Running Title

High-throughput analysis of microbial communities

Title

Methods for high-throughput comparative analyses of natural microbial communities

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Abstract

One of the most commonly applied metagenomics approaches is the amplification and sequencing of the highly conserved ribosomal RNA (rRNA) genes from organisms in a complex microbial community. Ribosomal RNA surveys, typically using the 16S rRNA gene for prokaryotic identification, provide information about the total diversity and taxonomic affiliation of organisms present in a sample. We cover the creation of 16S rRNA metagenomic surveys of microbial communities using high-throughput techniques. It is meant to be an outline for those not familiar with 16S rRNA surveys using Illumina technology, and highlights some important considerations in study design and implementation. We begin by outlining the best

practices for minimizing artifacts and errors during library construction. We address issues specific to Illumina short read and paired-end data.

Introduction

This chapter will outline the molecular methods used in the Alm lab to identify and compare microbial communities using high-throughput sequencing technologies, specifically the Illumina platforms (San Francisco, CA). We will outline our methods for library construction.

Library construction

Identifying microorganisms present in a natural community using a sequencing-based 16S rRNA survey begins with the construction of a library. A library is a collection of DNA fragments that represents the sequence diversity in a sample. These fragments are enriched from the rest of the community genomic DNA by PCR using primers which match to the microbial population or gene of interest. However, these sequences must be manipulated in a platform-specific way for sequencing. The complete molecular construct contains the genomic DNA sequences from the enrichment reaction, sequences that identify the sample it originated from (i.e. index or barcode sequences) and sequences required by the platform to adhere library fragments to the solid matrix and provide a priming site for the sequencing reaction (Fig. 1). Adding a barcode sequence to the molecular construct identifying which sample the library originated from allows for hundreds of libraries to be sequenced in the same reaction, commonly called multiplexing.

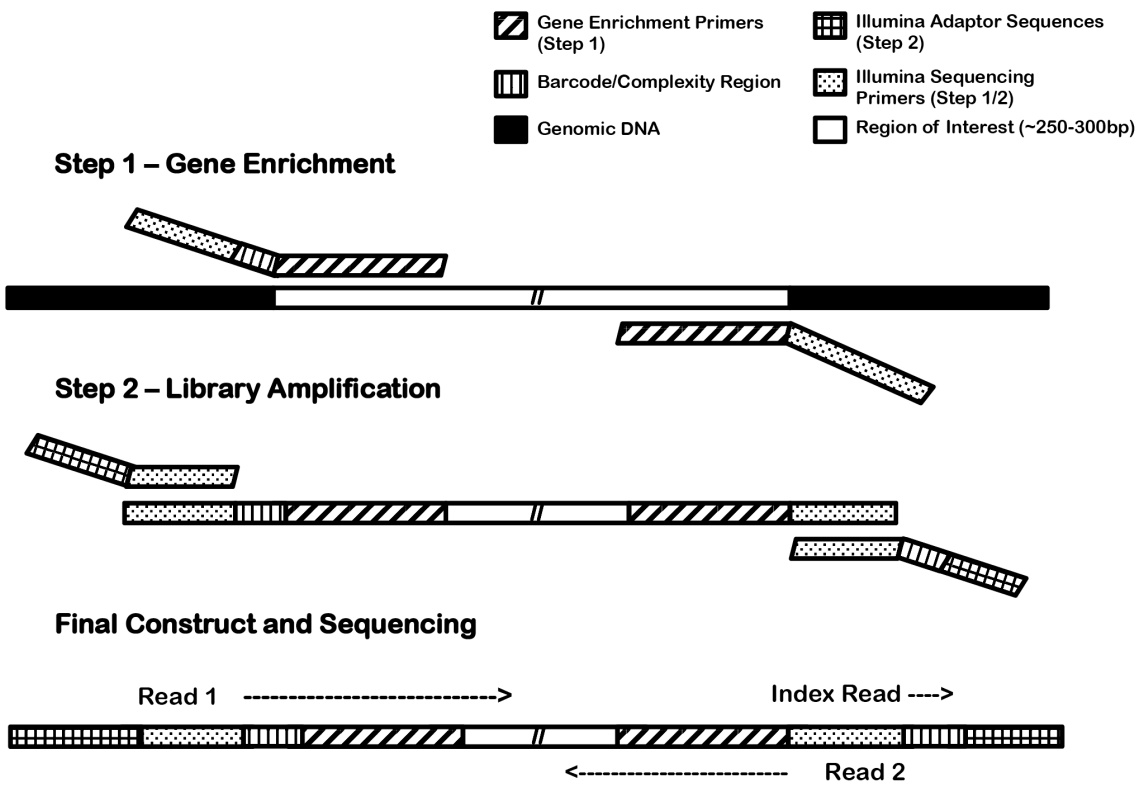

Figure 1. Overview of two-step 16S rRNA library construct. The gene enrichment step (Step 1) involves amplification of genomic DNA of interest (i.e. 16S rRNA gene) using region-specific primers. Library amplification (Step 2) involves the addition of the barcode or indexing sequence, which should be unique for each library. The final construct includes all of the adapter and cluster binding sites required by Illumina sequencing (hatched), along with sequencing primer binding sites (dotted) and the region of genomic DNA that was enriched (white).

There can be multiple different designs for these molecular constructs, depending on the researchers needs and priorities. One approach maximizes useable read lengths by using the same primer for both amplification and sequencing (Knight et al., 2011). We have developed an alternative approach which maximizes the flexibility of the library construct by separating the amplification of genomic DNA of interest from the addition of Illumina-specific adapters and indexing sequences (Blackburn, 2010). With this two-step PCR approach, the same indexing oligos can be used to sequence multiple different amplicons (e.g. different areas of the 16S rRNA gene or other functional genes). For example, a 96-well plate of synthesized oligos, each containing a unique index sequence can be used to sequence both bacterial 16S and eukaryotic 18S sequences. Additionally, the first few bases that are read by the sequencer can be manipulated, which provide additional indexing capacity since this region can be used as a second barcode. Alternatively, this region may be used to improve sequencing quality for amplicon-only runs (for more information, reference the section on *Multiplexing and sequencing*).

With a two-step approach, the complete molecular construct is created in a step-wise manner (Fig. 1). Primers targeting the community genomic DNA for the enrichment step are synthesized with a binding site for the primers used during library amplification. After the first PCR reaction (Step 1), the region of interest has been enriched and the amplicon has incorporated a sequence on either end that matches part of the Illumina specific sequences. This overlap is the priming site for a second PCR reaction (Step 2) that incorporates the Illumina adapters and the

indexing or barcode sequences into the molecular construct. For dual indexing capacity, an additional barcode sequence can be added between the template specific primers and the sequences overlapping the adapters.

There are steps necessary to build the molecular construct from the two-step approach described above. These are:

- Normalization
- Amplification from community genomic DNA
- Addition of Adapter and Indexing Sequences
- Purification and multiplexing

Normalizing samples before PCR cycling

Proper PCR amplification is an important part of obtaining results that minimize methodological errors. Over-cycling can promote chimeric molecules by promoting the extension of partial amplification products when primer concentrations drop at the end of the cycle (Qiu, et al., 2001). Over cycling also tends to normalize amplicon concentrations for different templates altering their relative abundances (Polz & Cavanaugh, 1998). Therefore, we use an initial real-time PCR to visualize the reaction curves and ensure that each sample is not cycled past the exponential or log phase of the reaction. Not only can this improve data quality, but it can also be used to normalize input DNA concentrations when they might be different, ensuring that different samples are cycled similarly.

Real-time PCR is a reaction similar to subsequent PCR reactions in library construction, but visualized using an optical monitor during thermal cycling. Real-

time PCR was carried out using with a CFX 96 Real-Time System (BioRad, Hercules, CA) with the following conditions: 0.5 units of Phusion (New England Biolabs, Ipswich, MA) with 1 x High Fidelity buffer, 200 μM of each dNTP, 0.3 μM of each forward and reverse primer primers and approximately 40 ng of mixed DNA template were added for each 25 μL reaction. We chose to use the V4 region of the 16S rRNA gene because these primers provide broad taxonomic coverage (Wang & Qian, 2009) and good taxonomic assignments (Soergel, Dey, Knight, & Brenner, 2012). The sequence we used for the first step amplification targets the position 515 (5' -ACACG ACGCT CTTCC GATCT YRYRG TGCCA GCMGC CGCGG TAA - 3') and 786 (5'- CGGCA TTCCT GCTGA ACCGC TCTTC CGATC TGGAC TACHV GGGTW TCTAA T-3') of the *E. coli* 16S rRNA gene, respectively (Knight, et al., 2011). Additionally, 5 X SYBR Green I nucleic acid stain (Molecular Probes, Eugene, OR) was added as a reporter for double stranded DNA abundance. Samples were cycled with the following conditions: denaturation at 98 °C for 30 sec annealing at 52 °C for 30 sec and extension at 72 °C for 30 sec. This is carried out for 40 cycles and the threshold value was set manually to a point just above the background (Fig. 2). Using the cycle number at which the curve for each sample crossed this threshold ($C_{t,sample}$), samples are normalized to the most dilute sample (highest $C_{t,sample}$ value), with a C_t less than or equal to 20 cycles ($C_{t,lowest}$). Normalization is done by estimating the concentration of each sample relative to the most dilute sample. Templates are diluted according to the following equation:

$$1.75^{-(C_{t,sample} - C_{t,lowest})}$$

Once the samples are diluted accordingly, the first step PCR will use $C_{t,lowest}$ cycles for all diluted samples.

Amplification of the 16S rRNA gene from community DNA

There are two aspects of the first step reaction that can minimize artifacts; cycling conditions and running multiple reactions. The diluted template should be amplified under conditions similar to the real-time PCR and be limited to the cycle number identified as $C_{t,lowest}$. Although SYBR is not added, the rest of the reagents and cycling temperatures and times should be identical to the conditions in the real-time PCR reaction. Previous studies have demonstrated that the maximum amount of diversity is recovered when samples are split into multiple reactions and cycled separately (Lahr & Katz, 2009), since skewed representation of sequences can results from jackpot effects that arise in single PCR reactions. For example, cycling each library in four- 25μL replicates will improve the recovery of the total diversity of the original sample. These separate reaction replicates (often done across different PCR plates), should be pooled before beginning purification step.

Addition of sequencer (platform-specific) specific adapters and indexing sequences

Platform specific adapters and indexing sequences are added to the amplicon through a second step PCR reaction (Fig. 1). The first step reactions are purified with Agencourt AMPure XP- PCR purification system (Beckman Coulter, Brea, CA), which can be used in small batches, or in a 96-well format, using a 96-well magnetic plate, following the manufacture's protocol. The primers in the first step reaction

contain an overlapping sequence, which provides the primer-binding site for incorporating the full platform-specific sequences and a sample specific barcode sequence during the second step reaction. The conditions for the second step PCR are similar to the first step, although 4 μl of the purified first step reaction was used as a template and 0.4 μM of each PE-III-PCR-F (5'- AATGA TACGG CGACC ACCGA GATCT ACACT CTTTC CCTAC ACGAC GCTCT TCCGA TCT- 3') and the barcoded reverse primer (5' - CAAGC AGAAG ACGGC ATACG AGAT**N NNNNN NNN**CG GTCTC GGCAT TCCTG CTGAA CCGCT CTTCC GATCT -3' where N's are represent the indexing sequencing specific for each sample) was used with 9 cycles. The concentration of other reagents was the same. Samples were cycled with the following conditions: denaturation at 98 °C for 30 sec annealing at 83 °C for 30 sec and extension at 72 °C for 30 sec. Samples are again cycled in 4 x 25 μL reactions for 7-9 cycles, which is sufficient to allow the adapters and indexing sequences to become incorporated in to the final construct. All reaction products are cleaned with the Amp-Pure magnetic beads in a manner similar to the first step reaction.

Multiplexing and sequencing

A final real-time PCR step is an accurate and high-throughput method for multiplexing samples together for sequencing. The relative concentration is determined for all samples in a set (usually 96 samples at a time) using the C_t values as described above. Samples are then diluted to the same relative concentration and pooled with equal representation. Primers used for this round of real-time PCR should be those that anneal to the Illumina adapters which mimic binding to the

solid matrix. This informs of the concentration of product that will actually be sequenced in each sample and confirms that adapters have been properly added. Pooled samples are finally checked using an Agilent Bioanalyzer, which provides a quantitative histogram of the size of library fragments. Illumina sequencing requires final fragments of 200-650 bp. Often, PCR reactions are not 100% efficient, and adjustments can be made to more accurately pool samples by assuming samples increase by 1.85-fold (for example) for each cycle. If all of the samples are of a similar origin (i.e. all obtained from human stool) they can often be pooled together without a final real-time PCR, assuming uniform concentrations across all samples.

Sequencing should be done with the addition of some amount of complexity in the sample when sequencing with Illumina platforms. Whether using MiSeq or HiSeq or older technology (e.g. GAII), additional diversity improves the quality of the resulting sequence. There are two options for added diversity: to spike in a small amount of a non-amplicon sample (e.g. 20-50% phiX, standard used for QC), or generate diversity with complexity regions of different lengths. We are considering incorporating staggered primers to improve the quality from amplicon only runs by varying the length of the complexity region, although it has not been tested. Along with standard paired-end sequencing reads, a specific barcode (indexing) read can be performed for the appropriate number of barcode bases (i.e. 8 bases in our example). This is done before preparing for the reverse read. For further details please reference Illumina's Index Read protocols.

Control samples are useful for optimizing program parameters and trouble-shooting. For large projects, especially those spanning multiple sequencing lanes,

control samples ensure reproducibility across lanes. A control sample may be a completely defined, mixed community (i.e. mock community) of either bacteria or DNA templates or a sample that is re-sequenced in each of the sequencing lanes (i.e. re-sequenced control), or both. The benefit of a mock community is that the diversity and sequences are known prior to sequencing, which can help with troubleshooting and optimizing program parameters during processing. The re-sequenced control can help to assess reproducibility across sequencing runs. We recommend sequencing both with every lane. For mock, communities, our templates are typically linearized, purified plasmid DNA that allow use to know exactly what the input sequence and relative template concentration should be.

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