Journal of Visualized Experiments

A Method to Define the Effects of Environmental Enrichment on Colon Microbiome Biodiversity in a Mouse Colon Tumor Model

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE57182R3
Full Title:	A Method to Define the Effects of Environmental Enrichment on Colon Microbiome Biodiversity in a Mouse Colon Tumor Model
Keywords:	Mind-Body Medicine; Environmental Enrichment; Colon Cancer; microbiome; Stool; Wound Repair
Corresponding Author:	Melinda Angus-Hill Huntsman Cancer Institute Salt Lake City, UNITED STATES
Corresponding Author's Institution:	Huntsman Cancer Institute
Corresponding Author E-Mail:	melinda.angus-hill@hci.utah.edu
First Author:	Andrew K. Fuller
Other Authors:	Andrew K. Fuller
	Benjamin D. Bice
	Ashlee R. Venancio
	Ambur M. Staab
	Stephanie J. Georges
	Julio R. Hidalgo
	Annika V. Warncke
Author Comments:	I would like to have this article in press (if accepted) by the end of December, 2017 as my laboratory will be moving after that time.
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	12-31-2017

HUNTSMAN
CANCER INSTITUTE
UNIVERSITY OF UTAH

UNIVERSITY OF UTAH
HEALTH SCIENCES

2000 CIRCLE OF HOPE
SALT LAKE CITY, UTAH 84112-5550
OFFICE 801 585 0303
FAX 801 585 0900

November 27, 2017

Dr. Vineeta Bajaj, PhD.
Review Editor, JoVE
1 Alewife Center, Suite 200
Cambridge, MA 02140

Dear Dr. Vineeta Bajaj,

We would like to submit our revised manuscript titled: "**A Method to Define the Effects of Environmental Enrichment on Colon Microbiome Biodiversity in a Mouse Colon Tumor Model**" by Andrew K. Fuller *et al*. for your consideration for publication. Thank you for your editorial comments. The revised manuscript is aligned with comments and revision priorities. We hope you agree that the manuscript is a stronger candidate for publication in JoVE.

We were invited to submit a protocol aimed at microbiota analysis from stool samples. We have written a protocol to describe this method. In the revision, we address the majority of Editorial concerns by clarifying the written document to improve the technical details, discussion and flow.

Thank you for your advice regarding highlights. We have cut some more and we are now below the 2.75 pg. filming threshold.

We hope that if accepted, the article can be in press by the end of December, as my laboratory will be moving at that time, and those who will be filmed will be staying in Utah. This will make film production difficult.

Thanks for your efforts to help us clarify the manuscript, both editorially and by email. Please contact me if there are additional questions.

Yours sincerely,

Melinda L. Angus-Hill, Ph.D.
Assistant Professor, Department of Medicine
Investigator, Huntsman Cancer Institute
2000 Circle of Hope
Salt Lake City, UT 84112
Office Phone: 801-213-4240

TITLE:

A Method to Define the Effects of Environmental Enrichment on Colon Microbiome Biodiversity in a Mouse Colon Tumor Model

AUTHORS AND AFFILIATIONS:

Andrew K. Fuller[1,2], Benjamin D. Bice[1,2], Ashlee R. Venancio[1,2], Ambur M. Staab[1,2], Stephanie J. Georges[1,2], Julio R. Hidalgo[1,2], Annika V. Warncke[1,2], Melinda L. Angus-Hill[1,2]

[1]Division of Gastroenterology, Hepatology, and Nutrition, Department of Internal Medicine, University of Utah, Salt Lake City, UT 84132, USA

[2]Huntsman Cancer Institute, University of Utah, Salt Lake City, UT 84112, USA

EMAIL ADDRESSES:

Andrew K. Fuller (andrew.fuller@hsc.utah.edu)
Benjamin D. Bice (bice.utah@gmail.com)
Ashlee R. Venancio (avenanci@ymail.com)
Ambur M. Staab (ambur.staab@gmail.com)
Stephanie J. Georges (sjeangeo@gmail.com)
Julio R. Hidalgo (julio.hidalgo@hci.utah.edu)
Annika V. Warncke (annikawarncke@hci.utah.edu)
Melinda L. Angus-Hill (melinda.angus-hill@hci.utah.edu)

CORRESPONDING AUTHOR:

Melinda L. Angus-Hill (melinda.angus-hill@hci.utah.edu)
Tel: (801)-213-4240

KEYWORDS:

Mind-Body Medicine, Environmental Enrichment, Colon Cancer, Microbiota, Stool, Wound Repair

SUMMARY:

Environmental Enrichment (EE) is an animal housing environment that is used to reveal mechanisms that underlie the connections between lifestyle, stress, and disease. This protocol describes a procedure that uses a mouse model of colon tumorigenesis and EE to specifically define alterations in microbiota biodiversity that may impact animal mortality.

ABSTRACT:

Several recent studies have illustrated the beneficial effects of living in an enriched environment on improving human disease. In mice, environmental enrichment (EE) reduces tumorigenesis by activating the mouse immune system, or affects tumor bearing animal survival by stimulating the wound repair response, including improved microbiome diversity, in the tumor microenvironment. Provided here is a detailed procedure to assess the effects of environmental enrichment on the biodiversity of the microbiome in a mouse colon tumor model. Precautions

regarding animal breeding and considerations for animal genotype and mouse colony integration are described, all of which ultimately affect microbial biodiversity. Heeding these precautions may allow more uniform microbiome transmission, and consequently will alleviate non-treatment dependent effects that can confound study findings. Further, in this procedure, microbiota changes are characterized using 16S rDNA sequencing of DNA isolated from stool collected from the distal colon following long-term environmental enrichment. Gut microbiota imbalance is associated with the pathogenesis of inflammatory bowel disease and colon cancer, but also of obesity and diabetes among others. Importantly, this protocol for EE and microbiome analysis can be utilized to study the role of microbiome pathogenesis across a variety of diseases where robust mouse models exist that can recapitulate human disease.

INTRODUCTION:

Environmental enrichment (EE) studies utilize complex housing parameters to affect social stimulation (large housing cages, larger groups of animals), cognitive stimulation (huts, tunnels, nesting materials, platforms) and physical activity (running wheels). EE has been utilized by many labs to understand the effects of increased activity and improved social and cognitive interactions on disease initiation and progression using a wide array of mouse models, including barbering induced alopecia, Alzheimer's disease, Rett syndrome, and several tumor and digestive disease models[1-6].

Several mouse models have been developed to study colon tumorigenesis in mice. Perhaps the most well-defined model is the *Apc^{Min}* mouse. The *Apc^{Min}* mouse was developed in the laboratory of William Dove in 1990[7], and has been used as a mouse model of mutations in the *APC* gene that are commonly associated with human colorectal cancer. In contrast to humans harboring *APC* mutations, *Apc^{Min}* mice primarily develop small intestinal tumors, with very rare occurrence of colon tumors. However, a *Tcf4^{Het}* allele with a single knockin-knockout heterozygous mutation in *Tcf4*, vastly increases colon tumorigenesis when combined with the *Apc^{Min}* allele[8]. Recently, this mouse model of colon tumorigenesis has been used to determine the effects of EE on colon tumorigenesis[6]. In the Bice *et al*. study, the physiological and phenotypic effects of EE on males and females of four different mouse lines (*wild-type* (*WT*), *Tcf4$^{Het/+}$ Apc$^{+/+}$, Tcf4$^{+/+}$ Apc$^{Min/+}$,* and *Tcf4$^{Het/+}$ Apc$^{Min/+}$*)) were defined. Perhaps the most interesting finding was that EE significantly increases the lifespan of both male and female colon tumor-bearing animals. This demonstrated that EE may reduce at least some of the symptoms associated with colon tumorigenesis, and improve animal health. Remarkably, this improved lifespan in males is not a direct result of reduced tumorigenesis, and instead was linked to the initiation of a tumor wound healing response, including improved microbiome biodiversity[6].

Several EE specific studies have been published with interesting results. However, from a technical standpoint, important results are often not translatable to other laboratories. Maintaining identical EE methodologies between different laboratories is an incredibly complex issue, not only due to enrichment devices and housing used, but also bedding, food, ventilation, breeding, genetics, activity in the room, and animal protocol requirements, among others[9-11]. One example is animal integration, where animals must be stably integrated into the mouse colony, therefore normalizing genetic background and diet composition, to avoid non-treatment

related effects. Further, many EE studies have been completed prior to the realization of the importance of the microbiome in disease, and the way that common mouse husbandry practices can affect the composition of the gut microbiome[10,12].

Breeding strategy and animal placement in EE can increase stress if not performed properly. Since EE studies utilize large numbers of both male and female animals and multiple genotypes, experimental setup can be difficult given the requirement for animals from several litters to be combined. Therefore, a breeding and weaning strategy was developed to allow for combining of weaned animals of the correct genotype from different litters. The primary rationale for this was to normalize the microbiota among litters and to reduce stress when animals were moved to the experimental environment. The microbiome was transmitted from the dam[10]. To provide microbial diversity to the colony, females were purchased from Jackson Labs and integrated into the colony for one month before the experiment began[9,10,12]. To further normalize microbiome biodiversity between animals, females were co-housed prior to breeding. Following breeding, communal housing during rearing and the ability to escape nursing pups improved the stress levels of maternal care[13,14], possibly furthering microbiome normalization. To prevent non-EE related effects on the microbiome, this communal housing of all experimental animals prevented fighting and additional stress that occurred when combining several males from different litters into one experimental cage. Finally, equal numbers of animals of all genotypes were included in the cages. This provided the opportunity for improved microbiota biodiversity across genotypes, and removed the contribution of coprophagia (the animal's tendency to consume stool) or possible genotype-specific behavioral differences to the overall study.

This protocol provides a strategy that expands previous EE studies to include known aspects of microbiome research, including microbiota transmission and animal colony integration for microbiota normalization, to enable more uniform microbiome populations between experimental animals. Heeding these precautions is essential due to the ability of non-treatment related microbiota differences to confound study findings. Eliminating non-EE related microbiota changes will enable researchers to specifically define the role of EE on microbiota composition during disease development and progression.

PROTOCOL:

All methods described here were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah.

1. Experimental Design and EE and Control Cage Setup

Note: For reference, an outline of the experimental design is illustrated (**Figure 1**).

1.1. Set up control (NE) and EE cages (**Figure 2**).

1.1.1. To set up NE cages, use autoclaved conventional control cages (**Table 1**) that lack enrichment devices.

1.1.2. For large cages, drill one hole per cage that is large enough to accommodate a grommet and tunnel (**Table of Materials**).

1.1.3. To set up pup rearing and EE cages, connect two large autoclaved cages with a sterilized grommet secured tunnel and 2 sterilized platforms to increase floor space (**Table 1**). For EE experiments, provide sterilized running wheels, tunnels, igloos, huts, crawl balls, and nesting material within the EE cages.

1.1.4. Place both EE and NE cages in a ventilated rack to provide equal ventilation.

Note: Two large cages with 2 platforms (**Table 1**) allow for a maximum of 12 pregnant females per pup rearing setup[15] (see **Table of Materials**, step 3.2 in document, and **Table 2**).

1.1.5. Feed mice *ad libitum* irradiated standard chow and autoclaved reverse osmosis water.

1.1.6. Provide mice with sterile bedding materials.

Note: All manipulations of empty cages and cages with animals must be done in the hood to prevent contamination. For cage manipulation of large cages, cover the hole in the cage with an adhesive film.

1.2. Prepare animals for breeding. Group house 15 2-month old females in a single large cage (**Table 1**) for 2 weeks prior to mating. Separately house 2-month old littermate males for 2 weeks prior to mating.

Note: The number of females for breeding is dependent on the experiment and the number of animals required. In this study, 15 females were utilized to obtain 12 plugged females, which is the maximum number allowed in the pup rearing setup described in 1.1.2 (**Table 2**).

1.3. For breeding, combine sire and dam animals, 1 male per 2 females. The first check in the morning should be for vaginal plugs, which may be a visual sign that the animals have mated during the night.

Note: House males and females together until each female has plugged. A vaginal plug can be identified visually, if it is external.

1.3.1. To detect internalized plugs, a probe is inserted into the vaginal opening and if the vaginal plug is present, the probe is not easily inserted ([16]; see section 4.3.6).

Note: Record the morning a plug is detected as ½ day, since the mating occurred in the night.

1.3.2. Once females have vaginal plugs, transfer them to a large pup rearing cage for group housing with other mated females (Setup in step 1.1.2 without enrichment devices).

1.3.3. Replace mated females with new unmated group housed females for mating and continue mating to obtain a maximum number of litters in a 7-day period.

Note: All animals must have a delivery date within 7 days of each other.

1.4. Allow pregnant females to give birth in group housing so that all litters are raised within one large pup rearing cage (Setup in step 1.1.2 without enrichment devices).

1.4.1. Keep track of pup numbers and birthdates, and begin genotyping pups at 7 days of age.

1.4.1.1 Tattoo pups on their toes at 7 days of age with a number code to identify them[13,17].

1.4.1.2 Clean the toe with 70% ethanol and gently insert a micro-tattoo device containing ink into the skin surface parallel to the toe[17] (see **Table of Materials**).

1.4.1.3 Collect tissue with scissors for genotyping from the tail tips by incising a small piece of tissue from 7-day old neonates.

1.4.2. Isolate genomic DNA from tissue and perform PCR using a conventional HotSHOT method as described in [18].

1.4.3. Separate animals by sex at 14-21 days into large cages with mothers.

Note: Ensure that the older pups are able to feed on their own, and that younger pups continue to nurse until old enough to feed on their own.

1.4.4. At 21-28 days, distribute male and female animals separately by genotype into NE or EE environments, making sure to keep equal proportions of each genotype per cage (**Figure 2A**).

Note: Ensure that the total number of animals allowed in each NE or EE cage is based on the maximum number allowed by IACUC (**Table 2**). The NE cages have at most 5 animals (**Table 2**). In EE cages, for social stimulation, no fewer than 20, and with space restrictions, no more than 41 animals should be allowed in the EE cage (**Table 2**).

2. Stool Collection at 16 Weeks of Age

2.1. Begin stool collection 1 to 2 days prior to sacrifice, and separately collect stool on the day of sacrifice during dissection using sterile tools.

Note: Collecting stool at the same time 1-2 days prior to collection may help to avoid the loss of a sample due to the possibility that no stool is present at the time of collection.

2.1.1. To collect stool from live animals, carefully scruff the animal over a clean cage. Collect stool using sterile forceps into a sterile microfuge tube.

Note: Animals will typically eliminate stool when immobilized, which allows for rapid stool collection directly into a sterile microfuge tube. If an animal does not immediately defecate when immobilized, place it into a clean cage and wait for the animal to defecate (typically up to 1 h).

2.1.2. Collect stool on the day of sacrifice.

2.1.2.1. For euthanizing the animal, place the animal in a bell jar containing a small container with a cotton ball soaked in isoflurane. Once cessation of breathing is observed (usually after 2 min), lay the animal on its back to allow colon dissection.

2.1.2.2. Apply 70% ethanol to the mouse abdomen.

2.1.2.3. Lift the skin anterior to the urethral opening with forceps, and use scissors to cut along the ventral midline until reaching the ribcage, and cut from the base of the first incision towards each leg. Fold back the skin and use scissors to cut through the peritoneal wall in the same pattern.

2.1.2.4. Use forceps to grasp the distal colon at the anus to dissect and detach the distal colon from the rectum. While pulling the colon vertically with forceps, use scissors to cut through the mesentery to release the colon.

2.1.2.5. Cut the colon just below the cecum and lay it on filter paper. Use forceps to lift the top of the colon tube, opening the lumen to allow one side of open scissors to be inserted. Cut longitudinally, distal to proximal, and splay open the colon lengthwise.

2.1.2.6. Collect stool from the distal colon into a sterile microfuge tube using sterile forceps.

2.2. Store stool in a microfuge tube at -80 ˚C until time of bacterial DNA isolation.

Note: On the day of sacrifice, in addition to the stool, collect other samples such as whole blood, serum, plasma, normal and tumor tissue from colon and small intestine, microsomes, adipose tissue, *etc*. to address defined questions in the study.

3. Genomic DNA Isolation from Stool

Note: Utilize a commercial kit to isolate microbial DNA from stool following a stool pathogen detection protocol. Remove samples directly for the -80 ˚C freezer and store on dry ice while weighing.

3.1. Transfer up to 220 mg of stool to a clean microfuge tube containing 1.4 mL of room temperature (RT) stool lysis buffer (see **Table of Materials**).

3.2. Vortex sample for 1 min to thoroughly homogenize solids (**Figure 2B**). Heat the suspension to 95 °C for 5 min to lyse all bacteria (including Gram-positive bacteria).

3.3. Vortex samples for 15 s and then centrifuge at 20,000 x g for 1 min to pellet the stool solids. Transfer supernatant to a 2-mL microfuge tube. Add one tablet to each sample to absorb PCR inhibitors, vortex until the tablet is dissolved, and incubate the sample at RT for 1 min.

3.4. Centrifuge the sample at 20,000 x g for 3 min and transfer the supernatant to a new microfuge tube. Centrifuge at 20,000 x g for 3 min. Aliquot 15 µL of proteinase K (20 mg/mL stock) into a new 1.5 mL microfuge tube. Pipette 200 µL of the sample into the tube containing proteinase K.

3.5. Add 200 µL of guanidinium chloride lysis buffer to the tube, vortex thoroughly for 15 s (see **Table of Materials**) and incubate the sample at 70 °C for 10 min. Add 200 µL of ethanol (96-100%) to the tubes and mix well by vortexing.

3.6. Place a silica based spin column in a 2-mL collection tube and apply the samples to the column. Close the lid and centrifuge for 1 min at 20,000 x g.

3.7. Transfer the column to a new 2 mL collection tube and add 500 µL of wash buffer 1 to the column, cap the column and centrifuge for 1 min at 20,000 x g. Transfer the column to a new 2 mL collection tube and add 500 µL of wash buffer 2 to the column, close the cap and centrifuge at 20,000 x g for 3 min.

3.8. With the cap closed, transfer the column to a new 2 mL collection tube, and centrifuge for an additional 1 min at 20,000 x g to remove residual wash buffer. Transfer the column to a 1.5 mL labeled microfuge tube and elute sample by adding 200 µL of elution buffer containing EDTA to the membrane (see **Table of Materials**).

3. 9. Close the cap and incubate at RT for 1 min. Centrifuge the sample for 1 min at 20,000 x g. Discard the column.

4. DNA Concentration Determination and Sample Preparation for PCR

Note: Utilize a fluorometer and a commercially available dsDNA fluorescent assay to determine genomic DNA concentration in each sample (see **Table of Materials**). The fluorescent dye must bind double stranded DNA specifically.

4.1. Prepare a 1:200 dilution of each sample (1 µL of each sample in 199 µL dsDNA master mix) and a 1:50 dilution of standards. Analyze on a fluorometer using the dsDNA setting.

Note: A high volume of DNA in PCR can be inhibitory, therefore, the volume of DNA used must not be more than 10% of the final volume of the PCR. A fluorometer enables accurate

measurement of DNA in the sample, as only DNA bound to the fluorescent dye will fluoresce, eliminating the possible contribution of contaminants to the final calculated DNA concentration. This level of accurate quantitation is essential for the downstream sequencing application.

4.2. Prepare PCR templates diluted to 5 ng/μL with the appropriate volume of 10 mM Tris, pH 8.5 to make working template stocks of each sample.

4.3. Store samples at -20 °C.

5. Design Primers to the 16S Desired V Regions

5.1. Design primers to selectively amplify the desired V 16S rRNA regions.

5.2. Analyze primers with Probe Match, from the Ribosomal Database Project[19], to determine the approximate hit rate for various phyla.

Note: For V1-V3 regions, the current study used published primers Bosshard forward[20], which bind at position 8 within the V1 region, and 533 reverse[21], which binds at position 533 within the V3 region. Primers must include overhang adapter sequences for indexing.

5.3. When designing primers, include adapter sequences at the 5' ends of each primer, as recommended for 16S metagenomics sequencing library preparation[22] (**Table 3**).

5.4. Synthesize these large primers with cartridge purification. Reconstitute desiccated primers and dilute a PCR working stock to 1 μM in 10 mM Tris, pH 8.5.

6. Amplicon PCR to Amplify the V Region(s) with Overhang Adapter Sequences Attached[22].

6.1. Set up the amplicon PCR reaction mix as described in **Table 4.**

6.2. Place an adhesive clear PCR plate seal on the plate and run the amplicon PCR using the parameters in **Table 5**.

6.3. (Optional) Run amplicon PCR products on an agarose gel or a high sensitivity DNA assay that enables quantitative measurement of amplicon size (see **Table of Materials**).

Note: The amplicon size from this study is 550 bp (**Figure 3A**).

7. PCR Cleanup Using Magnetic Beads[22]

7.1. Centrifuge the amplicon PCR plate quickly at 1,000 x g for 1 min to collect condensation.

Note: PCR tube strips can be used instead of PCR plates to minimize contamination. Discard tube lids and never reuse.

7.2. Vortex the magnetic beads to evenly disperse them, and add 20 µL of magnetic beads to each amplicon PCR well, then pipette the entire volume up and down slowly 10 times.

7.3. Incubate at RT for 5 min. Place the PCR plate on a magnetic stand for 2 min until magnetic beads are collected and the supernatant is clear. Remove and discard the supernatant.

7.4. Wash beads with 200 µL fresh 80% ethanol while the PCR plate is on the magnetic stand and incubate for 30 s at RT on the magnetic stand. Carefully remove the supernatant.

7.5. Repeat the wash for a second time. Now, use a fine pipette tip to remove any residual ethanol from the wells and allow air drying for 10 min.

7.6. Remove the PCR plate from the magnetic stand and add 52.5 µL of 10 mM Tris pH 8.5 to each well. Pipette up and down 10 times to suspend beads and incubate at room temperature for 2 min.

7.7. Transfer the PCR plate to the magnetic stand to collect magnetic beads and transfer 50 µL of the supernatant to a clean PCR plate. Place an adhesive clear PCR plate seal on the plate and store at -20 ˚C for up to one week.

8. Preparation of a Plate Scheme for Index PCR

Note: To generate a V1-V3 library, a second PCR was performed with an index kit (see **Table of Materials**). A default indexing scheme was used to map out unique dual index combinations for each sample (**Figure 3B** and [23]).

8.1. Ensure that each sample has a unique combination of 2 index primers (*i.e.*, dual indexing).

9. Perform Index PCR to Attach Barcodes to the Adaptor Sequences as Described[22].

9.1. Transfer 2.5 µL of PCR amplicons (clean amplicons) to a new 96 well plate and place in an index plate fixture to aid in indexing.

9.2. Arrange the index 1 and index 2 primers as in the example of the prepared plate graphic (**Figure 3B**).

Note: Visual cues are provided to avoid primer mix-ups: index 2 primer tubes should have white caps and clear solution, while index 1 primer tubes should have orange caps and yellow solution.

9.3. Assemble the index PCR Mix reaction as described in **Table 6.** Mix by pipetting up and down 10 times. Cover with an adhesive clear PCR plate seal and centrifuge to collect at 1,000 x g at room temperature for 1 min.

9.4. Run the index PCR using the parameters in **Table 7**.

10. Purify Final PCR Library

Note: This PCR clean-up is identical to step 7 above, and uses magnetic beads to perform PCR Clean-Up of the index PCR[22].

10.1. Centrifuge the PCR plate from step 10 quickly at 1,000 x g for 1 min to collect condensation.

10.2. Vortex the magnetic beads to evenly disperse them, then add 20 μL of magnetic beads to each amplicon PCR well, then pipette the entire volume up and down slowly 10 times to mix.

10.3. Incubate at RT for 5 min.

10.4. Place PCR plate on a magnetic stand for 2 min until magnetic beads are collected and supernatant is clear. Remove and discard the supernatant.

10.5. Wash beads with 200 μL fresh 80% ethanol while the PCR plate is on the magnetic stand and incubate for 30 s at room temperature on the magnetic stand. Carefully remove the supernatant.

10.6. Repeat the wash for a second time.

10.7. Following the second wash, use a fine pipette tip to remove any residual ethanol from the wells and allow air drying for 10 min.

10.8. Remove the PCR plate from the magnetic stand and add 52.5 μL of 10 mM Tris pH 8.5 to each well. Pipette up and down 10 times to suspend beads and incubate at room temperature for 2 min.

10.9. Transfer the PCR plate to the magnetic stand to collect magnetic beads and transfer 50 μL of the supernatant to a clean PCR plate. Place an adhesive clear PCR plate seal on the plate and store at -20 ˚C for up to one week.

10.10. (Optional) Run index PCR products on an agarose gel or a high sensitivity DNA assay that enables quantitative measurement of amplicon size (see **Table of Materials**).

Note: The final indexed library size from this study was 668 bp (**Figure 3C-D**).

11. Quantify, Normalize, and Pool the Indexed Libraries for Sequencing

11.1. Determine the DNA concentration of each sample with a fluorometer and a dsDNA fluorescent assay kit (see **Table of Materials**).

11.1.1. Prepare a 1:200 dilution of the sample (1 µL of each sample in 199 µL dsDNA master mix, which includes buffer and reagent) for each of the indexed samples and standards (190 µL dsDNA master mix and 10 µL of standard). Analyze on a fluorometer using the dsDNA setting.

11.2. Following the DNA concentration calculation, normalize the libraries by calculating the average library size. Do this by summing adapter lengths, index lengths, and V amplicon size from primers, and view products by agarose gel to be certain the actual size is similar to the calculated size (**Figure 3C**, see [22]).

11.2.1. Alternatively, utilize a high sensitivity DNA assay that enables quantitative measurement of DNA integrity, amplicon size, and concentration (Table of Materials, **Figure 3D**).

Note: In this study, the average library size was calculated based on summing adapter lengths, index lengths, and V1-V3 amplicon size from primers. The average size was 668 bp.

11.2.2. Concentrations of samples are normalized using the formula in **Table 8**.

11.3. Dilute samples to 4 nM and pool 5 µL from each 4-nM sample into a single tube for sequencing.

12. Sequence the Library using a Next Generation Sequencing System and Parse the Data

12.1. Sequence the library.

Note: For this study, the University of Utah High Throughput Genomics Core performed library denaturation and sample sequencing (as described in [6,22]).

12.2. Parse the data.

Note: To separate data from pooled samples, index reads were identified and separated (as described in [22]).

12.3. Generate FASTQ files and utilize this for subsequent data analysis.

13. Analyze Sequenced Data from the 16S Amplicon Library

Note: This step is performed as described in Bice *et al*., 2017[6].

13.1. Install freely available data analysis tools (see **Table of Materials**; [24]).

13.2. Assemble demultiplexed fastq files from the sequenced data (as described in [25,26]). Discard all unassembled sequences.

13.3. Perform analyses following a *de novo* open taxonomic unit (OTU) picking protocol (as described in [27]).

13.3.1 Bin sequences into a single fastq file by sampleID and group sequences with 97% or greater similarity into OTUs, as described in [28]. Align representative sequences of core set with minimum sequence length of 150 and 75% percent identity[29,30]. Assign taxonomy as described[28].

Note: Samples can be binned and analyzed[31], followed by taxonomic assignment and OTU table construction[32].

13.3.2. Create a mapping file that identifies descriptive names and characteristics of samples to link to sample identification and validate the mapping file[33,34].

13.3.3. Make an OTU network that links OTUs to sample descriptions using a mapping file[35].

13.3.4. Calculate taxonomy summaries in terms of relative abundance by summarizing taxa through plots[36].

13.3.5. Explore alpha diversity of samples at uniform sequencing depth appropriate to samples. To define the appropriate depth for alpha diversity, summarize total counts observed in each sample by using the biome summarize-table command, as described in [37].

REPRESENTATIVE RESULTS:

Several studies have demonstrated that the practice of mind-body medicine improves health outcomes. Similarly, in mice, environmental enrichment improves outcomes including improved lifespan and tumor wound repair[6]. Therefore, an EE procedure was developed with the aim of defining the role of microbiota in this phenotype while first normalizing the microbiome prior to the initiation of the experiment (**Figure 1**). Importantly, all breeding animals are integrated into the mouse colony for at least one month prior to the commencement of breeding, and newborn pups are co-housed with mothers in one large cage to normalize microbiota transmission prior to the experiment. When animals are between 21 and 28 days old, equal numbers of each genotype are weaned into their respective housing, either EE or NE environments (**Figure 2A**). At 16 weeks, stool from all animals is collected and homogenized (**Figure 2B**), followed by bacterial DNA isolation. Finally, 16S amplicons are amplified from stool microbiome DNA and barcode indexed to allow for sequencing of all microbiome libraries simultaneously (**Figure 3**). The unique sequences identified in WT and tumor bearing animals in both NE and EE conditions are shown in **Figure 4**. Interestingly, EE does not improve biodiversity in WT animals, but vastly increases biodiversity in tumor bearing animals (**Figure 4**), demonstrating that this method allows biodiversity improvements. This increase in biodiversity can be attributed to the increased presence of the phylum Proteobacteria, with significant increases in the classes

Alphaproteobacteria and Betaproteobacteria, and decreases in pathogenic Gammaproteobacteria (**Figure 5**; **Supplemental Table 1**). The largest increase is in the Betaproteobacteria class is the genus *Sutterella*, a likely commensal involved in secreted IgA degradation (**Figure 6**, also see [38]).

FIGURE AND TABLE LEGENDS:

Figure 1: A representation of the experimental timeline. The short bands represent 7-day windows, as most of the protocol is accomplished in 7-day increments. This also aids in visualization of the range of pup ages across the experiment.

Figure 2: **EE and NE housing conditions and stool homogenates (as described in the protocol).**

Figure 3: 16S Microbiome Library Preparation. (**A**) Unpurified PCR amplicon products derived from stool genomic DNA. (**B**) Indexing plate graphic designed as per the software used (**Table of Materials**, [23]). The dual index combinations, I7 (Index 1; Row) and I5 (Index 2; Column), are shown for each sample. Each index is 8 bp in length. The sample numbers refer to the respective mouse numbers from EE studies. (**C**) Unpurified Index PCR products. (**D**) Quality analysis of final purified and pooled 16S libraries. (**A,C,D**) Black arrows denote 550 bp amplicon and 668 bp indexed library. Red arrows denote non-specific products that are eliminated following purification as shown in **D.** Upper and Lower markers are size markers added to the sample for size reference.

Figure 4: Changes in alpha diversity following EE of *Tcf4$^{Het/+}$ Apc$^{Min/+}$* animals. Alpha diversity of WT and *Tcf4$^{Het/+}$ Apc$^{Min/+}$* tumor bearing animals. At 20,000 reads, NE and EE of WT, p=0.64 and NE and EE of *Tcf4$^{Het/+}$ Apc$^{Min/+}$*, p=0.03 using two-sample t-test with Welch correction. Adapted from Bice *et al*., 2017[6].

Figure 5: EE-mediated changes following EE of *Tcf4$^{Het/+}$ Apc$^{Min/+}$* animals. R-ggplot2 generated box-whisker plots denoting changes in abundance of the phylum Proteobacteria and classes Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria. Outliers are noted as circles. **p=0.005 using two-sample t-tests with Welch correction. Error bars calculated using standard error of the mean (SEM). Adapted from Bice *et al*., 2017[6].

Figure 6: Changes in the relative abundance of *Sutterella* following EE of *Tcf4$^{Het/+}$ Apc$^{Min/+}$* animals. Outliers are noted as circles. **p=0.005 using two-sample t-test with Welch correction. Error bars calculated using SEM. Adapted from Bice *et al*., 2017[6].

Supplemental Table 1: Classification of Bacteria Isolated from Stool Collected from NE and EE mice. Classification across genotypes at the (**A**) Phylum, (**B**) Class, (**C**) Order, (**D**) Family, and (**E**) Genus level. Comparisons of NE and EE of the same genotype or WT to *Tcf4$^{Het/+}$ Apc$^{Min/+}$*. P-values are calculated using a two-sample t-test with Welch correction. Adapted from Bice *et al*., 2017[6].

Table 1: EE and NE Cage sizes and Floor Space.

Table 2: Allowed Numbers of Animals in Cages Based on Floor Space[15].

Table 3: **Amplicon PCR Primers.**

Table 4: **Amplicon PCR Mix.**
Table 5: Amplicon PCR Program Set Up.
Table 6: **Index PCR Mix.**
Table 7: **Index PCR Program Set Up.**
Table 8: **Formula for Normalizing Before Pooling Samples**

DISCUSSION:

This procedure allows for the analysis of microbiota isolated from stool following environmental enrichment of normal or tumor bearing animals. Because these are large experiments which involve breeding to obtain many animals of different sexes and genotypes, normalizing the microbiome between animals prior to commencement of the experiment is essential to avoid non-EE related effects on microbiome biodiversity.

For consistency between NE and EE conditions, the breeding process is conducted to ensure that all mice initially have exposure to the same microbes and, therefore, are expected to have similar microbiome contents. It is possible, and likely, that mouse genotype affects microbiome composition. For this reason, mouse numbers per genotype are maintained between NE and EE conditions to be certain that any animal that is consuming stool will encounter a similar diversity of the microbiome.

Several difficulties are apparent when designing EE experiments. First, the total number of animals needed for the experiments is dependent upon the experimental details, but the total number is also limited by the number of animals allowed in the cage. For example, historical data surrounding mouse survival in a preliminary EE experiment were used to calculate the number of animals to define the mechanism underlying improved survival observed in previous experiments. From this data, a total of 17 animals in the comparison group were required for an 80% power to detect a difference in survival in a two-sided t-test where alpha=0.05. So, 4-5 animals in the control group vs. 20-24 (or up to 41) animals per cage in the experimental group must accommodate a power calculation. Therefore, several control group cages are needed along with the experimental cage. Further, with complex genotypes, it is difficult to obtain sufficient animal numbers of every genotype, which necessitates the large numbers of breeding females required. However, with other models where fewer transgenic differences are present, more animals of the same genotype can be analyzed in this system and fewer breeders are necessary. In the United States, 12 pregnant females can be housed in the 633 in^2 of space (**Table 2**). The issue with this is that as pups get older, they take up more space. Given that male pups are separated from female pups at 14-21 days, an approved exception to the space rule in certain countries may be feasible. Otherwise, male and female pups can be genotyped and selected, and then separated at a younger age with mothers to stay below the maximum mouse numbers. It is essential to gain approval for these studies and to adhere to local rules on space restrictions. Finally, with microbiota, the number of animals needed to detect even small differences in microbiota composition is difficult to calculate *a priori*. While in this study, significant differences in the microbiota were found with 4 animals per group, it is possible that increasing that number of animals would reveal microbiota that are more variable between EE mice or are only slightly altered by EE.

This method article describes the particular equipment and bedding that are used, which on the surface may not appear essential. However, several non-obvious issues that affect consistency can be encountered and need to be addressed prior to embarking on these very large, expensive, and time-consuming studies. One major issue is cage ventilation. With large numbers of animals in a cage, ventilation becomes an issue, and is an issue that most researchers do not take into account when attempting to provide consistent environments between control and experimental cages. All cages in the described setup are placed in a ventilated cabinet to equalize ventilation across the experimental and control cages. This cannot be accomplished when using cages that do not fit in a ventilated cabinet. Other means to normalize ventilation between experimental and control animals could be tested and consistently applied, but these methods are not explored in this study. Similar consistency issues arise with bedding. In the colon cancer model system used in this study, animals that have digestive disease will ingest certain types of bedding, especially corn cob bedding, leading to digestive blockages and illness. It is important to keep this in mind if the animals are known to ingest bedding when not otherwise occupied, as in the control environment. This phenomenon and the subsequent inconsistent health effects will profoundly affect all data.

The ribosomal 16S gene has been used as a means to study bacterial populations. It contains nine regions that express genetic variability, V1-V9, and interspersed conserved regions that remain relatively unchanged between bacterial species[39]. The V1-V3 region, in particular, provides the highest probability of species-level identification[39]. Similar V1-V3 studies on Colorectal Cancer (CRC) and Advanced Colorectal Adenoma found changes in three phyla of interest: Bacteroidetes, Firmicutes, and Proteobacteria[21,40,41]. It has also been reported that exercise can shift the microbial population and lead to an increase in Firmicutes[42]. For this reason, this study identified microbiome populations by using V1-V3 primers following a 16S metagenomic library prep protocol[22] to potentially define the effects of environmental enrichment on these phyla known to be altered in adenoma and CRC. This procedure can be modified to amplify and sequence other variable regions of the 16S rRNA genes. One way is to use Probe Match to understand the phylogenetic classification of microbiota present in the sample that will be identified by the probe. In this way, probes can be targeted to specifically define and phylogenetically classify microbes of interest. This allows a different characterization of the microbiota present in the stool samples, and may reveal additional EE-dependent alterations in the microbiome of tumor bearing mice that may affect disease progression.

Using this procedure, the genus *Sutterella* was identified as the most altered genus following EE of tumor bearing animals. This procedure can be adapted to accommodate studies that utilize any method meant to analyze the effects of a perturbation on microbiome composition in genetically modified models of human disease. For example, in place of EE, mice could be inoculated with *Sutterella* to define whether *Sutterella* inoculation is sufficient to increase microbial biodiversity and wound repair in 16-week-old male tumor bearing animals.

Undoubtedly, the most unique aspect of this protocol is the concern over normalizing microbiota prior to EE and maintaining microbiome diversity throughout the EE studies. Since microbiome

studies are continually improving, it is likely that more robust methods for characterizing the microbiome will arise, and the microbiome characterization in this protocol will become obsolete. For example, with the current study, the probes used to amplify the 16S rRNA have bias, depending on the probes that are chosen, and do not characterize all of the bacteria present in the microbiome. While the methods used to survey and characterize the microbiome will undoubtedly improve, the basic foundation of designing and running EE experiments while keeping normalization of the microbiome in mind will remain an essential facet of EE experiments.

ACKNOWLEDGMENTS:

We thank B. Dalley in the University of Utah Genomics core for library sequencing, and K. Boucher in the University of Utah Biostatistics core for statistical advice, and access to these technical cores supported by National Cancer Institute award P30 CA042014. The project described was supported by the National Cancer Institute Grants P01 CA073992 and K01 CA128891 and the Huntsman Cancer Foundation.

DISCLOSURES:

The authors declare they have no conflicts of interest.

REFERENCES:

1 Bechard, A., Meagher, R., & Mason, G. Environmental enrichment reduces the likelihood of alopecia in adult C57BL/6J mice. *Journal of the American Association for Laboratory Animal Science : JAALAS.* **50** (2), 171-174, https://www.ncbi.nlm.nih.gov/pubmed/21439209 (2011).

2 Jankowsky, J.L. *et al.* Environmental enrichment mitigates cognitive deficits in a mouse model of Alzheimer's disease. *J Neurosci.* **25** (21), 5217-5224, doi:10.1523/JNEUROSCI.5080-04.2005 (2005).

3 Kondo, M. *et al.* Environmental enrichment ameliorates a motor coordination deficit in a mouse model of Rett syndrome--Mecp2 gene dosage effects and BDNF expression. *Eur J Neurosci.* **27** (12), 3342-3350, doi:10.1111/j.1460-9568.2008.06305.x (2008).

4 Reichmann, F., Painsipp, E., & Holzer, P. Environmental enrichment and gut inflammation modify stress-induced c-Fos expression in the mouse corticolimbic system. *PLoS One.* **8** (1), e54811, doi:10.1371/journal.pone.0054811 (2013).

5 Cao, L. *et al.* Environmental and genetic activation of a brain-adipocyte BDNF/leptin axis causes cancer remission and inhibition. *Cell.* **142** (1), 52-64, doi:10.1016/j.cell.2010.05.029 (2010).

6 Bice, B.D. *et al.* Environmental Enrichment Induces Pericyte and IgA-Dependent Wound Repair and Lifespan Extension in a Colon Tumor Model. *Cell reports.* **19** (4), 760-773, doi:10.1016/j.celrep.2017.04.006 (2017).

7 Moser, A.R., Pitot, H.C., & Dove, W.F. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science.* **247** (4940), 322-324, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=2296722 (1990).

8 Angus-Hill, M.L., Elbert, K.M., Hidalgo, J., & Capecchi, M.R. T-cell factor 4 functions as a tumor suppressor whose disruption modulates colon cell proliferation and tumorigenesis. *Proc Natl Acad Sci U S A.* **108** (12), 4914-4919, doi:10.1073/pnas.1102300108 (2011).
9 Holmdahl, R. & Malissen, B. The need for littermate controls. *Eur J Immunol.* **42** (1), 45-47, doi:10.1002/eji.201142048 (2012).
10 Ubeda, C. *et al.* Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice. *J Exp Med.* **209** (8), 1445-1456, doi:10.1084/jem.20120504 (2012).
11 Spor, A., Koren, O., & Ley, R. Unravelling the effects of the environment and host genotype on the gut microbiome. *Nat Rev Microbiol.* **9** (4), 279-290, doi:10.1038/nrmicro2540 (2011).
12 Fujiwara, R., Watanabe, J., & Sonoyama, K. Assessing changes in composition of intestinal microbiota in neonatal BALB/c mice through cluster analysis of molecular markers. *Br J Nutr.* **99** (6), 1174-1177, doi:10.1017/S0007114507862349 (2008).
13 Castelhano-Carlos, M.J., Sousa, N., Ohl, F., & Baumans, V. Identification methods in newborn C57BL/6 mice: a developmental and behavioural evaluation. *Lab Anim.* **44** (2), 88-103, doi:10.1258/la.2009.009044 (2010).
14 Curley, J.P., Davidson, S., Bateson, P., & Champagne, F.A. Social enrichment during postnatal development induces transgenerational effects on emotional and reproductive behavior in mice. *Frontiers in behavioral neuroscience.* **3**, 25, doi:10.3389/neuro.08.025.2009 (2009).
15 National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals., Institute for Laboratory Animal Research (U.S.), & National Academies Press (U.S.). National Academies Press,, Washington, D.C., xxv, 220 p (2011).
16 Silver, L.M. *Mouse genetics : concepts and applications.* Oxford University Press, New York (1995).
17 Chen, M., Kan, L., Ledford, B.T., & He, J.Q. Tattooing Various Combinations of Ears, Tail, and Toes to Identify Mice Reliably and Permanently. *Journal of the American Association for Laboratory Animal Science : JAALAS.* **55** (2), 189-198, https://www.ncbi.nlm.nih.gov/pubmed/27025811 (2016).
18 Truett, G.E. *et al.* Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques.* **29** (1), 52, 54, https://www.ncbi.nlm.nih.gov/pubmed/10907076 (2000).
19 Cole, J.R. *et al.* Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* **42** (Database issue), D633-642, doi:10.1093/nar/gkt1244 (2014).
20 Bosshard, P.P., Zbinden, R., & Altwegg, M. Turicibacter sanguinis gen. nov., sp. nov., a novel anaerobic, Gram-positive bacterium. *Int J Syst Evol Microbiol.* **52** (Pt 4), 1263-1266, doi:10.1099/00207713-52-4-1263 (2002).
21 Chen, W., Liu, F., Ling, Z., Tong, X., & Xiang, C. Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. *PLoS One.* **7** (6), e39743, doi:10.1371/journal.pone.0039743 (2012).

22 Illumina. *16S Metagenomic Sequencing Library Preparation: Preparing 16S ribosomal RNA Gene Amplicons for the Illumina MiSeq System*. https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf.

23 Illumina. Illumina Experiment Manager. https://www.illumina.com/informatics/research/experimental-design/illumina-experiment-manager.html.

24 Caporaso, J.G. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods.* **7** (5), 335-336, doi:10.1038/nmeth.f.303 (2010).

25 Aronesty, E. ea-utils: Command-line tools for processing biological sequencing data. *Expression Analysis, Durham, NC.* (2011).

26 Knight, R., Caporaso, J.G. QIIME: Multiple Paired Ends Script. http://qiime.org/scripts/multiple_join_paired_ends.html

27 Knight, R., Caporaso, J.G. QIIME: De-Novo OTU Picking Protocol. http://qiime.org/scripts/pick_de_novo_otus.html

28 Edgar, R.C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics.* **26** (19), 2460-2461, doi:10.1093/bioinformatics/btq461 (2010).

29 Caporaso, J.G. *et al.* PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics.* **26** (2), 266-267, doi:10.1093/bioinformatics/btp636 (2010).

30 DeSantis, T.Z. *et al.* Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol.* **72** (7), 5069-5072, doi:10.1128/AEM.03006-05 (2006).

31 Knight, R., Caporaso, J.G. QIIME: Multiple Split Libraries Fastq Script. http://qiime.org/scripts/multiple_split_libraries_fastq.html.

32 Knight, R., Caporaso, J.G. QIIME: De Novo Otus Script. http://qiime.org/scripts/pick_de_novo_otus.html

33 Knight, R., Caporaso, J.G. QIIME: Links to Sample Identification. http://qiime.org/documentation/file_formats.html.

34 Knight, R., Caporaso, J.G. QIIME: Validation of Mapping File. http://qiime.org/scripts/validate_mapping_file.html.

35 Knight, R., Caporaso, J.G. QIIME: Link of OTUs to Sample Description Using Mapping File. http://qiime.org/scripts/make_otu_network.html.

36 Knight, R., Caporaso, J.G. QIIME: Summarize Taxa Through Plots. http://qiime.org/scripts/summarize_taxa_through_plots.html.

37 Knight, R., Caporaso, J.G. QIIME: Biome Summarize Table Command. http://biom-format.org/documentation/summarizing_biom_tables.html.

38 Moon, C. *et al.* Vertically transmitted faecal IgA levels determine extra-chromosomal phenotypic variation. *Nature.* **521** (7550), 90-93, doi:10.1038/nature14139 (2015).

39 Chakravorty, S., Helb, D., Burday, M., Connell, N., & Alland, D. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *J Microbiol Methods.* **69** (2), 330-339, doi:10.1016/j.mimet.2007.02.005 (2007).

40 Chen, H.M. *et al.* Decreased dietary fiber intake and structural alteration of gut microbiota in patients with advanced colorectal adenoma. *Am J Clin Nutr.* **97** (5), 1044-1052, doi:10.3945/ajcn.112.046607 (2013).
41 Zhu, Q. *et al.* Analysis of the intestinal lumen microbiota in an animal model of colorectal cancer. *PLoS One.* **9** (6), e90849, doi:10.1371/journal.pone.0090849 (2014).
42 Evans, C.C. *et al.* Exercise prevents weight gain and alters the gut microbiota in a mouse model of high fat diet-induced obesity. *PLoS One.* **9** (3), e92193, doi:10.1371/journal.pone.0092193 (2014).

Figure 1

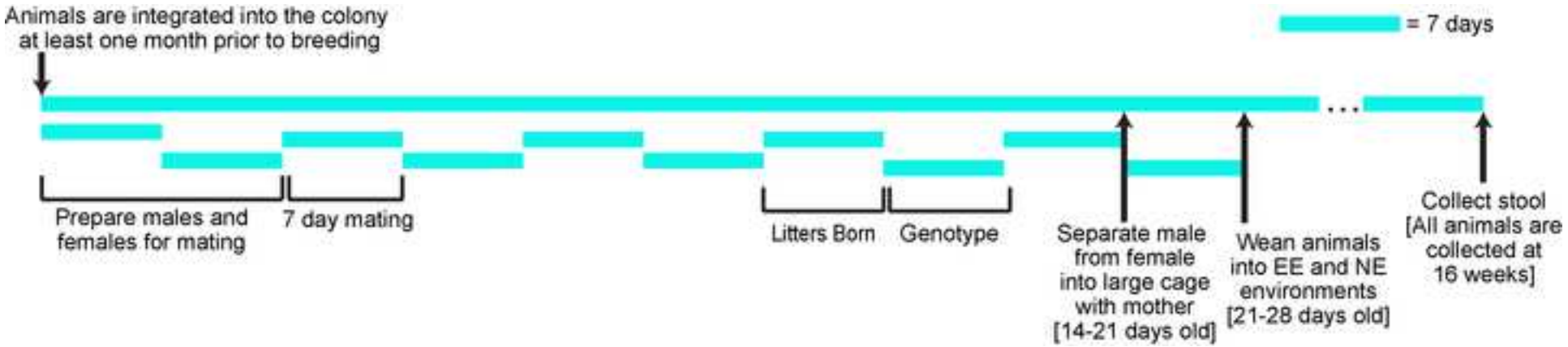

Figure 2

A.

Environmentally Enriched (EE) Environment

B.

Homogenized Stool Samples

Male EE Male NE

Tcf4$^{Het/+}$ *Apc*$^{Min/+}$

Figure 3

Click here to download Figure Jove_Figure 3.tif

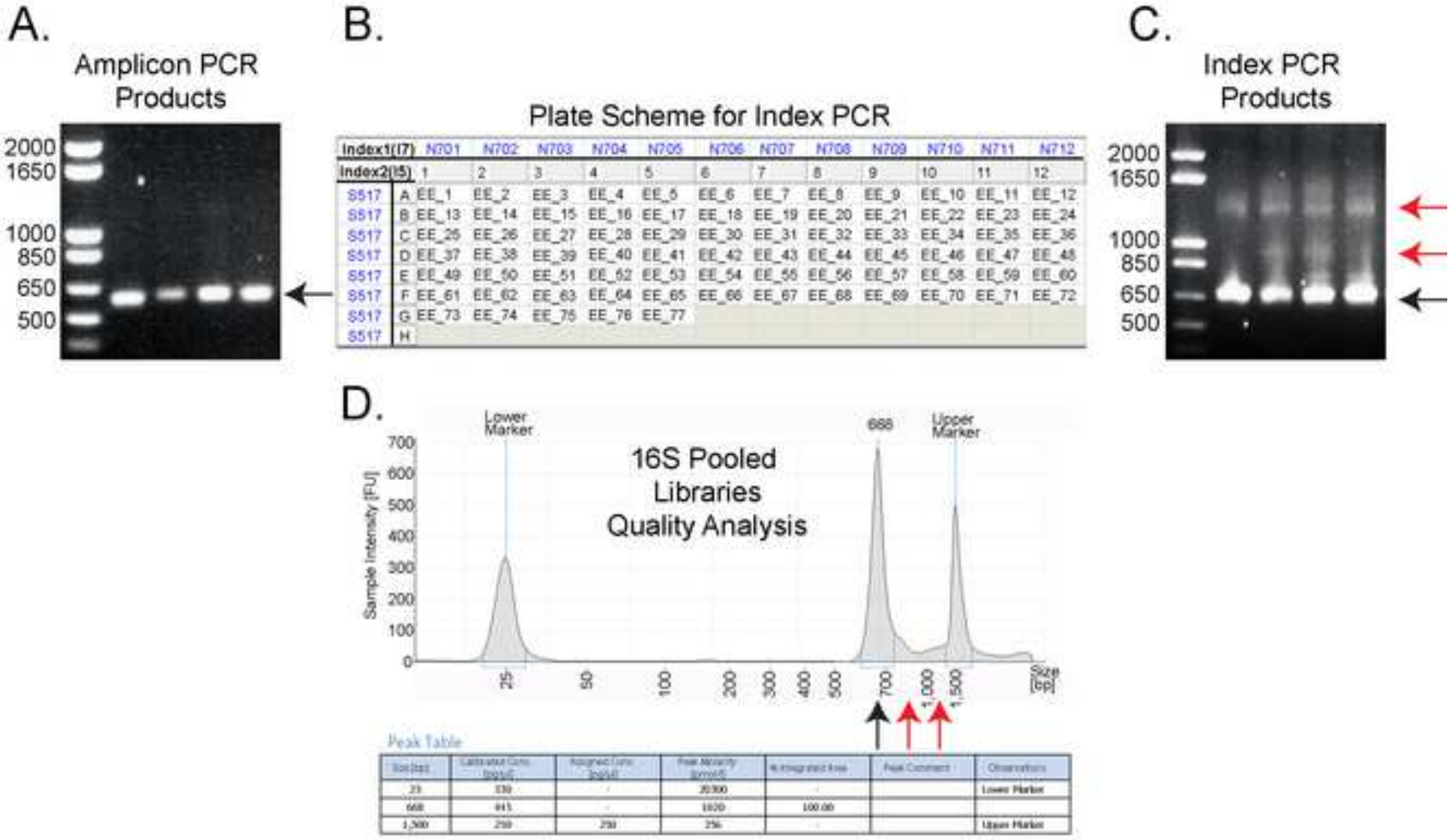

Figure 4

Click here to download Figure Jove_Figure 4.tif

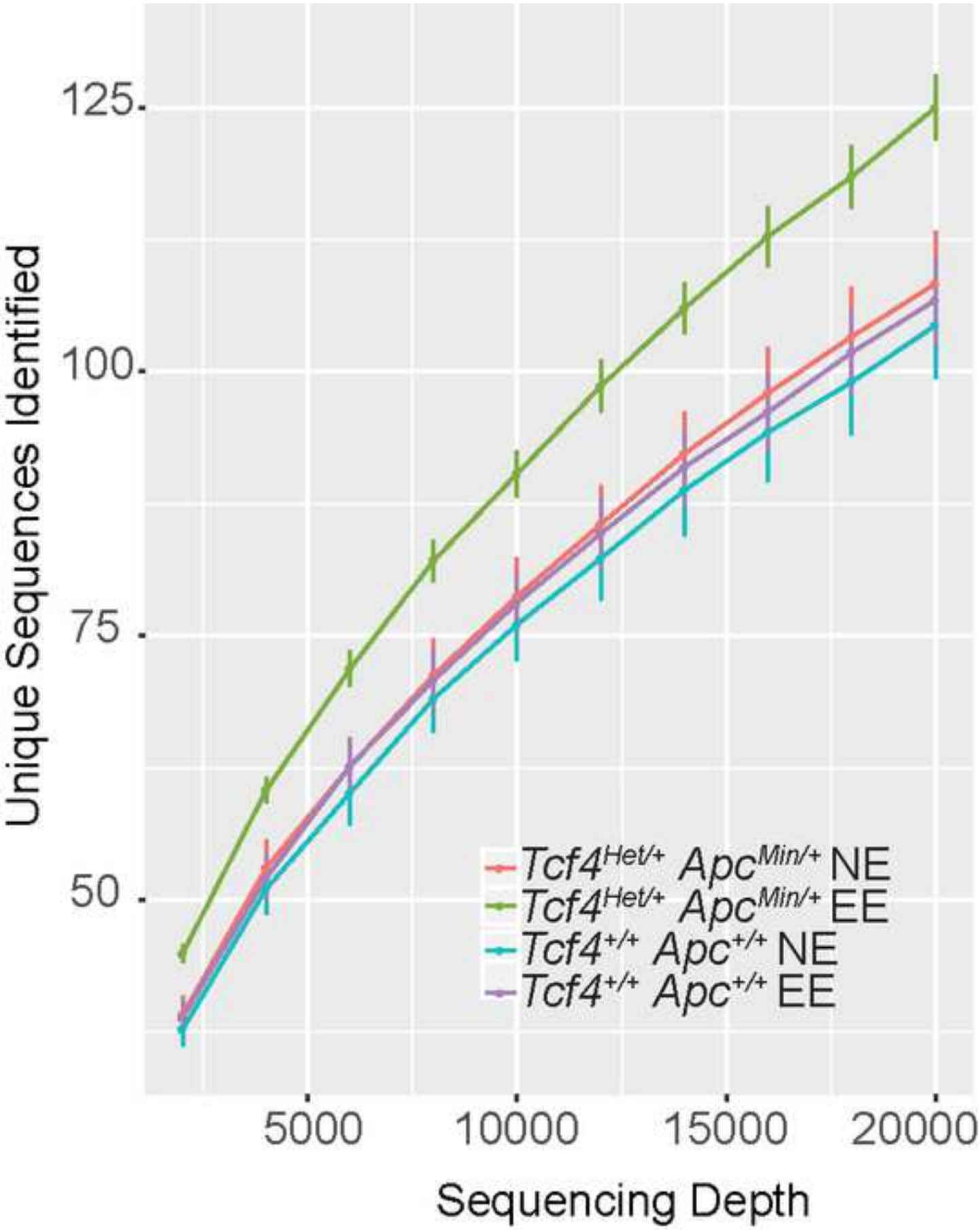

Figure 5

Click here to download Figure Jove_Figure 5.tif

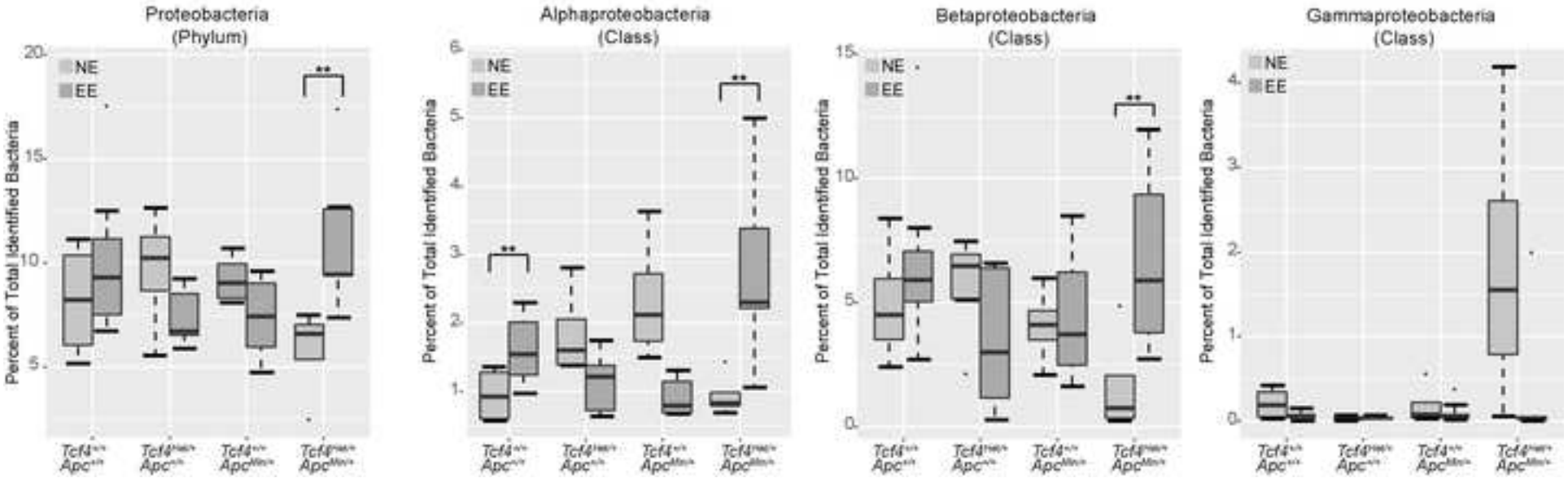

Figure 6

Click here to download Figure Jove_Figure 6.tif

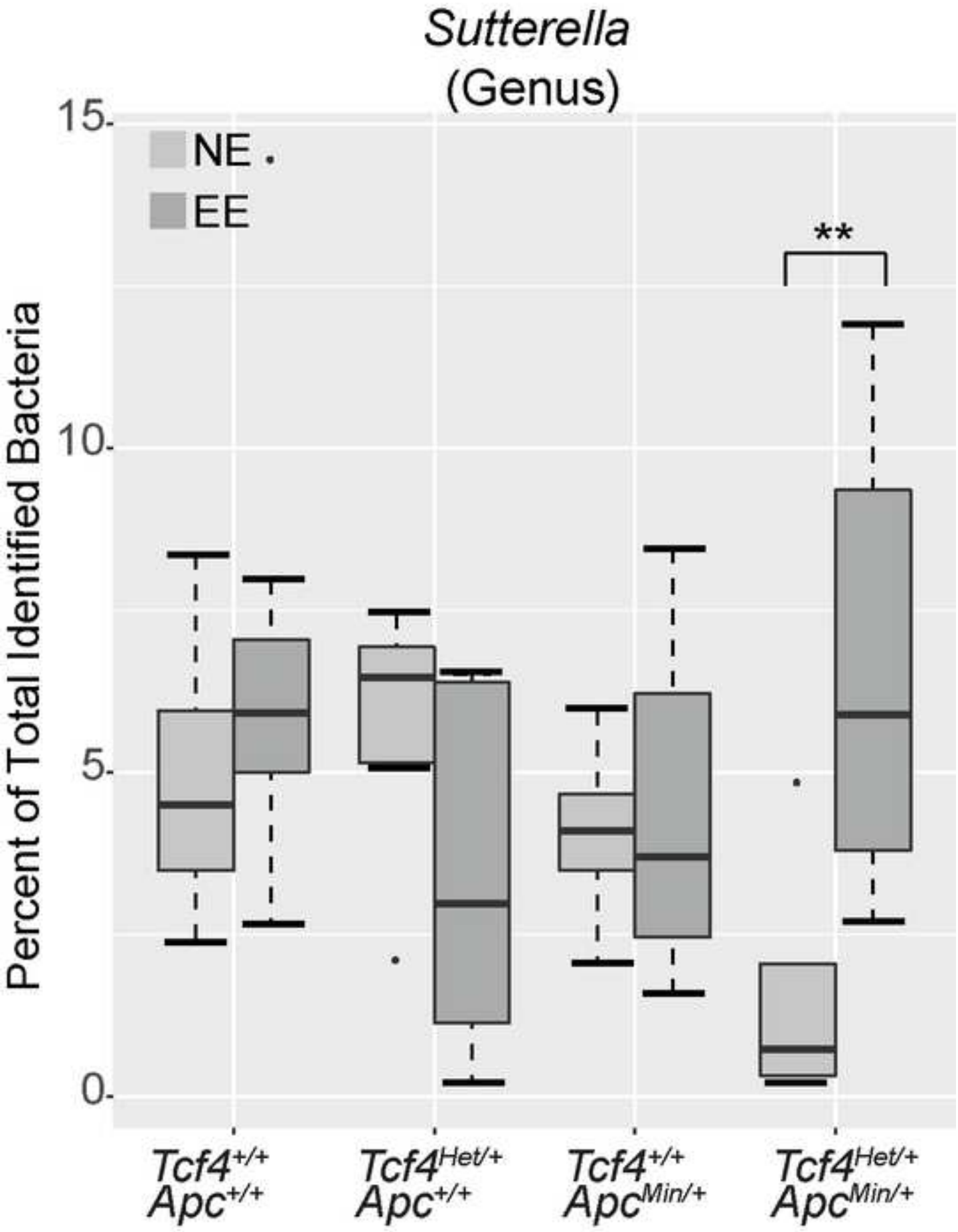

Table 1

Click here to download Table JoVE_Table1_Fuller.xlsx

Item	Total Area ($inch^2$)	Total Area (cm^2)	Cage Size (inch) L x W x H
One Control Cage (NE)	68.25	440.32	10.5 x 6.5 x 5.5
One Large Cage (EE)	264.36	1706.32	13.87 x 19.06 x 7.75
Two Large Cages (EE)	528.72	3412.64	2@ 13.87 x 19.06 x 7.75
Two Platforms (EE)	93	600	2@ 11.8 x 3.94 x 2.95
Two Large Cages with Two Platforms (EE)	621.72	4013	2@ 13.87 x 19.06 x 7.75 + 2@ 11.8 x 3.94 x 2.95

Cage Size (cm) L x W x H
26.67 x 16.51 x 13.97
35.24 x 48.42 x 19.69
2@ 35.24 x 48.42 x 19.69
2@ 30 x 10 x 7.5
2@ 35.24 x 48.42 x 19.69 + 2@ 30 x 10 x 7.5

Table 2

Animals Allowed in Cage	Required Inches Squared Per Animal	EE Cage Area (Inches^2)	Total Animals allowed
Up to 25	12	622 in^2 (4013 cm^2)	up to 25
25+	15	622 in^2 (4013 cm^2)	up to 41
Female with Litter	51	622 in^2 (4013 cm^2)	up to 12

Amplicon PCR Primers

Forward	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**agagtttgatcMtggctcag**-3'
Reverse	5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TTACCGCGGCTGCTGGCAC** -3'

Locus specific sequences are shown in bold and the non-bold is the overhang adapter sequence.

Amplicon PCR reaction set up

	Volume
Microbial DNA (5ng/µl)	2.5 µl
Forward Primer (1 µM; from step 5.1.2)	5.0 µl
Reverse Primer (1 µM; from step 5.1.2)	5.0 µl
2X HotStart Ready Mix	12.5 µl
Total	25.0 µl

Amplicon PCR set up

95 °C for 3 minutes
25 Cycles of:
95 °C for 30 seconds
55 °C for 30 seconds
72 °C for 60 seconds
72 °C for 3 minutes
Hold at 4 °C

Index PCR barcode assembly

DNA	2.5 µl
Index Primer 1 (N7XX)	2.5 µl
Index Primer 2 (S5XX)	2.5 µl
2X HotStart Ready Mix	12.5 µl
PCR grade water	5 µl
Total	25 µl

Index PCR Setup

95 °C for 3 minutes
8 cycles of:
95 °C for 30 seconds
55 °C for 30 seconds
72 °C for 30 seconds
72 °C for 5 minutes
Hold at 4 °C
Store at -20 °C

Table 8 Click here to download Table Jove_Table8_Fuller.xlsx

Sample Concentration Formula for Pooling

$$\frac{(\text{DNA concentration in ng/µl})}{(660\ \text{g/mol} \times \text{average library size})} \times 106 = \text{Concentration in nM}$$

An example from this study is:

$$\frac{(85.2\ \text{ng/µl})}{(660\ \text{g/ mol} \times 668\ \text{bp})} \times 10\text{^}6 = 193.3\ \text{nM}$$

materials

Click here to download Materials Table Materials Table_Fuller.xlsx

Name of Material/ Equipment	Company	Catalog Number
Teklad Diets/Harlan Labs Chow	Harlan Labs	3980X
Cell-Sorb Plus bedding	Fangman Specialties	82010
AIMS Tattooing System For Neonates	AIMS	NEO-9
Zyfone One Cage 2100 AllerZone Mouse Micro-Isolator System Complete with cage, AllerZone filter top and modular diet delivery system	Lab Products	82120ZF
Zyfone One Cage 2100 Life Span Enrichment Device	Lab Products	82109ZF
Zyfone One Cage 2100 Cage 13-7/8" Length X 19-1/16" Width X 7-3/4" Depth	Lab Products	82100ZF
Zyfone One Cage 2100 AllerZone Micro-Isolator filter top	Lab Products	82101ZF
Tunnel	Bio-Serv	K3323 or K3332
Grommet to connect Tunnel to cages	Fabricated by the University of Utah Machine Shop	n/a
Fast-track wheel	Bio-Serv	K3250 or K3251
Mouse Igloo	Bio-Serv	K3328, K3570 or K3327

Mouse Igloo floor	Bio-Serv	K3244
Mouse Hut	Bio-Serv	K3272, K3102 or K3271
Crawl Ball	Bio-Serv	K3330 or K3329
Bio-hut	Bio-Serv	K3352
Adhesive film	VWR	60941-072
Laminar Flow Ventilated Rack	Techniplast	Bio-C36
1.5 mL Microfuge Tube- RNAse and DNAse free	Any supplier	
QIAamp DNA Stool MiniKit	Qiagen	51504
Waterbath (capable of heating to 95)	Any supplier	
Waterbath (capable of heating to 70 degrees)	Any supplier	
Ethanol (200 proof)	Sigma Aldrich	E7023
Fluorometer: Qubit	ThermoFisher Scientific	Q33216
Qubit dsDNA broad Range Assay Kit	ThermoFisher Scientific	Q32850
EB Buffer or 10 mM Tris pH 8.5	Qiagen	19086
Experiment specific primers	Any Supplier	
PCR grade water	Any supplier	
2X KAPA HiFi HotStart Ready Mix	Kapa Biosystems	KK2601

Agarose for running diagnostic gels	Any supplier	
TapeStation High Sensitivity D1000 Screen Tape Trace	Agilent	5067-5583
Agencourt AMPure XP Magnetic Beads	Beckman Coulter	A63880
Magnetic stand	Life Technologies	AM10027
Library Preparation Guide	Illumina	
Unique Dual Indexing	Illumina	Illumina Experiment Manager Software
Nextera XT 96 Index Kit	Illumina	FC-131-1002
MicroAmp Optical 96-well reaction plate	Applied Biosystems/ThermoFisher	N8010560
TruSeq Index Plate Fixture	Illumina	FC-130-1005
Adhesive clear plate seal	Applied Biosystems /ThermoFisher	4360954
Sequencing by MiSeq with v3 reagents and dual 300 bp reads	Illumina	MS-102-3003

PhiX Control Kit	Illumina	FC-110-3001
Proteinase K (600 mAU/ml)	Qiagen	19131
Data Analysis Tools	Qiime	QIIME software Tools
Step 13.2.	Qiime	FastQ Join method
Step 13.3.	Qiime	De-Novo OTU picking protocol
Step 13.3.1.		Open Taxonomic Units (OTUs) using Uclust
Step 13.3.1.	Pynast	Pynast
Step 13.3.1.	Pynast	Pynast_Greengenes

13.3.1. Note:	Qiime	Multiple Split Libraries
13.3.1. Note:	Qiime	Pick de novo OTUs script
Step 13.2.2.	Qiime	Create a mapping file
Step 13.2.2.	Qiime	Validate a mapping file
Step 13.3.3.	Qiime	Link the OTU to sample description to mapping file
Step 13.3.4.	Qiime	Summarize Taxa through plots
Step 13.3.5.	Qiime	Biome Summarize table

Comments/Description
Standard irradiated chow formulated by Dr. Mario Capecchi in collaboration with Harlan Labs.
Autoclave prior to use.
https://animalid.com/neonate-rodent-tattoo-identification/32. Other animal grade tattoo systems and inks can be used with similar results including the Aramis Micro Tattoo Kit.
Each EE cage requires one of each catalog # 82120ZF, 82100ZF, and 82101ZF, as well as two of 82109ZF. Food is only in one side.
Each EE cage requires one of each catalog # 82120ZF, 82100ZF, and 82101ZF, as well as two of 82109ZF. Food is only in one side.
Each EE cage requires one of each catalog # 82120ZF, 82100ZF, and 82101ZF, as well as two of 82109ZF. Food is only in one side.
Each EE cage requires one of each catalog # 82120ZF, 82100ZF, and 82101ZF, as well as two of 82109ZF. Food is only in one side.
Connect cages together and use for enrichment
Be certain the material is resistant to chewing and autoclavable
Use with mouse igloo and floor
Use with Fast-track wheel and floor

Use with mouse Igloo and Fast-Track

Wood pulp hut used for sheltering and nesting
Use to temporarily cover drilled hole in large cage to prevent mice from escaping
The cabinet we used in this study is not currently supplied. The Bio-C36 is very similar.

This kit supplies reagents for 50 DNA preparations. Stool Lysis Buffer=ASL; Guanidinium Chloride Lysis Buffer= AL; Wash Buffer 1 with Guanidinium Chloride= AW1; Wash Buffer 2= AW2; Elution Buffer with EDTA=AE

For 94 degree incubation of stool samples to lyse cells.

For 70 degree incubation of stool samples

For Amplicon Amplification (1.25 mL allows 100 rxns).

TapeStation or Bioanalyzer instruments are common in Institutional Genomics Cores to analyze library quality . Alternatively a Bioanalyzer DNA1000 Chip (Agilent, 5067-1504) can be used.
Magentic beads For PCR cleanup- 5 mL will clean 250 PCR reactions

Illumina. 16S Metagenomic Sequencing Library Preparation: Preparing 16S ribosomal RNA Gene Amplicons for the Illumina MiSeq System. https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf.

Freely available at: https://support.illumina.com/sequencing/sequencing_software/experiment_manager/downloads.html
Used to add barcodes to amplicons

Applied Biosystems/ThermoFisher Microamp adhesive film

Equivalent to 20 mg/ml of proteinase K. Supplied with QiaAmp kit

Installation may differ based on your system and the QIIME website describes several options (http://qiime.org/install/install.html). For this study, MacQIIME software package 1.9.1 was utilized (compiled by Werner Lab, SUNY, http://www.wernerlab.org/software/macqiime

(http://code.google.com/p/ea-utils). For this study Multiple join paired ends was used http://qiime.org/scripts/multiple_join_paired_ends.html. Aronesty, E. ea-utils: Command-line tools for processing biological sequencing data. Expression Analysis, Durham, NC. (2011).

http://qiime.org/scripts/pick_de_novo_otus.html.

Edgar, R.C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics.* **26** (19), 2460-2461, doi:10.1093/bioinformatics/btq461 (2010).

Caporaso, J.G. et al. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics. 26 (2), 266-267, doi:10.1093/bioinformatics/btp636 (2010).

DeSantis, T.Z. et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 72 (7), 5069-5072, doi:10.1128/AEM.03006-05 (2006). Greengenes version 13_8 was used in this study

http://qiime.org/scripts/multiple_split_libraries_fastq.html.

http://qiime.org/scripts/pick_de_novo_otus.html

http://qiime.org/documentation/file_formats.html.

http://qiime.org/scripts/validate_mapping_file.html.

http://qiime.org/scripts/make_otu_network.html.

http://qiime.org/scripts/summarize_taxa_through_plots.html.
http://biom-format.org/documentation/summarizing_biom_tables.html In this study, all samples were rarified to 20,000 OTUs followed by analysis using alpha rarefaction script in QIIME.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: A method to define the effects of Environmental Enrichment on Colon microbiome biodiversity in a colon tumor mouse model

Author(s): Andrew K. Fuller, Benjamin D. Bica, Ashlee R. Venancio, Amber M. Staab, Stephanie J. Georges, Julio R. Hidalgo, Annika R. Warncke, Melinda L. Angus-Hill

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: Melinda L. Angus-Hill

Department: Dept. of Medicine

Institution: Huntsman Cancer Institute / University of Utah

Article Title: A method to define the effects of Environmental Enrichment on colon microbiome biodiversity in a Mouse Colon tumor model

Signature: [signature] Date: 8/21/17

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Editorial comments:

1. The editor has formatted the manuscript to fit journals style. Please retain the same.

We have retained the formatting. The numbers along the side are mostly correct, but some numbers are skipped between pages. I have no idea how to fix this problem.

The references were reformatted by endnote but were changed to be in the same style as the manuscript.

The affiliations part of the manuscript combined Gastroenterology with Huntsman cancer institute. These were separarated (this is accurate as they are not the same entities).

2. The editor has brought down the highlight to fit 3 pages as of now, please try to work on it and bring it down to 2.75 pages which is a hard cut limit for filming.

Thanks for the help with this for the last revision. We have unhighlighted more, and by my calculation, keeping formatting, this is now between 2.5 and 2.75.

3. Please remove all commercial language from the manuscript. Even if the free version of a software is used, we cannot publish the names since these are trademark and commercial.

All commercial language is removed as directed in the manuscript.

4. Please address specific comments marked in the manuscript attached.

All comments are addressed in the manuscript.

JOURNAL PUBLISHING AND LICENSING AGREEMENT

Elsevier Inc.

Article:	**Environmental Enrichment Induces Pericyte and IgA-Dependent Wound Repair and Lifespan Extension in a Colon Tumor Model**
Corresponding author:	**Dr. Melinda L. Angus-Hill**
E-mail address:	**melinda.angus-hill@hci.utah.edu**
Journal:	**Cell Reports**
Our reference	**CELREP3732**
PII:	**S2211-1247(17)30481-3**
DOI:	**10.1016/j.celrep.2017.04.006**

YOUR STATUS

» I am one author signing on behalf of all co-authors of the manuscript

DATA PROTECTION & PRIVACY

» I do wish to receive news, promotions and special offers about products and services from Elsevier Inc. and its affiliates worldwide

LICENSE OF PUBLISHING RIGHTS

I hereby grant to Elsevier Inc. (hereinafter the "Journal") an exclusive publishing and distribution license in the manuscript identified above and any tables, illustrations or other material submitted for publication as part of the manuscript (the "Article") in print, electronic and all other media (whether now known or later developed), in any form, in all languages, throughout the world, for the full term of copyright, and the right to license others to do the same, effective when the Article is accepted for publication. This license includes the right to enforce the rights granted hereunder against third parties.

SUPPLEMENTAL MATERIALS

With respect to Supplemental Materials that I wish to make accessible through a link in the Article or on a site or through a service of Elsevier Inc., Elsevier Inc. shall be entitled to publish, post, reformat, index, archive, make available and link to such Supplemental Materials on a non-exclusive basis, in all forms and media (whether now known or later developed) and permit others to do so. "Supplemental Materials" shall mean additional materials that are not an intrinsic part of the Article, including but not limited to experimental data, e-components, encodings and software, and enhanced graphical, illustrative, video and audio material.

SCHOLARLY COMMUNICATION RIGHTS

I understand that I retain the copyright in the Article and that no rights in patents, trademarks or other intellectual property rights are transferred to the Journal. As the author of the Article, I understand that I shall have: (i) the same rights to reuse the Article as those allowed to third party users of the Article under the CC BY-NC-ND License, as well as (ii) the right to use the Article in a subsequent compilation of my works or to extend the Article to book length form, to include the Article in a thesis or dissertation, or otherwise to use or re-use portions or excerpts in other works, for both commercial and non-commercial purposes. Except for such uses, I understand that the license of publishing rights I have granted to the Journal gives the Journal the exclusive right to make or sub-license commercial use.

USER RIGHTS

The publisher will apply the *Creative Commons Attribution-Noncommercial-NoDerivative Works 4.0 International License* (CC BY-NC-ND) to the Article where it publishes the Article in the journal on its online platforms on an Open Access basis. For further information, see http://www.elsevier.com/about/open-access/open-access-options.http://www.elsevier.com/about/open-access/open-access-options.

The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0.http://creativecommons.org/licenses/by-nc-nd/4.0.

REVERSION OF RIGHTS

Articles may sometimes be accepted for publication but later rejected in the publication process, even in some cases after public posting in "Articles in Press" form, in which case all rights will revert to the author. See http://www.elsevier.com/locate/withdrawalpolicy. http://www.elsevier.com/locate/withdrawalpolicy.

REVISIONS AND ADDENDA

I understand that no revisions, additional terms or addenda to this License Agreement can be accepted without's express written consent. I understand that this License Agreement supersedes any previous agreements I have entered into with the Journal in relation to the Article from the date hereof.

COPYRIGHT NOTICE

The publisher shall publish and distribute the Article with the appropriate copyright notice.

AUTHOR REPRESENTATIONS / ETHICS AND DISCLOSURE / SANCTIONS

I affirm the Author Representations noted below, and confirm that I have reviewed and complied with the relevant Instructions to Authors, Ethics in Publishing policy, Declarations of Interest disclosure and information for authors from countries affected by sanctions (Iran, Cuba, Sudan, Burma, Syria, or Crimea). Please note that some journals may require that all co-authors sign and submit Declarations of Interest disclosure forms. I am also aware of the publisher's policies with respect to retractions and withdrawal (http://www.elsevier.com/locate/withdrawalpolicy).I affirm the Author Representations noted below, and confirm that I have reviewed and complied with the relevant Instructions to Authors, Ethics in Publishing policy, Declarations of Interest disclosure and information for authors from countries affected by sanctions (Iran, Cuba, Sudan, Burma, Syria, or Crimea). Please note that some journals may require that all co-authors sign and submit Declarations of Interest disclosure forms. I am also aware of the publisher's policies with respect to retractions and withdrawal (http://www.elsevier.com/locate/withdrawalpolicy).
For further information see the publishing ethics page at http://www.elsevier.com/publishingethics and the journal home page. For further information on sanctions, see https://www.elsevier.com/trade-sanctionsFor further information see the publishing ethics page at http://www.elsevier.com/publishingethics and the journal home page. For further information on sanctions, see https://www.elsevier.com/trade-sanctions

Author representations

- The Article I have submitted to the journal for review is original, has been written by the stated authors and has not been previou
- The Article was not submitted for review to another journal while under review by this journal and will not be submitted to any oth
- The Article and the Supplemental Materials do not infringe any copyright, violate any other intellectual property, privacy or other r entity, or contain any libellous or other unlawful matter.
- I have obtained written permission from copyright owners for any excerpts from copyrighted works that are included and have cre the Article or the Supplemental Materials.
- Except as expressly set out in this License Agreement, the Article is not subject to any prior rights or licenses and, if my or any of institution has a policy that might restrict my ability to grant the rights required by this License Agreement (taking into account th communication rights permitted hereunder), a written waiver of that policy has been obtained.
- If I and/or any of my co-authors reside in Iran, Cuba, Sudan, Burma, Syria, or Crimea, the Article has been prepared in a persona capacity and not as an official representative or otherwise on behalf of the relevant government.
- If I am using any personal details or images of patients, research subjects or other individuals, I have obtained all consents requir and complied with the publisher's policies relating to the use of such images or personal information. See http://www.elsevier.com for further information.If I am using any personal details or images of patients, research subjects or other individuals, I have obta required by applicable law and complied with the publisher's policies relating to the use of such images or personal information. Se http://www.elsevier.com/patientphotographs for further information.
- Any software contained in the Supplemental Materials is free from viruses, contaminants or worms.
- If the Article or any of the Supplemental Materials were prepared jointly with other authors, I have informed the co-author(s) of th Agreement and that I am signing on their behalf as their agent, and I am authorized to do so.

For information on the publisher's copyright and access policies, please see http://www.elsevier.com/copyright.For information on the publisher's copyright and access policies, please see http://www.elsevier.com/copyright.

☑ **I have read and agree to the terms of the License Agreement.**

7th April 2017 T-copyright license-v4/2016

Click here to access/download

Supplemental Coding Files

Supplemental Table 1_ Fuller et al.xlsx