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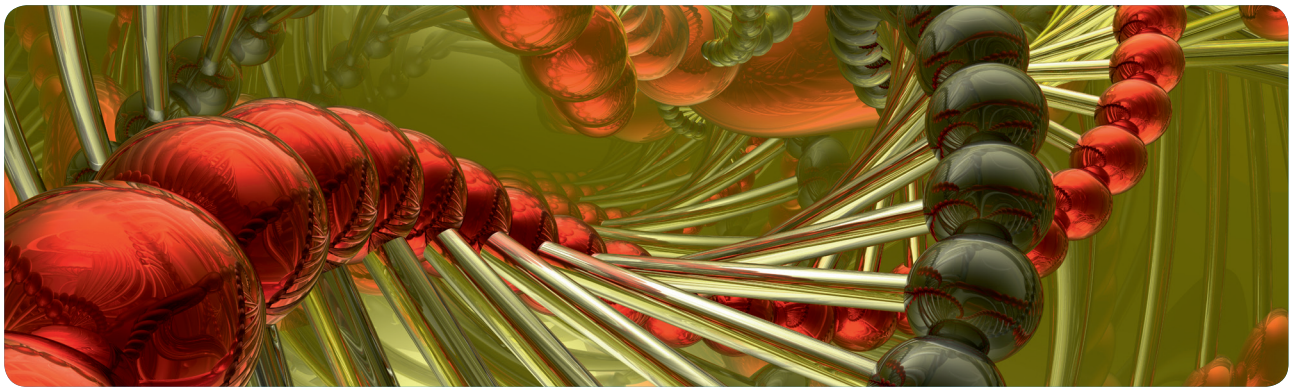
Application Note

One-Step versus Two-Step PCR for 454 Amplicon Sequencing



Introduction

Amplicon deep-sequencing using next generation sequencing (NGS) technologies has become an important and widely used tool to study the diversity in cell populations and/or to track the diversity in microbial communities. Common to all amplicon sequencing projects is that several PCR generated amplicons can be pooled for sequencing due to the high sequencing throughput of NGS technologies using barcoded amplification primers. These fusion primers consist of the adaptor sequence specific for the NGS platform, the barcode sequence (MID) and the locus-specific sequence (Figure 1). The barcode sequence is used to sort the sequence reads to individual samples after sequencing the pools.

Basically two different approaches for the PCR amplification of fragments are used in practice. In the first approach (one-step PCR) the fragment is amplified directly by using the 454 fusion primers. In the second approach (two-step PCR) the fragment is amplified in a first round using locus-specific primers and in a second round with the 454 fusion primers. In the present Application Note the two protocols were compared for their reproducibility during PCR amplification and their suitability to monitor the diversity in microbial communities.

Material and Methods

Two regions of the 16S rDNA (V123 and V345) were amplified from 5 environmental samples by using either the one-step or two-step protocol. The amplicons generated by each protocol were pooled and sequenced on 1/16 of a 454 picotiter plate (PTP). Sequencing results were analysed using an in-house analysis pipeline partially based on the mothur package (Schloss et al. 2009). The number of reads per operational taxonomic unit (OTU) was recorded for each sample and pairwise distances among communities were calculated using the Morisita-Horn index in R. Pairwise distances among communities were then used to perform cluster analysis. Moreover, diversity measures of communities were compared for the same sample using the one-step or two-step protocol.

Figure 1. Example of the structure of a 454 fusion primer including the 454 adaptor, the multiplex identifier (MID) also called barcode sequence as well as the template-specific part of the primer. The barcode is used to bioinformatically deconvolute multiplexed samples after sequencing. Depending on the template-specific sequence the fusion primers are 50-65 bases long (for more details and instructions see also the application note „454 Amplicon Sequencing“).

Results and Discussion

The two-step PCR approach resulted in much more stable amplification of PCR products (Figure 2). In contrast, the one-step protocol revealed a failure rate of > 45% for the performed PCR reactions. In order to exclude that the two-step PCR protocol leads to a bias, the two different approaches were compared to each other regarding the community structure of

five different samples. Pairwise community differences were calculated using the Morisita-Horn index because it is less sensitive to missed species at low frequencies (e.g. singletons) than other indices (Wolda, 1981). The results clearly indicate that the differences between the communities of the same sample either amplified with the one-step or the two-step protocol were low (Figure 3). Moreover, results implicate that the two-step protocol is in favour over the one-step protocol since it generally results in an equal or higher number of OTUs (Figure 4).

However, the final number of amplification cycles have to be carefully adjusted for each different metagenomic sequencing project. It is important to avoid that the amplifications already reach a plateau in the first PCR round when applying the locus-specific primers.

Conclusions

Based on the data of this study as well as our experience from other projects it can be concluded that the two-step PCR approach is preferred over the traditional one-step PCR approach: The two-step protocol **(i) represents a more stable PCR amplification system, (ii) often allows the detection of equal or higher numbers of OTUs (iii) and notably does not bias PCR amplification** (see also Berry et al. 2011). Therefore, we strongly suggest to use the two-step protocol when tackling metagenomic sequencing projects by applying the 454 FLX technology.

References

Schloss, P.D., et al. (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol **75**: 7537-7541.

Wolda, H. (1981) Similarity indices, sample size and diversity. Oecologia **50**: 296–302.

Berry, D, et al. (2011) Barcoded Primers Used in Multilex Amplicon Pyrosequencing Bias Amplification. Appl Environ Microbiol **77**: 7846-7849.

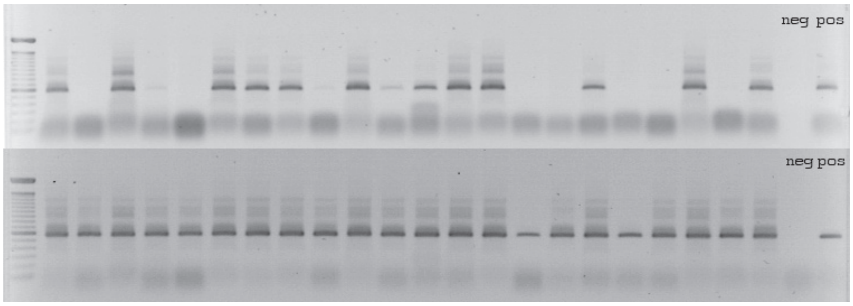


Figure 2. Comparison of PCR efficiencies for a fraction of samples using either a one-step PCR (above) or a two-step PCR protocol. A positive and negative control are shown on the right and the size standard is shown on the left. The results clearly show that the two-step PCR approach is more robust and reliable than the one-step protocol.

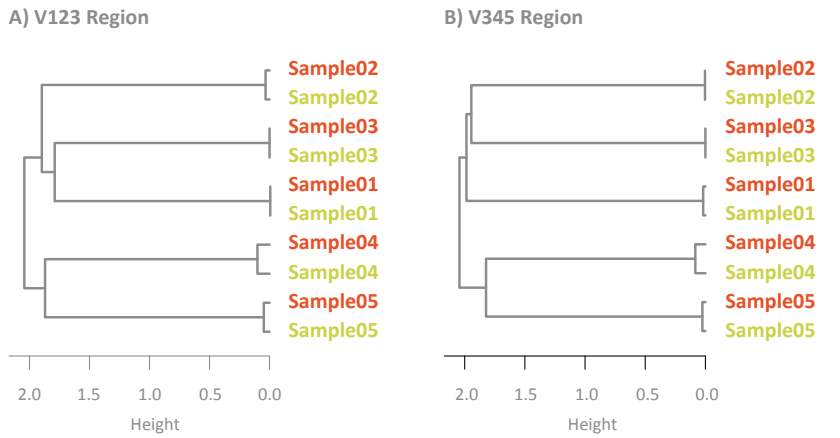


Figure 3. Cluster analysis based on pairwise community distances (Morisita-Horn) for five microbiota amplified either with a one-step protocol (red) or a two-step protocol (green). Figure 3A. Cluster analysis based on the V123 region of the 16S rDNA. Figure 3B. Cluster analysis based on the V345 region of the 16S rDNA.

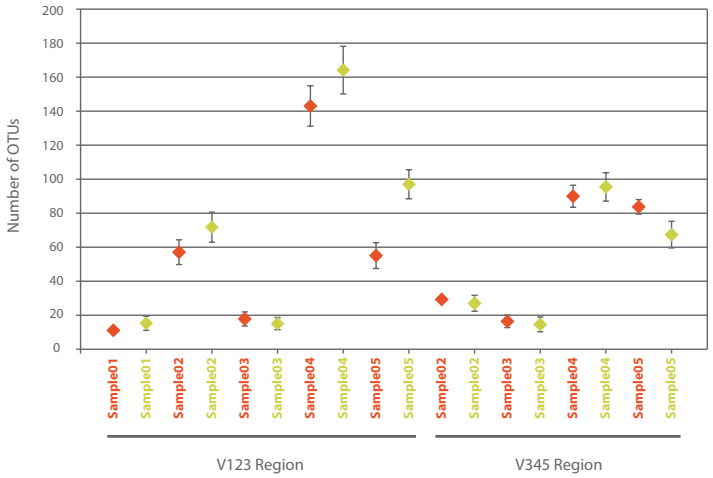


Figure 4. Number of OTUs detected in the amplicons for different samples, the one- and two-step PCR approach as well as the two different regions. The number of OTUs and its 95% confidence intervals were calculated for 1,500 reads to normalize the different number of reads per sample. Sample01 was excluded for the V345 region because the number of reads for the one-step PCR was < 1,500 reads. One-step protocol in red; two-step protocol in green.