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Whitepaper

How the PathogenDx Microarray Technology Can Distinguish "*Bonafide*" Bacterial 16S DNA Sequences from "16S-like" Sequences Found in Cannabis Chloroplast and Mitochondria DNA.

A patent application of PathogenDx, Inc., entitled **"Microarray Based Multiplex Pathogen Analysis and Uses Thereof"**, United States Patent Publication #20180251822 has recently published onSeptember 6, 2018. This invention describes a method to PCR amplify bacterial and fungal microbial samples followed by microarray analysis of the resulting PCR products.

The bacterial aspect of the analysis is particularly interesting, in that as is now well-known from the literature that cannabis and other flowering plants present residual "bacterial16S like" sequences in both their chloroplast and mitochondrial genomes (1,2,3). One useful aspect of the invention described in Application 20180251822 is analysis of bacterial samples via testing of their 16S DNA, especially bacterial samples which have been recovered from plant matter and analyzed without enrichment culture. Under those culture-free conditions, residual plant tissue may be recovered along with the contaminating bacterial cells of interest. Consequently, a major focus of Application 20180251822 is methodology to selectively amplify the "true" bacterial 16S DNA in these recovered samples, under conditions where "bacterial16S like" DNA within plant chloroplast and mitochondria is not amplified and consequently, remains invisible during microarray hybridization analysis thereafter.

Some in the cannabis community may be interested to better understand the process described in PathogenDx's Application 20180251822, with a special focus on such 16S DNA discrimination. The process is relatively simple and is based on the general structure of the PDx microarray assay described in Application 20180251822.

Overview of the [Tandem PCR + Microarray] assay that is used in PathogenDx products. Sample preparation and analysis in these assays is relatively simple and follows the same overall process:

1. **Harvest of a microbial cell pellet,** usually from a plant wash, typically by centrifugation or filtration.
2. **Lysis of the resulting microbial pellet,** to release the DNA content of the cells.
3. **PCR amplification of the raw pellet lysate, "PCR #1",** to generate a "Primary Amplicon". The PCR#1 reaction amplifies a ≈500bp region of bacterial 16S DNA, but not the "16S like" sequences.
4. **PCR amplification of the Primary amplicon, "PCR #2",** to generate a "Secondary Amplicon".The PCR#2 reaction is nested within the 500bp domain to generate a ≈225bp sub-region of 16S DNA.

The orientation of those two PCR reactions on the underlying bacterial 16S DNA gene is shown in Figure 1, which is a summary of the data described in Application 20180251822.

1. **PCR #1 is driven by PCR primers** identified as Seq 1 (forward PCR primer) and Seq2 (reverse PCR primer).
2. **PCR #2 is driven by PCR primers** identified as Seq 19 (forward PCR primer) and Seq20 (reverse PCR primer).

The PCR primer pair which drives PCR #2 (Seq19,20, red boxes in Figure 1) each bind to sites which do not vary among most bacteria, i.e. they are "Universal" 16S DNA sequence domains. The resulting "Secondary Amplicon" is thus well suited as a target analyte for hybridization to an array of DNA hybridization probes, comprising a microarray, derived from the well-known sequence diversity within the 16S region within the domain defined by PCR #2.

The general location of such known 16S sequence diversity is identified in Figure 1 as "Microarray Probe Hybridization Sites" (Middle of Figure 1, in black) with the Sequence ID for several of those hybridization probes, as originally specified in Application 20180251822 (in black) positioned close by to each location.

The same "Universal" 16S sequence structure to which Seq 19 & Seq 20 each bind (red boxes) is also seen in the eukaryotic16S-like sequence elements which persist in the mitochondrial and chloroplast genomes of cannabis and hemp and also in other plants (1,2,3). Consequently, in the presence of cannabis DNA contamination in a bacterial DNA sample, the eukaryotic 16S-like sequences is amplified by a simple "Universal" PCR reaction such as that driven by Seq19,20. Such ambiguity is well-discussed in the literature (1,2,3 and references therein).

To eliminate the risk of specious cannabis "16S-like" DNA contamination in the PCR reactions described in Application 20180251822, we have chosen to develop the assay as a two-step tandem PCR reaction series. In that series, a first PCR reaction is performed on the raw bacterial lysate, i.e. **"PCR #1"** in Figure 1, to generate **"Primary Amplicon".** The PCR #1 reaction is driven by the PCR primer pair Seq1, Seq2 (blue boxes) which are those described in Application 20180251822.

Detailed sequence analysis reveals that Seq 1 (blue box, left) is common to both bacterial 16S and the cannabis eukaryotic "16S-like" sequences.

Thus, it is Seq 2 (blue box, right) which confers the needed PCR sequence specificity.

The origin of the needed sequence specificity is elucidated in the lower right hand corner of Figure 1. There, it is shown that the sequence structure of the reverse PCR primer for PCR #1 (Seq 2, blue box) is exactly complementary to the 16S DNA sequence of most bacterial species of interest in cannabis microbiology, i.e. "Seq 2 Priming Site, Bacteria" (Top).

Conversely, the analogous **"Seq 2 Priming site in Cannabis sativa Chloroplast"** (Middle) and the **"Seq 2 Priming site in Cannabis sativa Mitochondria"** (Lower) have both been designed to display a well-defined mismatch at the 3' terminus of the primer-template complex. Such 3' terminal mismatches are well-known in PCR practice to render such primer-template complexes unable to support PCR under ordinary conditions of high specificity PCR thermal cycling. **The Seq 2 Priming site in *Cannabis sativa* Chloroplast (Middle)** is seen to additionally possess in internal 2bp mismatch which would provide for additional inactivation of its function as a reverse PCR primer for PCR#1.

The combined use of [Tandem PCR + Microarray Hybridization] enhances the specificity obtained from the Tandem PCR pair alone. This is due to the fact that, over the course of evolution, the eukaryotic 16S-like sequences in both chloroplast and in mitochondria have diverged significantly from those seen in modern bacterial species, especially within the region of high 16S sequence diversity, i.e. the **"Microarray Probe Hybridization Sites"** identified in the middle of Figure 1, middle.

In particular, as a result of that substantial 16S sequence divergence over time, eukaryotic 16S-like DNA sequence obtained from amplification of either *C.sativa* chloroplast or *C.sativa* mitochondrial DNA do not match, in most cases, with the sequence designs in place for the microarray probes specific for *bonafide* bacterial 16S DNA, thus providing for a second-tier of discrimination beyond that obtained from just the Tandem PCR reactions (#1, #2) themselves.

Summary.

Data from multiple customer labs using well-characterized microbe-free cannabis samples, have confirmed that the combination of bacterial 16S specific Tandem PCR, coupled to bacterial 16S specific microarray hybridization (Figure 1) provide in the aggregate for highly-specific bacterial detection and speciation, based on 16S DNA analysis, under conditions where false positive signals do not occur due to accidental contamination with cannabis DNA.

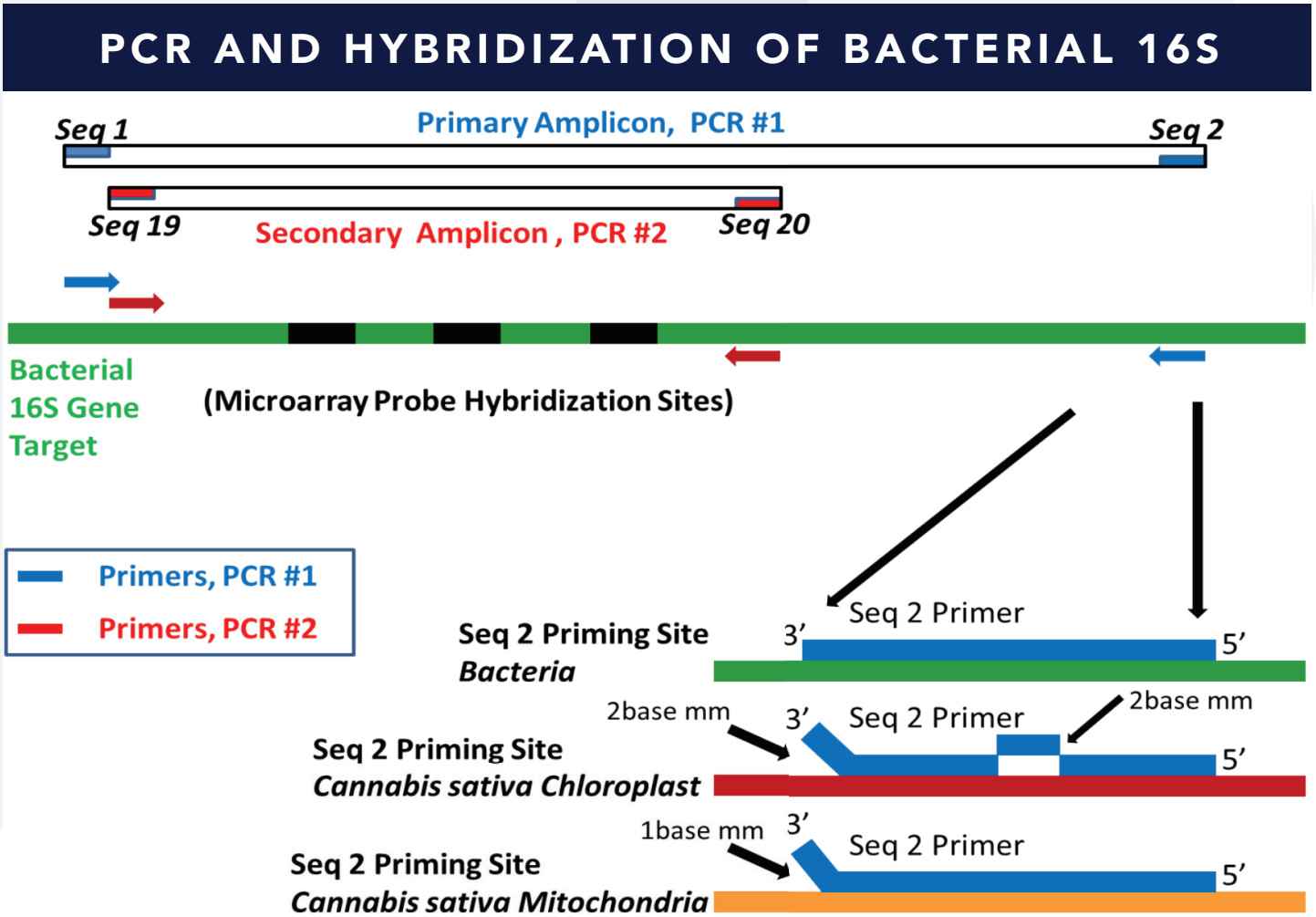

Figure 1. The Structure of the PCR Reactions Used for Bacterial Analysis in Application 20180251822

References.

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