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# Isolation of Commensal *Escherichia coli* Strains from Feces of Healthy Laboratory Mice or Rats

Tingting Ju and Benjamin P. Willing*

Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Canada
*For correspondence: willing@ualberta.ca

**[Abstract]** The colonization abundance of commensal *E. coli* in the gastrointestinal tract of healthy laboratory mice and rats ranges from $10^4$ to $10^6$ CFU/g feces. Although very well characterized, the family that *E. coli* belongs to has a very homogeneous 16S rRNA gene sequence, making the identification from 16S rRNA sequencing difficult. This protocol provides a procedure of isolating and identifying commensal *E. coli* strains from a healthy laboratory mouse or rat feces. The method can be applied to isolate commensal *E. coli* from other laboratory rodent strains.
**Keywords:** Commensal, *Escherichia coli*, Isolation, Laboratory rodents

**[Background]** *Escherichia coli* is a Gram-negative, facultative anaerobe which constitutes only a minor fraction of the vertebrate gut microbiota, but plays a key role in microbial interaction, immune modulation and metabolic functionalities (Tenaillon *et al.*, 2010). Being one of the best-characterized model microorganisms, commensal *E. coli* strains have been increasingly studied to unravel the mechanisms through which gut commensal microbes adapt to the unique niche and impact host physiology. However, the high homology among different strains raises difficulties in identification and characterization of commensal *E. coli* based on a 16S rRNA sequencing approach. Thanks to the development of next-generation sequencing techniques and large-scale analyses of whole genomes, we are able to identify commensal *E. coli* strains isolated from the gastrointestinal tract of different hosts according to the presence of virulence genes in the genome. In this protocol, we show an approach to isolate and identify commensal *E. coli* strains from a laboratory mouse or rat using selective culture media and whole genome sequencing. However, it should be noted that the presence of commensal *E. coli* in laboratory animals depends on the vendor and environmental conditions of the facility.

## Materials and Reagents

1. Gloves and masks (KCWW, Kimberly-Clark, catalog number: 52817; Cardinal Health, Insta-Gard, catalog number: AT7511-WE)
2. 1.5 ml centrifuge tube (sterile) (Fisher Scientific, catalog number: 05-408-129)
3. Wide bore tips, 0-200 µl (Corning, Axygen®, catalog number: T-1005-WB-C)
4. Thin-wall PCR tubes (Fisher Scientific, catalog number: 14-230-225)
5. Tips, 0.1-10 µl, 0.1-1 ml (sterile) (Fisher Scientific, catalog numbers: 02-707-474; 02-707-480)
6. Cell spreader (Fisher Scientific, catalog number: 08-100-11)
7. Petri dishes (100 x 15 mm) (Fisher Scientific, catalog number: FB0875713)

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8. 15 ml conical sterile polypropylene centrifuge tubes (Thermo Fisher Scientific, Nunc™, catalog number: 339650)
9. Sterile 0.22 µm filter (Corning, catalog number: 431219)
10. 1 ml syringe (BD, catalog number: 309659)
11. A healthy NIH Swiss mouse (Harlan Laboratories Inc., Indianapolis, IN) and Sprague-Dawley (SD) rat (Charles River Canada, St. Constant, QC)
12. 70% ethanol diluted from 100% ethanol (Commercial Alcohols, catalog number: P016EAAN)
13. Agarose (Thermo Fisher Scientific, Invitrogen™, catalog number: 16500500)
14. Tris-Acetate-EDTA (TAE) (50x stock) (Fisher Scientific, catalog number: BP13321)
15. SYBR Safe DNA gel stain (Thermo Fisher Scientific, Invitrogen™, catalog number: S33102)
16. 6x DNA loading dye (Thermo Fisher Scientific, Thermo Scientific™, catalog number: R0611)
17. 1 kb Plus DNA ladder (Thermo Fisher Scientific, Thermo Scientific™, catalog number: SM1331)
18. GeneJET gel extraction and DNA cleanup micro kit (Thermo Fisher Scientific, Thermo Scientific™, catalog number: K0832)
19. PureLink genomic DNA mini kit (Thermo Fisher Scientific, Invitrogen™, catalog number: K182001)
20. Nextera XT DNA library preparation kit (Illumina, catalog number: FC-131-1096)
21. Nextera XT DNA library preparation index kit (Illumina, catalog number: FC-131-1002)
22. Nextera XT DNA library preparation kit (Illumina, catalog number: FC-131-1096)
23. Nextera XT DNA library preparation index kit (Illumina, catalog number: FC-131-1002)
24. Qubit™ 1x dsDNA HS assay kit (Thermo Fisher Scientific, Invitrogen™, catalog number: Q33230)
25. PhiX control kit (Illumina, catalog number: FC-110-3001)
26. MiSeq reagent kit V3 (Illumina, catalog number: MS-102-3003)
27. Sodium phosphate dibasic ($Na_2HPO_4$) (Fisher Scientific, catalog number: S373-500)
28. Potassium phosphate monobasic ($KH_2PO_4$) (Fisher Scientific, catalog number: P285-500)
29. Sodium chloride (NaCl) (Fisher Scientific, catalog number: S271-500)
30. Potassium chloride (KCl) (Fisher Scientific, catalog number: P217-500)
31. Hydrochloric acid (HCl) (Fisher Scientific, catalog number: A144-500LB)
32. MacConkey agar (BD, catalog number: 212123)
33. Luria-Bertani (LB) broth (Sigma-Aldrich, catalog number: L3022)
34. Glycerol (Fisher Scientific, catalog number: BP229-1)
35. DNA Taq polymerase with 50 mM $MgCl_2$ (Thermo Fisher Scientific, Invitrogen™, catalog number: 10342020)
36. Oligo primers 27F/1492R (Weisburg *et al.*, 1991)
    27F: 5'-AGAGTTTGATCMTGGCTCAG-3'
    1492R: 5'-TACGGYTACCTTGTTACGACTT-3'
37. Deoxynucleotide triphosphates (dNTPs) (Thermo Fisher Scientific, Invitrogen™, catalog number: 10297-018)

 

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38. PCR grade water (Thermo Fisher Scientific, Invitrogen™, catalog number: AM9932)
39. Sodium hydroxide (NaOH) (Fisher Scientific, catalog number: SS266-1)
40. 1x phosphate buffered saline (PBS) (pH 7.4) (see Recipes)
41. MacConkey agar (see Recipes)
42. LB broth (see Recipes)
43. Glycerol stock of bacterial isolates (see Recipes)
44. PCR reaction mix (see Recipes)
45. 0.1 N NaOH (see Recipes)

## Equipment

1. Forceps (sterilized by autoclave)
2. Vortex (Fisher Scientific, catalog number: 02215365)
3. Pipettes [*e.g.*, P1000 (Eppendorf, catalog number: 3120000062), P200 (Eppendorf, catalog number: 3120000054), P10 (Eppendorf, catalog number: 3120000020)]
4. Thermal cycler (Thermo Fisher Scientific, Applied Biosystems, model: GeneAMP PCR System 9700)
5. Microwave (RCA, catalog number: RMW733)
6. Gel electrophoresis system (Fisher Scientific, model: FB300)
7. UV transilluminator with photo documentation (Azure Biosystems, model: Azure c200)
8. Incubator (37 °C) (Thermo Fisher Scientific, Thermo Scientific, model: Model 370)
9. Shaking incubator (37 °C) (Eppendorf, New Brunswick™, model: I26)
10. Qubit™ 3.0 fluorometer (Thermo Fisher Scientific, Invitrogen™, catalog number: Q33216)
11. Qubit™ assay tubes (Thermo Fisher Scientific, Invitrogen™, catalog number: Q32856)
12. Illumina MiSeq instrument (Illumina, model: MiSeq™ System, catalog number: SY-410-1003)
13. Autoclave (Beta Star Life Science Equipment, model: C2002BS)
14. -80 °C freezer (Thermo Fisher Scientific, Thermo Scientific™, model: Forma™ 900 Series, 989)

## Procedure

The protocol for isolating and identifying the gut commensal *E. coli* strains from laboratory mouse or rat feces includes the steps for fecal collection, bacterial culture and colony characterization (Figure 1).

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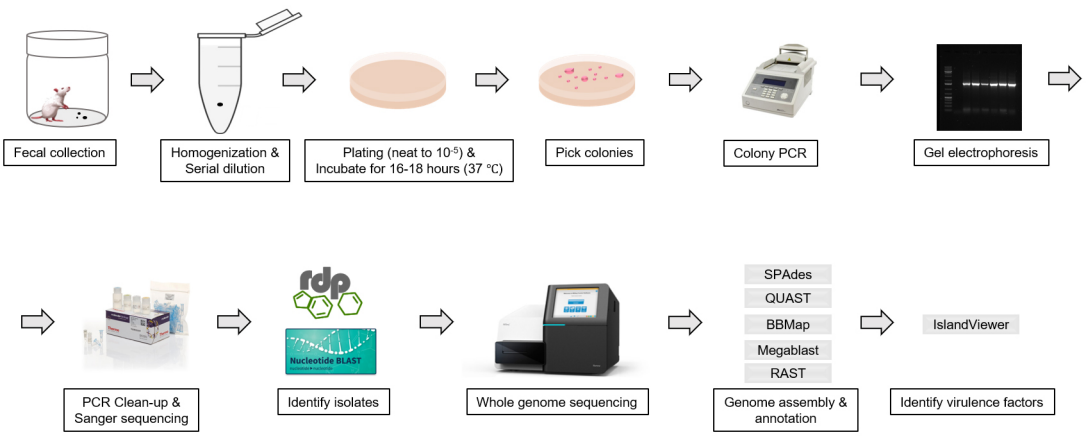


**Figure 1. Workflow scheme for the isolation and identification of commensal *E. coli* from mouse or rat feces**

A. Fecal collection

*Note: Personal protection equipment including gloves and masks are required.*

1. Put a healthy NIH Swiss mouse/SD rat into a clean cage and wait for defecation.
2. Use sterile forceps to transfer one fresh fecal pellet into a 1.5 ml centrifuge tube preloaded with 1 ml of 1x PBS.

   *Note: Recommended weight of one fresh fecal pellet: mouse, 30-50 mg; rat, 80-100 mg.*
3. Put the tubes with fecal pellet on the ice and immediately transfer them back to the lab to perform serial dilution and plating.

B. Serial dilution and plating

*Note: Wear gloves and use 70% ethanol as a disinfectant for cleaning the surfaces of working bench.*

1. Homogenize the fecal pellet thoroughly by vortexing for 30 sec at the maximum speed.
2. Add 900 µl of 1x PBS into four 1.5 ml centrifuge tubes. Perform 1:10 serial dilution from the fecal samples using the wide bore tips.
3. Use the cell spreaders to plate 100 µl of each dilution onto MacConkey agar plate ($10^{-1}$ to $10^{-5}$).
4. Incubate the plates aerobically at 37 °C for 16-18 h.

C. Colony PCR for *E. coli* identification by Sanger sequencing using 16S rRNA gene primers

1. MacConkey agar is a selective and differential medium for the isolation of coliform organisms. The potential *E. coli* colonies appear as red and round shaped colonies due to the capability to ferment lactose (Figure 2). Select potential colonies for the following PCR amplification.

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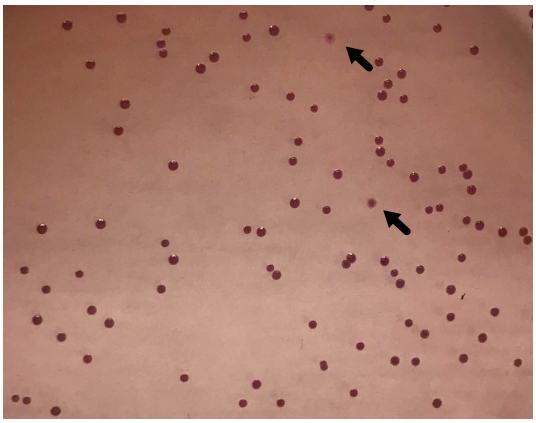

**Figure 2. Colony morphology of *E. coli* isolated from rat feces after growth on MacConkey agar.** The potential *E. coli* colonies appear as red with a round shape. The colonies pointed with black arrows are considered as negative colonies.

2. Add 50 µl of PCR reaction mix (see Recipes) into each PCR tube.
3. Use P10 tips to pick a small amount of the single colony and swirl in the PCR reaction. Mix the colonies and PCR reaction by pipetting up and down, and then discard the tips.
4. Complete the reaction in a thermal cycler following the PCR program (see Notes).
5. Prepare 40 ml of 1% agarose gel by dissolving 0.4 g agarose in 1x TAE and heating (microwave). Add 4 µl of SYBR Safe DNA gel stain into the gel and mix thoroughly. Cast the agarose gel in the apparatus and wait until the gel is solidified. Mix 5 µl of PCR reaction with 1 µl 6x DNA loading dye and apply the samples as well as the marker (2 µl, 1 kb Plus DNA ladder) to the gel. Run the electrophoresis for 30 min at 100 V. Take a picture to check the amplification using a UV transilluminator. Compare the size of the PCR product (about 1,465 bp) to the marker (Figure 3).

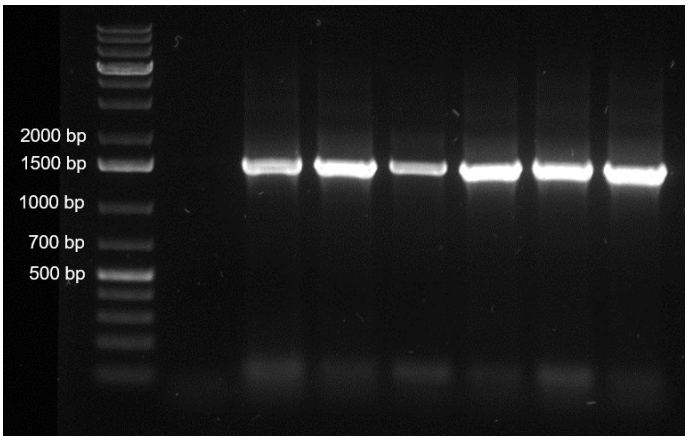


**Figure 3. Agarose gel electrophoresis image of the 16S rRNA genes amplified from six bacterial isolates.** Lane 1 indicates the negative control and the lane 2 to 7 indicate the PCR products amplified the 16S rRNA gene of the selected bacterial isolates. The size of the PCR product is around 1,465 bp.

bio-protocol www.bio-protocol.org/e2780 Vol 8, Iss 06, Mar 20, 2018 DOI:10.21769/BioProtoc.2780

6. PCR clean-up is performed using a GeneJET gel extraction and DNA cleanup kit following the manufacturer's instruction. The remaining PCR mix after the gel electrophoresis is used for this step. With the adjusted concentrations, the cleaned-up samples are further sequenced by Sanger sequencing using the 16S rRNA gene primers.
7. The 16S rRNA gene sequences are searched against the Ribosomal Database Project (RDP, released 11.4; http://rdp.cme.msu.edu/) and the NCBI nucleotide database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (see Data analysis).
8. The isolates that share higher than 98% 16S rRNA sequence identity with the type strain of *E. coli* are selected and cultured in LB broth (see Recipes) overnight in a shaking incubator (37 °C) to make glycerol stocks (25% glycerol, see Recipes) for preservation.

D. Whole genome sequences and annotation

1. The selected isolates are cultured overnight in 5 ml LB broth for genomic DNA extraction using a PureLink Genomic DNA Mini Kit following the manufacturer's instruction. The LB culture from the last Step C8 can be utilized for DNA extraction.
2. The whole genome sequencing is performed on an Illumina MiSeq platform. The isolated genomic DNA is fragmented to generate libraries using a Nextera XT DNA library preparation kit according to the manufacturer's instruction.
3. The concentrations of generated libraries are quantified by a Qubit™ 3.0 fluorometer using a Qubit™ 1x dsDNA HS assay kit according to the manufacturer's instruction. The quantified libraries are further normalized to 2 nM and pooled following the protocol of the Nextera XT DNA library preparation kit.
4. Denature the pooled libraries using 0.1 N NaOH and mix with 5% PhiX genomic DNA as a positive control.
5. The sequencing of denatured libraries is performed on an Illumina MiSeq instrument with 2 x 300 bp reads generated, using a MiSeq reagent V3 sequencing-by-synthesis kit.
6. The draft genome is assembled with the SPAdes assembler (Bankevich *et al.*, 2012). Genome assemblies are evaluated by Quality Assessment Tool for Genome Assemblies (QUAST) (Gurevich *et al.*, 2013). The tool of BBMap (Bushnell, 2014) is used to map the raw reads back to the contigs produced by SPAdes to obtain the information about the coverage for contigs. The algorithm of Megablast (Zhang *et al.*, 2000) is applied to blast the contigs against the reference bacterial genomes obtained from NBCI. Rapid Annotations using Subsystems Technology (RAST) (Aziz *et al.*, 2008) is used for genome annotation.
7. IslandViewer (Dhillon *et al.*, 2015) is used to predict toxin related virulence in the whole genome of the *E. coli* isolate. The genomes of the isolates submitted to IslandViewer are in the format of GENBANK. The isolates without identified hits of toxin virulence factor (VF)-related genes in the genome are considered to be commensal *E. coli* isolates.

 

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## Data analysis

After colony PCR and Sanger sequencing, the 16S rRNA sequences of bacterial isolates are aligned against the RDP and NCBI nucleotide database. The tools of Seqmatch and Classifier within the RDP database are used for assigning taxonomy. High-quality sequences of the type strains (the size ≥ 1,200 bp) are chosen in the settings of Seqmatch. The '16S ribosomal RNA sequences' database (Bacteria and Archaea) is used as the reference database with default settings when searching against the NCBI database.

## Notes

1. Thermal cycler condition
   Initial denaturation for 10 min at 94 °C
   35 cycles of 30 sec at 94 °C, 30 sec at 58 °C, and 1 min 40 sec at 72 °C
   Final extension for 7 min at 72 °C
   Hold at 4 °C

## Recipes

1. 1x phosphate buffer saline (PBS) (pH 7.4) (1 L)
   10 mM $Na_2HPO_4$
   1.8 mM $KH_2PO_4$
   137 mM NaCl
   2.7 mM KCl
   Adjust to pH 7.4 with HCl and sterilize by filter or autoclave before use
2. MacConkey agar
   Suspend 50 g of the powder in 1 L of $ddH_2O$. Mix thoroughly and boil for 1 min to completely dissolve the powder. Autoclave at 121 °C for 15 min. Cool down and dispense approximately 20 ml per Petri dish (100 x 15 mm in diameter). Store at 4 °C for up to a month
3. LB broth
   Stir to suspend 25 g of the powder in 1 L of $ddH_2O$. Autoclave for 15 min at 121 °C to sterilize. Store at 4 °C for up to a month
4. Glycerol stock of bacterial isolates (in 25% glycerol)
   a. Prepare 100 ml of 50% (v/v) glycerol
      Glycerol (100%) 50 ml
      Add $ddH_2O$ to100 ml
      Autoclave to sterilize
   b. Make glycerol stock from the broth culture of bacterial isolates
      Use sterile pipet tips, pipet 500 µl of bacterial broth culture into a sterile centrifuge tube

bio-protocol www.bio-protocol.org/e2780 Vol 8, Iss 06, Mar 20, 2018 DOI:10.21769/BioProtoc.2780

Add 500 µl of 50% autoclaved glycerol. Mix the solution by vortex and place the tubes into the -80 °C freezer

5. PCR reaction mix
   5 µl of 10x PCR buffer (1x final concentration)
   0.5 µl of 1 U/µl Taq polymerase
   2 µl of 50 mM $MgCl_2$
   2 µl of 10 µM Oligo primer 27F
   2 µl of 10 µM Oligo primer 1492R
   2 µl of 10 mM dNTP mix
   Add up to 50 µl of total volume with PCR grade water
6. 0.1 N NaOH solution
   Make a 1:10 dilution of 1 N NaOH (Fisher Scientific) using nuclease-free water. For example: In a sterile 15 ml centrifuge tube, add 9 ml of nuclease-free water into 1 ml of 1 N NaOH solution. Mix the solution by vortex and sterilize by filtration through a 0.22 µm filter with a syringe

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bio-protocol www.bio-protocol.org/e2780 Vol 8, Iss 06, Mar 20, 2018
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