Protocol for Metatranscriptomic analysis of Intestinal Microbiota.

Valeriy Poroyko (1), Alvaro G. Hernandez (2), John C. Alverdy(1), Donald C. Liu(1)
1 Department of Surgery, The University of Chicago, Chicago, IL
2 University of Illinois, Champaign, IL

Abstract

The objective of this publication is to provide the detailed protocol for metartancriptomisc studies of animal intestinal microbiota. The protocol describes isolation of high quality microbial community RNA from the mammalian intestinal content, subsequent mRNA enrichment, cDNA synthesis and sequencing. Twelve libraries were prepared pooled in equimolar concentrations into a single library and sequenced on one GS Titanium 70×75 picotiter plate, following this protocol. The total number of reads obtained for 12 libraries was 1,155,062 (average 96,000 per library) and the combined size of 12 libraries was 521 million bases (average 43 million bases per library). The reported size of non-ribosomal RNA library fraction is ~15%, the fraction of non-ribosomal reads is ~17%. Hence we described a robust technique for metranscriptomic studies of animal intestinal microbiota. The double stranded cDNAs, prepared following this protocol, are suitable for pyrosequencing (454, Illumina), clone library construction or could be used to archive and store metaranscriptomic samples.

1. Introduction

One of the stated goals of the Human Microbiome Project is not only characterize bacterial "community genomes", but also determine their corresponding messenger RNAs, proteins and metabolic products (Turnbaugh et al., 2007). Transcriptomics, study of the complete set of RNA transcripts produced by the genome at any one time, is thus an essential approach complementary to DNA-based meta-genomics (Bailly et al., 2007). Several attempts have been already made to study community gene expression using environmental RNA samples (Poretsky et al., 2005, Frias-Lopez et al., 2008) including the large scale pyrosequencing study of bacterial mRNA from an environmental marine sample (Frias-Lopez et al., 2008). The proposed protocol in this report describes in detail isolation of high quality microbial community RNA from the mammalian intestinal content, subsequent cDNA synthesis and sequencing. The constructed library is suitable for pyrosequencing (454, Illumina) or clone library construction. We have successfully utilized this protocol in pig animal model (Poroyko et al., 2010) using 454 Titanium pyrosequencing platform.

In this protocol we consider the importance of swift sample processing (Gilbert et al., 2008) to preserve the integrity of mRNA profiles. It was, also, found practical to avoid usage of RNA preserving reagents considering that the time required for reagent to penetrate bacterial cell walls has been shown to be sufficient for some species to undergo sporulation (Kenefic, 2006). The sampling protocol suggests harvesting the entire cecum and snap-freezing it within 10 minutes after the animal was sacrificed to assure minimal time of exposure to extracorporeal oxygen and protect against a drop of temperature.

The protocol is based on combination of 3 kits. The total bacterial RNA isolation was performed using the "RiboPure-Bacteria Kit" (#AM1925; Ambion, TX). This kit, tested on different bacterial species including *Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Yersinia pestis, Bacillus anthracis*

Rhodobacter sphaeroides, has shown high efficiency (Applied Biosystems 2004, Xu Y, et al., 2003, Drysdale M, et al., 2004, Cheng HP, Yao SY. 2004, Zhang D. et al., 2004, Prithiviraj B. et al., 2005, Di Cello F, et al., 2005). The "MICROBExpress Bacterial mRNA Purification Kit" (Ambion, TX) was used to deplete the pool of 16S and 23S rRNA molecules present in the sample. The consistent yield of mRNA makes RNA amplification unnecessary and allows usage of standard random primer cDNA synthesis protocol implemented in Double-Stranded cDNA Synthesis Kit (Invitrogen).

2 Detailed protocol

The usual precautions should be maintained while working with RNA. Use gloves, clean the equipment and work surfaces with RNaseZap.

2.1. Material general: latex gloves, RNaseZap (Ambion #AM9780), 100% ethanol, barrier nuclease free tips (10 ul, 100 ul, 1000ul), RNase-free Microfuge Tubes (1.5 ml) (Ambion, AM12400), RNase-free Microfuge Tubes (2.0 ml) (Ambion, AM12425), refrigerated and not refrigerated tabletop centrifuges, refrigerator +4^{0}C, freezers -20 ^{0}C, -70 ^{0}C. RNA concentration is determined by Nanodrop (Termo Scientific Inc., DE). RNA quality is measured by Agilent 2100 Bioanalyser (Agilent Technologies, CA).

2.2. Sample harvesting

Materials: portable Dewar flask, liquid nitrogen, 50ml sterile plastic centrifuge tubes, surgical equipment

1) Sacrifice the animal according to appropriate protocol.
2) Open abdominal cavity and harvest entire cecum (Fig 1A); place cecum in to 50 ml plastic centrifuge tube and immediately freeze in liquid nitrogen.
3) Store samples at -70C^0.

2.3 RNA isolation

Materials: portable Dewar flask, liquid nitrogen, Styrofoam container with dry ice, sterilized chilled pruner, handheld drill with 1/32 engraving cutter (Dremel), protective gaggles, Mini-Beadbeater-16 (BioSpec Products Inc., OK), RiboPure – Bacteria Kit (#AM1925; Ambion), dray bath 95 ^{o}C.

1) Remove samples from -70^{o}C storage and place in Dewar flask with liquid nitrogen. Carry the samples to workplace.
2) Prepare the 0.5 mL screw cap tubes with 250 ul Zirconia Beads (provided with the kit) as described in kit protocol step C1. Chill the tubes by placing them on dry ice.
3) Using prechilled sterile pruner cut the frozen cecum (Fig. 1B) in discs ~0.5-1cm of thickness. Remove the residual muscle tissues using pruner and handheld drill. Keep the sample deeply frozen during the procedure by dipping it in liquid nitrogen.
4) Cut the sample of cecal content in pieces and transfer the clamps of frozen sample (~150 ug) to the prechilled tube with Zirconia Beads (from step 2).
5) Add 350 ul RNAwiz to the sample, cap the tube. Place tube on dry ice.
6) Put frozen tubes in Mini-Beadbeater-16 (BioSpec Products Inc., OK). Beat cells: 2 rounds of 5 min beating.
7) Continue according to the **"**RiboPure – Bacteria Kit" (Ambion) kit protocol starting at the step **C6**.

It is essential to perform DNAse treatment as described in steps E1-E5 of the kit manual.

One preparation usually yields of 6-80 ug of total RNA in 100ul of EB buffer. It is recommended to perform 2 preparations for the sample and combine them to achieve higher yield. At this point is possible to stop and freeze RNA sample, store at -70 ^{o}C. Continue with RNA precipitation.

2.4. RNA precipitation

Materials: UltraPure Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) (Invitrogen), 3M sodium acetate (pH 5.5) and glycogen are parts of MICROB Express Bacterial mRNA purification Kit (#AM1905; Ambion)

1) Add the volume of UltraPure Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) (Invitrogen) equal to the volume of combined RNA samples (~200 ul). Mix by vortexing.
2) Centrifuge 5 min at maximum speed in bench top centrifuge. Collect supernatant in new 1.5 ml tube.
3) Precipitate the RNA by adding 0.1 volume of 3M sodium acetate (or 5M ammonium acetate), 5 ug glycogen, 3 volumes of 100% ethanol. Mix by vortexing.
4) Incubate the mixture overnight at -20 ^{o}C or 30-60 min at -70 ^{o}C.
5) Recover RNA by centrifugation at maximum speed in bench-top centrifuge for 30 minutes at 4 ^{o}C. Carefully remove and discharge supernatant.
6) Add 1 ml of ice cold 70% ethanol. Pellet the RNA by centrifuging at maximum speed for 10 min at 4 ^{o}C. Carefully remove and discharge supernatant.
7) Repeat step 6.
8) Briefly centrifuge the tube, aspirate any additional fluid and air-dry the sample for 10 minutes.
9) Dissolve the RNA in 15 ul TE pH 8.0. Take 2 ul aliquot to measure RNA concentration (2 ul) and quality (1 ul). Store sample at -70 ^{o}C.

2.5. mRNA purification.

Materials: Styrofoam container with ice, water bath 70 ^{o}C and 37 ^{o}C, MICROB Express Bacterial mRNA purification Kit (#AM1905; Ambion)

Perform the mRNA enrichment as described in kit protocol steps B1 – E3 using 10 ug of total RNA. Take aliquots to measure RNA concentration and quality. Store sample at -70 ^{o}C. Then the desired amount of mRNA (3 ug) is acquired continue with Double stranded cDNA synthesis, otherwise perform additional mRNA purification and combine samples until the right amount of mRNA is acquired, precipitate mRNA, measure RNA concentration and quality to monitor the enrichment procedure.

2.6. Double-Stranded cDNA Synthesis

Material: Styrofoam container with ice, water bath 70^{o}C, 45^{o}C, 16^{o}C, SuperScript™ Double-Stranded cDNA Synthesis Kit (Cat. No. 11917-010 (Invitrogen)).

Perform cDNA synthesis according to instruction provided with the kit. Use 3 ug of mRNA and 250 ng of random primer. Then the protocol accomplished dissolve library in 10 ul of TE pH 8.0. Store at -70 ^{o}C. At this point, the library is in a stable form and could be stored or used for sequencing.

2.7. Library size selection (optional)

In order to facilitate the efficiency of 454 sequencing platform it is recommended to perform size selection and enrich library for the longer reads.

Materials: TAE electrophoresis buffer, electrophoresis apparatus, low melting point agarose, 100bp DNA ladder (Invitrogen) or similar, Glucogen (#AM9510; Ambion), β-Agarase (New England BioLabs) water baths 65^{o}C and 42^{o}C.

1) Prepare 1% agarose gel using TAE buffer and low melting temperature agarose.
2) Mix the library with the appropriate volume of loading buffer and load the entire sample on a gel. Run the electrophoresis. Cut the region of 250-750bp (Region of 800bp-10kb could be collected as well). (Fig 2).
3) Weight the gel containing DNA.
4) Add 1/10 v/w of 10x β-Agarase I buffer and incubate mixture at 65^{o}C for 10 minutes or until the gel melts completely.
5) Transfer the tube with melted sample to 42 ^{o}C water bath. Let the temperature equilibrate.
6) Add 2 units of β-Agarase I enzyme per each 200 ug of 1% gel. Incubate 1 hour at 42^{o}C
7) Add 0.1 volume of 3M sodium acetate. Mix well. Chill on ice for 15minutes.
8) Centrifuge at maximum speed fro 15 minutes to pellet undigested gel.
9) Remove supernatant. Add 5 ug of glycogen and 2 volumes of ethanol mix by vortexing.
10) Incubate the mixture overnight at -20 ^{o}C or 30-60 min at -70 ^{o}C.
11) Precipitate by centrifugation at maximum speed for 30 minutes at 4 ^{o}C. Carefully remove and discharge supernatant.
12) Add 1 ml of ice cold 70% ethanol. Re pellet the library by centrifuging for 10 min at 4 ^{o}C. Carefully remove and discharge supernatant.
13) Repeat step 12.
14) Centrifuge the tube briefly, aspirate any additional fluid and air dry the sample for 10 minutes.
15) Resuspend the pellet in 23 ul TE buffer. Take aliquots to determine the library DNA concentration and quality. Store library at -70 ^{o}C.

At this point double stranded library is ready for sequencing using the method of choice.

2.8. Guideline for library multiplexing and sequencing (454 Titanium platform).

Use approximately 1 ug of size selected cDNAs to blunt-end adaptor ligation and conversion to a single-stranded template DNA library using the GS Titanium General Library Prep Kit (Roche Applied Science, Indianapolis, IN). Prepare libraries using barcode-containing adaptors in place of the standard Titanium adaptors, following Roche's instructions for preparation barcoded adaptors. Quantify libraries using Qubit reagents (Invitrogen, CA) and determine average fragment sizes by analyzing 1 µl of the sized cDNA samples on the Bioanalyzer (Agilent, CA) using a DNA 7500 chip. Pool the libraries in equimolar concentration into a single library. Process emulsion PCR, titration and sequencing on a GS FLX following the manufacturer's protocols (Roche Applied Science, Indianapolis, IN). Library reads could be sorted by barcode using SFF software tools, which also trims the barcode sequence in each read after sorting (Roche Applied Science, Indianapolis, IN).

3. Conclusion

Twelve libraries were constructed according to this protocol, pooled in equimolar concentration into a single library and sequenced using one GS Titanium 70×75 picotiter plate. The total number of reads obtained for 12 libraries was 1.155,062 and the combined size of 12 libraries was 521 mega bases. Table 1 shows the number of reads per library tag, the size of each library, the number of non-ribosomal RNA reads and the size of non-ribosomal libraries, as determined by MG-RAST (Meyer F., et al., 2008). The criteria for ribosomal RNA filtering are described previously (Poroyko et al., 2010).

Here we described the robust technique for metranscriptomic studies of animal intestinal microbiota. Although we only used Titanium platform to sequence 12 test libraries, the double stranded cDNA is a stable intermediate that is universally suitable for multiple sequencing platforms (such as Sanger capillary sequencing, Illumina, 454). It could thus be processed using variety of methods. The universal form of double stranded cDNA could also be used to archive and store metaranscriptomic samples in anticipation of advanced sequencing techniques that may emerge in the future.

Reference:

Applied Biosystems/Ambion. (2004) Bacterial Whole Genome Array Analysis. TechNotes 11(4) *Applied Biosystems/Ambion*. (2004), http://www.ambion.com/techlib/tn/114/8.html

Bailly et al., 2007 Bailly J, Fraissinet-Tachet L, Verner MC, Debaud JC, Lemaire M, Wésolowski-Louvel M, Marmeisse R. Soil eukaryotic functional diversity, a metatranscriptomic approach. *ISME J* 1 (2007), pp.632–642.

Cheng and Yao, 2004 Cheng HP, Yao SY The key Sinorhizobium meliloti succinoglycan biosynthesis gene exoY is expressed from two promoters. *FEMS Microbiol Lett* 231(1), (2004) pp.131-136

Di Cello et al., 2005 Di Cello F, Xie Y, Paul-Satyaseela M, Kim KS Approaches to bacterial RNA isolation and purification for microarray analysis of *Escherichia coli* K1 interaction with human brain microvascular endothelial cells. *J Clin Microbiol* 43(8), (2005), pp.4197-4199

Drysdale et al., 2004 Drysdale M, Bourgogne A, Hilsenbeck SG, Koehler TM atxA controls *Bacillus anthracis* capsule synthesis via acpA and a newly discovered regulator, acpB. *J Bacteriol* 186(2) (2004), pp.307-315

Frias-Lopez et al., 2008 Frias-Lopez J, Shi Y, Tyson GW, Coleman ML, Schuster SC, Chisholm SW, Delong EF. Microbial community gene expression in ocean surface waters. *Proc Natl Acad Sci U S A* 105(2008), pp.3805–3810.

Gilbert et al., 2008 Gilbert JA, Field D, Huang Y, Edwards R, Li W, Gilna P, Joint I. Detection of Large Numbers of Novel Sequences in the Metatranscriptomes of Complex Marine Microbial Communities. *PLoS ONE* 3(8) (2008), e3042. doi:10.1371/journal.pone.0003042

Kenefic 2006 Kenefic L. RiboPure™-Bacteria Kit From Ambion (2006), http://www.biocompare.com/Articles/ProductReview/514/RiboPure-Bacteria-Kit-From-Ambion.html

Meyer et al., 2008 Meyer F., Paarmann D., D'Souza M., Olson R., Glass E. M., Kubal M., Paczian T., Rodriguez A., Stevens R., Wilke A., Wilkening J., Edwards R. A. The Metagenomics RAST server - A public resource for the automatic phylogenetic and functional analysis of metagenomes *BMC Bioinformatics* 9 (2008), p386

Poretsky et al., 2005 Poretsky RS, Bano N, Buchan A, LeCleir G, Kleikemper J, Pickering M, Pate WM, Moran MA, Hollibaugh JT. Analysis of microbial gene transcripts in environmental samples. *Appl Environ Microbiol* 71: (2005) pp.4121–4126.

Poroyko et al., 2010 Poroyko V, White JR, Wang M, Donovan S, Alverdy J, Liu DC, Morowitz MJ. Gut Microbial Gene Expression in Mother-Fed and Formula-Fed Piglets. *PLoS ONE* 5(8), (2010) e12459. doi:10.1371/journal.pone.0012459

Prithiviraj et al., 2005 Prithiviraj B, Bais HP, Weir T, Suresh B, Najarro EH, Dayakar BV, Schweizer HP, Vivanco JM Down regulation of virulence factors of *Pseudomonas aeruginosa* by salicylic acid attenuates its virulence on *Arabidopsis thaliana* and *Caenorhabditis elegans*. *Infect Immun* 73(9), (2005), pp.5319-5328

Turnbaugh et al., 2007 Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. *Nature.* 449(7164) (2007), pp.804-10.

Urich et al., 2008 Urich T, Lanzén A, Qi J, Huson DH, Schleper C, Schuster SC. (2008) Simultaneous Assessment of Soil Microbial Community Structure and Function through Analysis of the Meta-Transcriptome. *PLoS ONE* 3(6) (2008), e2527. doi:10.1371/journal.pone.0002527

Xu et al., 2003 Xu Y, Mori T, Johnson CH. Cyanobacterial circadian clockwork: roles of KaiA, KaiB and the kaiBC promoter in regulating KaiC. *EMBO J.* 22(9) (2003), pp.2117-2126

Zhang et al., 2004 Zhang D, Manos J, Ma X, Belas R, Karaolis DK Transcriptional analysis and operon structure of the tagA-orf2-orf3-mop-tagD region on the Vibrio pathogenicity island in epidemic *V. cholerae*. *FEMS Microbiol Lett* 235(1), (2004) pp.199-207

Table 1 Library statistics

Library	MID Barcode	Number of reads	Size (bp)	Number of non-ribosomal RNA reads	Size of non-ribosomal RNA library (bp)
1	ACGAGTGCGT	97,379	42,875,895	18,066	7,044,627
2	ACGCTCGACA	109,740	51,003,969	13,924	5,689,712
3	AGACGCACTC	90,289	42,014,418	26,002	10,724,268
4	AGCACTGTAG	108,960	49,984,860	30,259	12,284,898
5	ATCAGACACG	87,406	40,825,289	19,556	8,131,577
6	ATATCGCGAG	87,257	39,716,120	24,865	9,996,482
7	CGTGTCTCTA	85,377	39,152,795	20,398	8,362,105
8	CTCGCGTGTC	90,230	41,224,534	7,700	3,040,174
9	TAGTATCAGC	93,935	42,253,063	17,803	7,275,400
10	TCTCTATGCG	93,073	42,481,079	6,196	2,494,900
11	TGATACGTCT	97,970	42,509,278	7,486	2,852,418
12	TACTGAGCTA	113,446	47,779,839	8,642	3,081,525

Figure 1. The piglet cecum: (A) Immediately after autopsy; (B) Frozen cecum cut open.

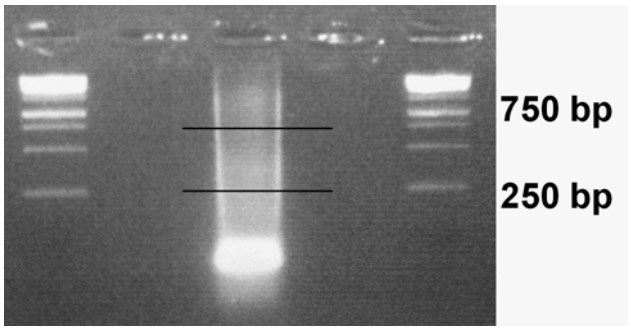

Figure 2. The cDNA of bacterial community metatranscriptome prior size selection. Region of interest 250-750bp is marked by lines. The TAE 1% agarose gel is used.