.e. For 1ug input DNA, use 160ul beads per reaction. For 48 samples, prepare 7.68ml of beads (48x160ul).

Note1: 160ul of beads can be added to individual tubes for each sample or the total 7.68ml can be split into 4 x 2ml tubes. This allows tubes to fit on 1.5ml/2ml rotating mixer.

Note2: The equation to calculate the number of beads required (Y) is highlighted in step 6.

- 9 Prepare 1X Bind/wash Buffer on ice by making a 1 in 5 dilution of NEBNext Bind/wash Buffer (5X) with DNase-free water.  
Volume of 1X bind/wash buffer needed:  
4ml per enrichment reaction if using separate tubes per reaction for MBD bead prep and wash steps.  
i.e. 4ml x 48 reactions= 192ml  
However, preparing a master mix of 20mls for MBD bead prep and wash steps is better and more economical for 48 reactions.  
Note: Keep 1X bind/wash buffer on ice throughout the MBD bead preparation. For the wash steps following the capture reaction; 1X bind/wash buffer can be at room temperature.

- 10 Prepare 0.1x Y $\mu$ l of MBD2-Fc protein and Y $\mu$ l of Protein A Magnetic Beads per reaction.  
i.e.  16  $\mu$ L with  160  $\mu$ L beads for a  1  $\mu$ g input per sample.

Note: The equation to calculate the number of beads required (Y) is highlighted in step 6.

i.e. For 48 samples, add 16ul MBD2-Fc protein to individual tubes per sample or add 768ul to the prepared 7.68ml of beads. (if using 4 x 2ml tubes, add 192ul to each tube).

- 11 Mix by pipetting up and down until the beads are completely homogeneous, at least 10 times. Mix the bead/protein mixture further by placing the tube(s) on a rotating mixer for  00:10:00 at  Room temperature.

- 12 Briefly spin the tube(s) and place on the magnetic rack for  00:05:00 (or until the beads have collected to the wall of the tube(s) and the solution is clear).

- 13 Carefully remove the supernatant with a pipette without disturbing the beads.

- 14 Add  $\text{1 mL}$  of 1X Bind/wash Buffer, cooled  $\text{On ice}$ , to the sample tube(s) to wash the beads. Pipette up and down until the beads are completely homogeneous, at least 10 times.
- 15 Mix the beads on a rotating mixer for  $00:03:00$  at  $\text{Room temperature}$ . 3m
- 16 Briefly spin the tube(s) and place on the magnetic rack for  $00:05:00$  (or until the beads have collected to the wall of the tube and the solution is clear). 5m
- 17 Carefully remove and discard the supernatant with a pipette without disturbing the beads.
- 18 Repeat steps 14–17 twice for a total of three washes to ensure the removal of unbound MBD2-Fc protein.
- 19 Remove the tube(s) from the rack and add (Y)ul of 1X Bind/wash Buffer (kept on ice) per reaction to resuspend the beads. Mix by pipetting up and down a few times.  
Note: The equation to calculate (Y) is highlighted in step 6.

## Capture methylated host DNA

- 20 Prepare 1  $\mu\text{g}$  input DNA in up to  $200 \mu\text{L}$  DNase free water in the tube/plate if not prepared at step 5 (TE Buffer pH 7.5 can also be used).

If using a 1ug DNA input, add 160 $\mu\text{l}$  of MBD2-Fc-bound magnetic beads to each DNA sample.  
For other DNA input amounts, add DNA (up to 200ul) to (Y)  $\mu\text{l}$  of MBD2-Fc bound magnetic beads.

Note: The equation to calculate the number of beads required (Y) is highlighted in step 6.

- 21 Add undiluted Bind/wash Buffer (5X) to the DNA/bead mix for a final concentration of 1X.  
Volume of 5X Bind/wash Buffer to add ( $\mu\text{l}$ ) = Volume of input DNA ( $\mu\text{l}$ )/4  
i.e. add 10  $\mu\text{l}$  of Bind/wash Buffer (5X) per reaction if the DNA input sample volume was 40  $\mu\text{l}$ .  
Pipette the sample up and down until the beads are completely homogenous, at least 10 times.
- 22 Agitate the tubes on a rotating mixer for  00:15:00 at  Room temperature . Pipette mix every 2 minutes if the samples are in a plate and can't be placed on the rotating mixer.  15m

## Collect enriched microbial DNA

- 23 Briefly spin the tubes/plate and then incubate at  Room temperature on the magnetic rack for  00:05:00 until the beads have collected to the wall and the solution is clear.  5m
- 24 Carefully remove the supernatant with a pipette without disturbing the bead and transfer it to a clean tube/plate. **Keep the supernatent** - this is enriched for microbial DNA and can be purified by bead clean-up. The sample can be stored at  -20 °C or can proceed directly to purification (bead clean-up).

## Bead clean-up

- 25 Vortex AMPure XP beads to resuspend.
- 26 Add 1.8X volume of resuspended AMPure XP beads to each sample. Mix well by pipetting up and down at 10 times, then incubate at  Room temperature for  00:05:00 .  5m

\* If the combined volume of beads and DNA does not exceed the capacity of the well, large numbers of bead clean-ups can be conducted in parallel on a microplate e.g. 0.8ml 96-well storage plates (#AB-0765 Thermo) . Alternately, the DNA/bead mix can be transferred to individual tubes for bead clean-up.

- 27 Place the tube/plate on the magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tubes or plate wells before placing on the magnetic stand.
- 28 After 00:05:00 (or when the solution is clear) carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets. 5m
- 29 Add 200 µL of freshly prepared 70% ethanol to the tubes/plate while in the magnetic stand. Ensure there is sufficient volume to cover the bead pellet. Incubate at Room temperature for 00:01:00, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets. 1m
- 30 Repeat Step 29 once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tubes/plate, place back on the magnetic stand and remove traces of ethanol with a p10 pipette tip.
- 31 Air dry the beads for 00:01:00 while the tubes/plate is on the magnetic stand with the lid open. Spin at full speed for 00:02:00 in order to collect residual 70% ethanol. Then place on the magnetic stand for 00:00:30 before pipetting the residual 70% ethanol and air-drying for 00:01:00. 4m 30s

\* 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. If beads turn brown and start to crack, they are too dry.
- 32 Remove the tubes/plate from the magnetic stand and resuspend the beads in 30 µl of 1X TE buffer. (NEB- TE Buffer (1X) #E7808AAVIAL, not supplied in kit)
- 33 Mix well by pipetting up and down 10 times. Incubate for at least 00:02:00 at Room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tubes or plate wells before placing back on the magnetic stand. 2m

- 34** Place the tubes/plate on the magnetic stand. After  00:05:00 (or when the solution is clear), transfer eluate to a new tube/plate. 5m
- 35** Samples can now be processed for library preparation using NEB NEBNext Ultra II FS DNA Library Prep Kit for Illumina with sequencing on the Illumina NextSeq2000.

## Validation of enrichment by qPCR

- 36** The input used for the qPCR reaction was  5 ng for both enriched and unenriched samples. The qPCR kit used was the Luna Universal qPCR Master Mix (NEBnext #M3003S). The input amount for using this kit is  $\leq$   100 ng.
- 37** For each sample (input and enriched, purified microbial DNA from tissue samples), aliquot  9  $\mu$ L to a 384 well plate in triplicate. Do this for both primers used (16s and RPL30 Human). The 16s rRNA gene control primer and RPL30 Human gene control primer are provided in the Microbiome DNA enrichment kit (NEBnext #E2612).
- 38** Add  0.25  $\mu$ L per sample of qPCR primers. Do this for both primers used (16s and RPL30 Human). Primers are supplied as 20  $\mu$ M each. Add  10  $\mu$ L of Luna Universal qPCR Master Mix per sample. Making a master mix of the primer and the Luna master mix is recommended. The total reaction volume will be about  20  $\mu$ L.
- 39** Perform qPCR with the following settings:

| A                    | B           | C          | D      |
|----------------------|-------------|------------|--------|
| Cycle Step           | Temperature | Time       | Cycles |
| Initial Denaturation | 95°C        | 60 seconds | 1      |
| Denaturation         | 95°C        | 15 seconds | 45     |
| Extension            | 60°C        | 30 seconds |        |
| Melt Curve           | 60-95°C     | Various    | 1      |

qPCR was carried out on the QuantStudio 12K Flex (Thermo Fisher). Analyse results as appropriate for your qPCR reagents and instrument.

- 40 There is no defined results to define successful enrichment. In general, for microbial DNA there should be minimal change in Cq value using the 16s primers in non-enriched input samples and enriched samples. For human DNA there should be a change in Cq value observed using the RPL30 human primers in enriched samples compared to unenriched samples.