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Title
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Publication Date
2003-04-04

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QUANTATITIVE PCR ASSAY FOR MARINE BACTERIA

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Monitoring the bacterial flora in coastal marine waters by conventional techniques has been difficult as most of the bacteria do not readily grow on culture plates and their morphologies are virtually identical in the microscope. Molecular techniques, particularly characterizing bacteria using polymerase chain reaction (PCR) amplification of their small subunit ribosomal RNA (SSU rRNA) genes, has dramatically improved the ability to identify bacteria from environmental samples. Identification of bacteria by PCR amplification is specific and very sensitive. However, it is exactly these properties of PCR amplification which make it difficult to determine the amount of each individual bacterial species in the population.

We have developed a quantitative PCR (QPCR) protocol that will allow the determination of the amount of a specific bacterial species in a sample. This protocol is based on the co-amplification of a modified internal standard sequence along with SSU rRNA sequences in the sample. The internal standard sequences in the PCR product are identified by gel electrophoresis following restriction endonuclease digestion. The amount of a specific SSU rRNA sequence in the sample is calculated by knowing the ratio of SSU rRNA to the internal standard sequence in the PCR product and the amount of internal standard sequence that was added to the original DNA sample (Fogel, *et al*., 1999). The protocol is very rapid and has the specificity and sensitivity of PCR amplification.

Recently we have developed a second QPCR protocol based on real time PCR analysis employing Taqman technology. The approach is more amenable to automation, however it requires sophisticated equipment and is susceptible to interference from inhibitors in the samples.

These protocols have been successfully applied in the laboratory and can be adapted for field application as an alternative to the conventional culture plate assays for marine bacteria. A major advantage of this approach is a dramatic decrease in the time required to assay the marine bacteria and an increase in the spectrum of bacterial species that can be detected (Brunk, *et al*., 1996). Thus, the assay can be used to determine the source of bacterial contamination by matching unique bacterial sequences from various potential pollution sources with the sequences present in the sample.

The primary objectives of this project were to:

1. To compare and evaluate the two QPCR techniques currently available, the internal standard assay and the Taqman assay relative to accuracy, reliability and cost effectiveness.

2. To refine primer/probe sets specific to known pollution sources, including sewage treatment plant effluents, storm drain runoff and human fecal material.

3. To explore potential interference with each of these QPCR assays from inhibitors of PCR amplification occurring in environmental samples.

4. To modify the laboratory protocols for sample collection and QPCR analysis as necessary in order for an effective QPCR analysis regularly performed in a general laboratory involved with environmental monitoring.

The majority of these objectives have been accomplished and provide a bases for general use of these protocols. The original objectives were not modified to a significant degree.

The overall project objective was to establish a protocol based on polymerase chain reaction (PCR) amplification of SSU rRNA sequences that can be used as an alternative to the culture plate based assay for monitoring coastal marine bacteria. We have developed two protocols that can used as general assays for the specific bacterial content of samples taken in the field. The internal standard assay use facilities of a normal laboratory that is setup for monitoring environmental bacterial samples, while the Taqman assay requires real time PCR analysis equipment.

The high sensitivity of PCR is a mixed blessing, it is valuable for detecting bacteria, but makes the determination of the abundance of different bacterial types difficult. PCR amplification usually saturates during the later cycles and amount of a specific sequence in the PCR final product is not always proportional to the amount of that sequence in the original sample. Also, different sequences may be amplified to different extents, in which case their abundance in the final PCR product does not reflect their abundance in the original sample (Brunk and Eis, 1998). Thus, quantitative PCR (QPCR) techniques are required to determine the abundance of specific SSU rRNA sequences in the original sample.

The most rigorous means of determining the abundance of a specific target sequence in the sample is to PCR coamplify a highly similar sequence, an internal standard, which can then be distinguished from the target sequence in the PCR product. The internal standard can simply be the target sequence with a modified unique restriction site with the internal standard differing from the target by only 2 or 3 nucleotides. The PCR amplification of these sequences are identical. Following PCR amplification two aliquots of the PCR product are analyzed, one cleaved with the restriction enzyme characteristic of the specific sequence and the other aliquot cleaved with the restriction enzyme characteristic of the internal standard. The cleaved products are analyzed by gel electrophoresis for full length and cleaved PCR product and the relative amount of cleaved PCR product in each digestion can be determined. Using this type of QPCR analysis the abundance of the target SSU rRNA sequence in the original sample can be readily calculated.

Recently a real-time analytic technique for QPCR analysis, Taqman (Trademark of ABI) analysis, has become available which provides remarkably rapid and accurate determination of target sequences in a sample. In essence Taqman analysis measures the increase in PCR produce for each cycle of the PCR amplification. When the PCR product is first detectable, the PCR reaction is still in the linear range and the amount of PCR product is proportional to the amount of target sequence in the original sample.

Although this QPCR analysis requires sophisticated and expensive instrumentation, it is a very rapid and accurate means of determining the amount of target DNA in a sample. However, we have found that environmental samples often contain inhibitors of PCR amplification. A second DNA target, that is not present in the environmental samples, can be added to each well as an internal normalization standard to monitor the efficiency of PCR amplification.

Our evaluation of these protocols indicates that the internal standard approach is reliable and can be performed in most monitoring laboratories with minimal additional equipment. A Taqman based approach requires sophisticated equipment and is more vulnerable to interference, however is more rapid and may be preferred for large analysis of standard bacterial contaminants.

We have continued to cooperate with the Environmental Monitoring Division of the Los Angeles Bureau of Sanitation in assisting them to develop molecular tools for monitoring coastal bacterial populations. We have recently described our approach and protocols in a chapter in *Environmental Molecular Microbiology: Protocols and Applications*" and a paper in "*Current Issues in Molecular Biology*", which will make these techniques widely available.

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