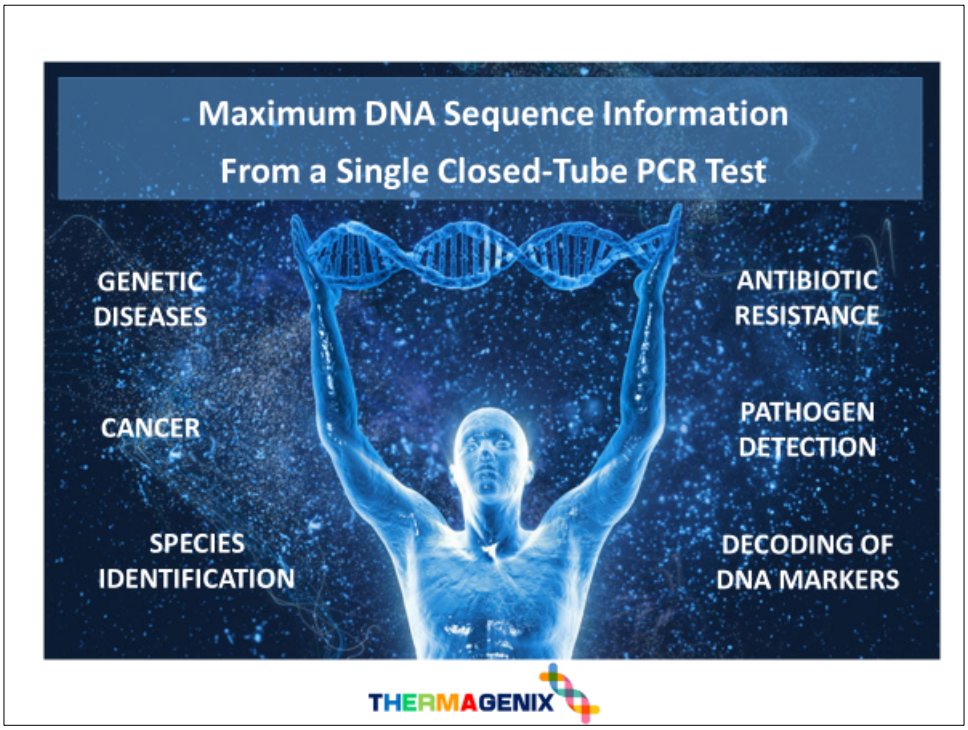
FASTDNA-ID™: A Portable, Accurate, and Cost-Effective Single-Tube Multiplex Platform for Identification of Any Sequence Variant in Defined DNA Segments Using a Common Set of Reagents

J. Aquiles Sanchez, Ph.D.
Principal Scientist – ThermaGenix, Inc.



INTRODUCTION

Rapid detection of DNA variations for identification of genetic diseases, pathogens, organisms, and DNA markers is complicated in cases where multiple individual variants occur at many possible sites in one or more target sequences several hundred nucleotides long. Next generation DNA sequencing (NGS) can score such multiplicity of DNA variants in a single test but NGS sequencing is currently impractical for rapid routine diagnostic use. An alternative approach uses monoplex or multiplex closed-tube polymerase chain reaction (PCR) amplification of short stretches of double-stranded DNA and sequence-specific hybridization probes. In this scenario, detection of multiple DNA variants in the same reaction typically requires the use of several sequence-specific probes of different colors. This approach is limited, however, by sequence-specific detection probes identifying only a subset of all possible DNA variants in their target sequences, interrogating only a limited number of nucleotides in the PCR

product, and/or having only a limited number of fluorescent colors that can be detected by most commercial fluorescent thermocyclers (maximum of four to six). High resolution melt analysis using DNA-binding fluorescent dyes overcomes these limitations by scanning stretches several hundred nucleotides long. However, DNA–binding dyes only fluorescence in one color and cannot be used to analyze several targets in the same tube, unless those targets melt at non-overlapping temperatures. Moreover, high-resolution melt analysis requires specialized equipment because sequence differences in whole amplicons cause only subtle changes in melt curves over a narrow range in temperature.

As a solution, ThermaGenix developed a new, highly informative PCR approach known as **FASTDNA-ID™**. **FASTDNA-ID™** uses a common set of reagents to read any variable sequences in any one or more specific target, independently of the complexity of the spectrum of DNA sequence variants. Multiple DNA segments can be read in the same single-tube test using set(s) of hybridization probes labeled with one or more fluorescent colors. **FASTDNA-ID™** provides comprehensive sequence information amenable to portable field-applications at a fraction of the time and cost required for DNA sequencing.

Strategies for Reading Variable Sequences Across Multiple DNA Segments

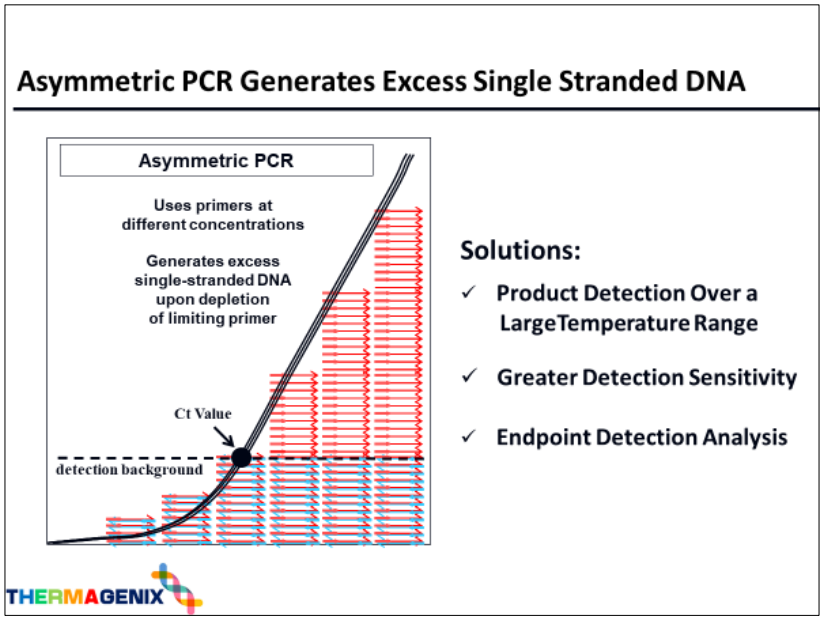
	One Test for All Variants	Single-Tube Test	Rapid Low-Cost	Portable On-Site
DNA Sequencing	YES	NO	NO	NO
Sequence-Specific Probes	NO	YES	YES	YES
THERMAGENIX FASTDNA-ID	YES	YES	YES	YES

THERMAGENIX

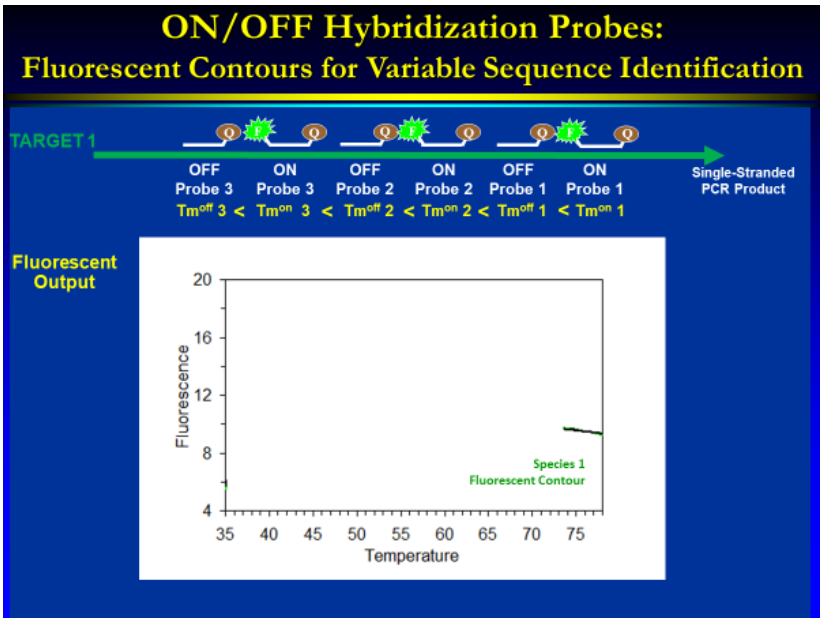
HOW DOES FASTDNA-ID™ WORK?

FASTDNA-ID™ combines amplification of single-stranded DNA through asymmetric PCR with ThermaGenix's proprietary hybridization probe technology known as ON/OFF hybridization probes. Asymmetric PCR uses different concentrations of amplification primers to generate excess single-strand DNA products once the primer at the lower concentration is depleted

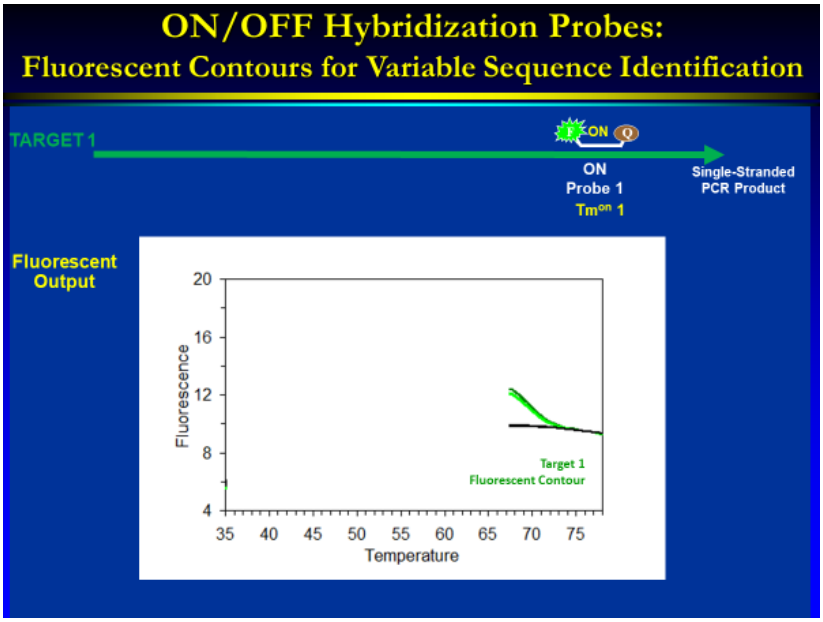
during amplification. The absence of a complementary strand to the single-stranded DNA products allows binding of ON/OFF probe sets to single-stranded amplicons over a large temperature range at the end of PCR. Lack of competition from a complementary product strand also allows hybridization probes to bind to completion to every available amplification product for maximum detection sensitivity.



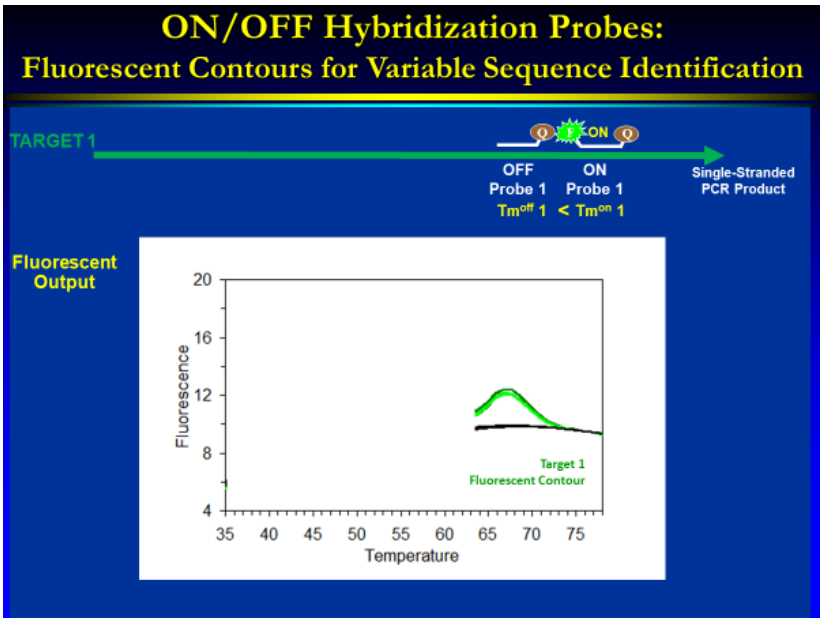
Sets of ON/OFF probe pairs of one or more colors hybridize along the length of the single-stranded PCR product(s) at increasingly lower temperatures to identify any DNA sequence variant along the amplicon. ON/OFF probes are low Tm probes that have a melting temperature (Tm's) at least 5°C below the primer Tm to prevent them from interacting with amplification products during PCR.



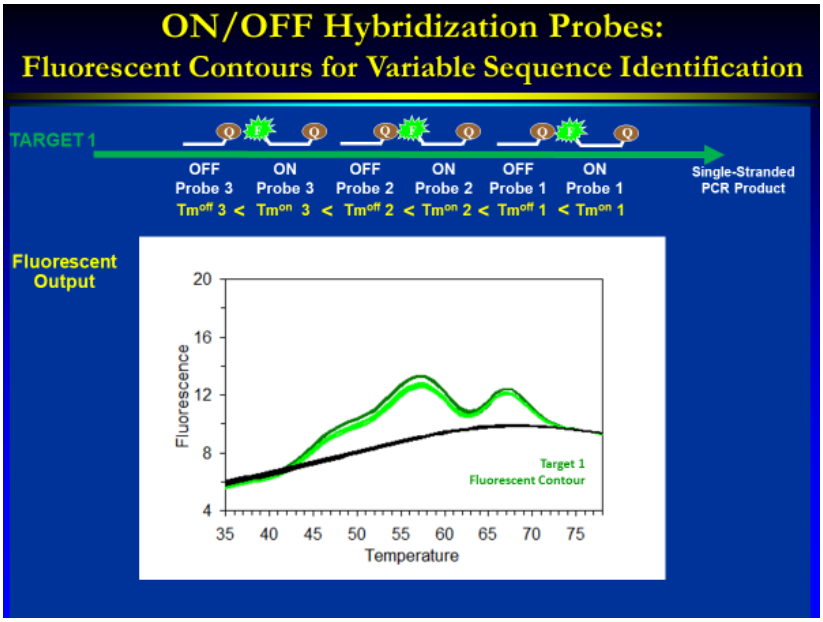
An ON-probe is a conventional linear mismatch-tolerant oligonucleotide probe labeled with a fluorophore at one end and a quencher moiety, such as a black hole quencher, at the other end. In the example below, as the temperature is lowered towards ambient at the end of PCR, the highest Tm ON probe hybridizes to its target. This forces the fluorophore apart from the quencher resulting in a fluorescence signal increase above background.



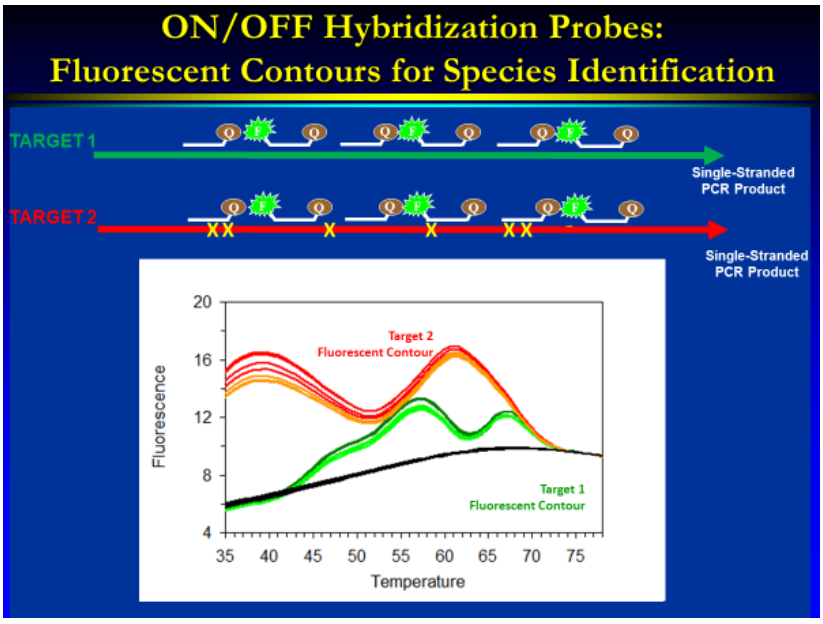
In contrast to ON-probes, an OFF-probe is a non-signaling linear oligonucleotide labeled with only a quencher moiety. In an ON/OFF probe pair, an OFF-probe binds adjacent to its paired ON-probe at a lower temperature such that the OFF-probe quencher absorbs energy from the fluorophore of its paired ON-probe, effectively turning ON-probe fluorescence signal off.



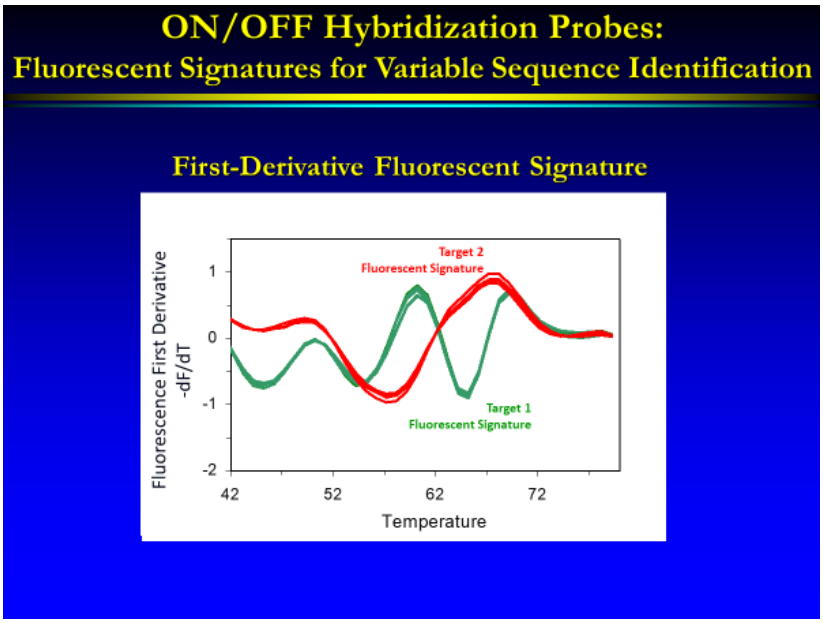
As the temperature is further lowered, the rest of the ON/OFF probes pairs of decreasing Tm bind to their target and generate a rising and lowering fluorescent contour pattern.



ON and OFF probes are relatively short, mismatch-tolerant, and interrogate only a portion of the target. As a result, targets with other variants that differ by as little as single base have distinct and consistently different fluorescent signatures.

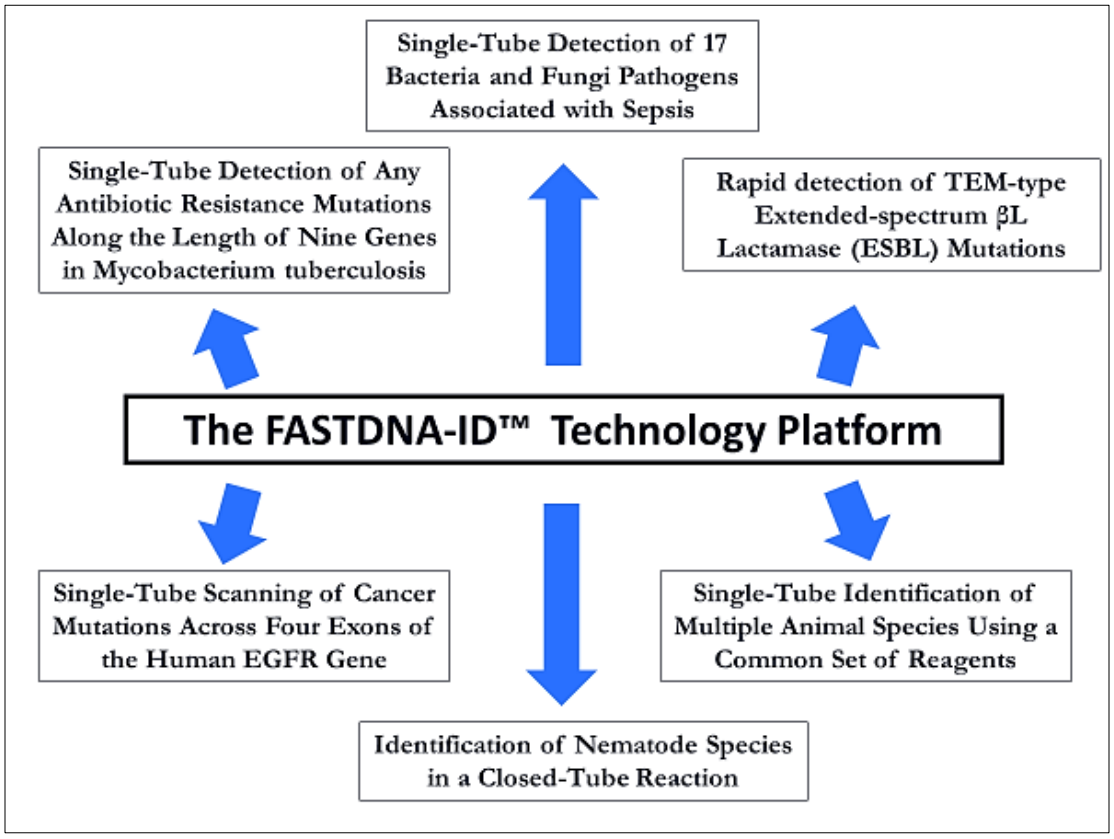


For convenience, each fluorescent contour is transformed into its first derivative curve, which we refer to as a fluorescent signature. Each fluorescent signature is characteristic of is underlying DNA sequence.



Fluorescent signatures using the same targets and probes are highly reproducible and exhibit single-nucleotide resolution. When an unfamiliar fluorescent signature is observed, its sequence can be determined to assign the fluorescent signature to the sequence for future identification without sequencing. Libraries of sequence-specific fluorescent signatures are mathematically coded and stored in a cloud-based database. An online algorithm automatically compares the fluorescent signature from a sample DNA to the reference library for immediate sequence information. Multiple pairs of probes of the same color or different colors can be used to analyze simultaneously analyze sequences several hundred nucleotides long. Different configurations of ON/OFF probe sets are possible for more detailed sequence analysis. Fluorescent signatures can be conveniently identified using the MIC PCR portable instrument from Bio Molecular Systems (Queensland, Australia).

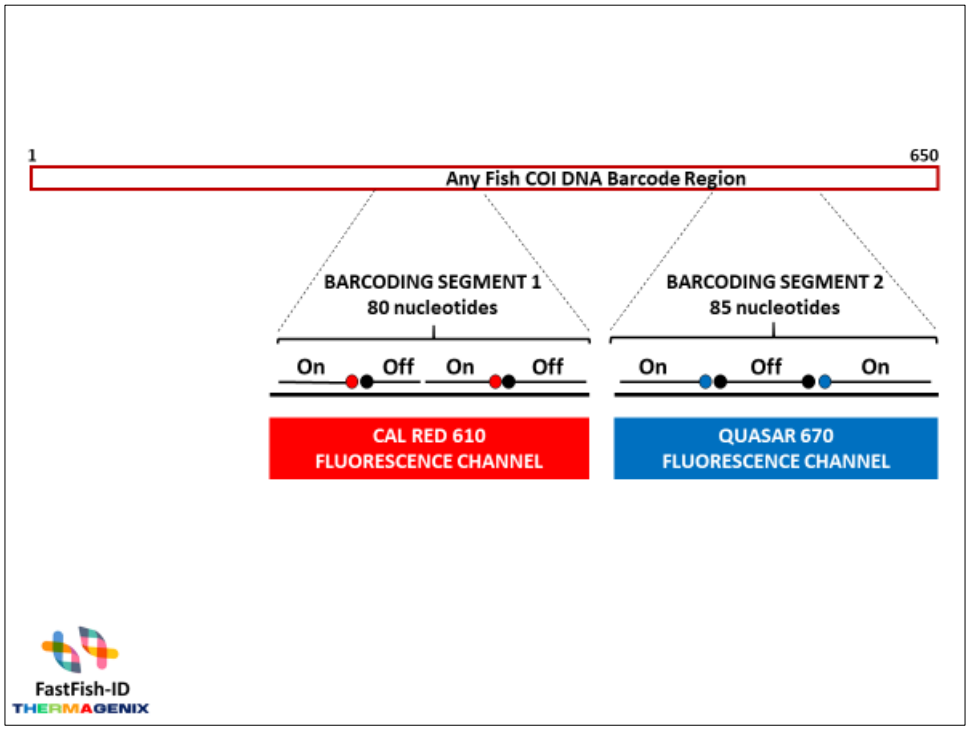
EXAMPLES OF FASTDNA-ID™ APPLICATIONS



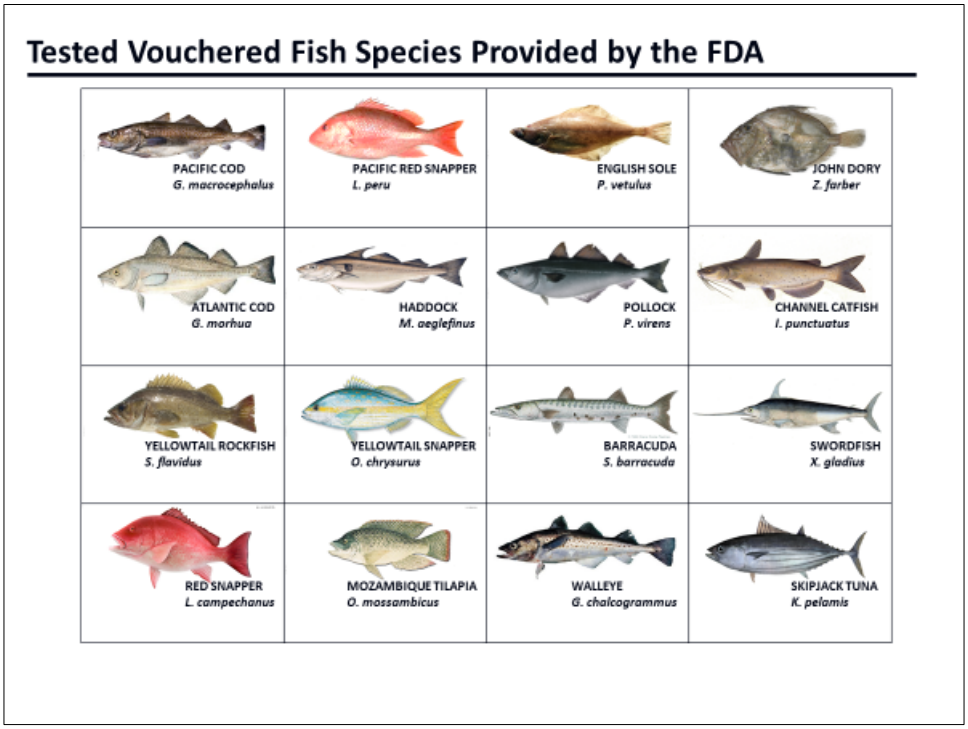
In each of the above applications, a single-set of ON/OFF probes per target was used to identify all possible DNA variants of said target in monoplex or multiplex amplification reactions.

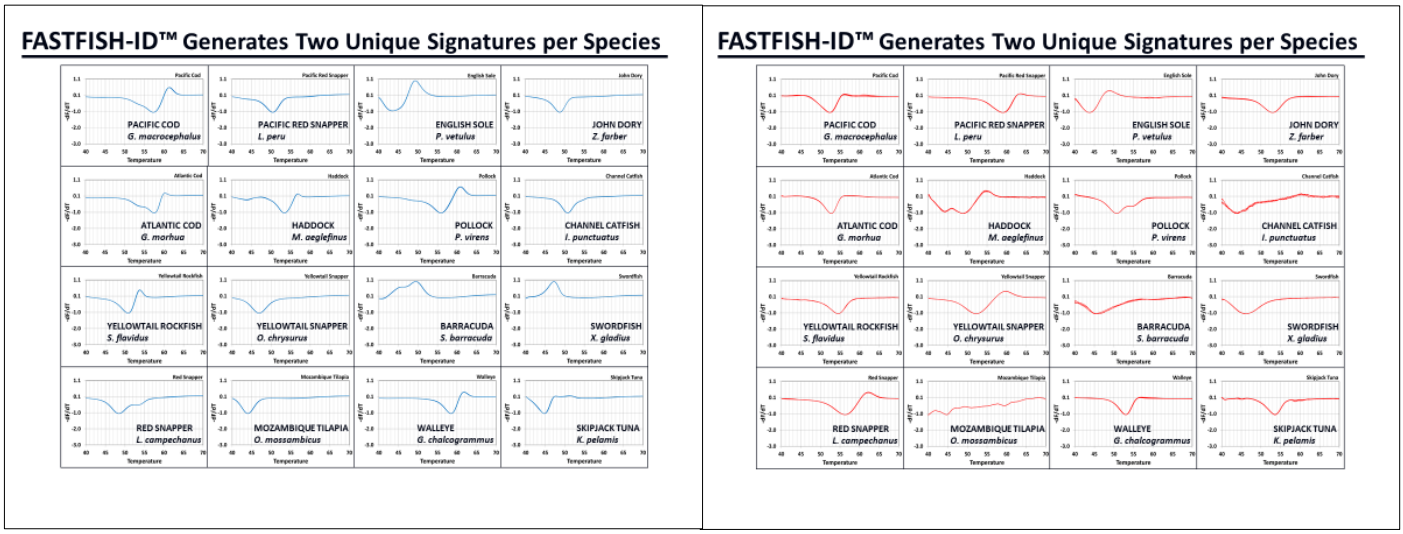
THERMAGENIX' FASTFISH-ID™ TEST:

AN EXAMPLE OF FLUORESCENT SIGNATURES GENERATED BY A SINGLE-SET OF REAGENTS FOR RAPID IDENTIFICATION OF DIFFERENT VARIABLE TARGET SEQUENCES CORRESPONDING TO DIFFERENT SPECIES OF COMMERCIAL FISH.



This example illustrates the use of sets of ON/OFF of different colors to identify DNA variants in the 650 base-pair CO1 DNA barcode region used as a standardized molecular tag for identification of animal species. Identification of commercial fish species by conventional DNA barcoding involves sequencing of CO1 DNA barcode region followed by comparison of the resulting DNA sequences to a reference library of species-species CO1 sequences at the Barcoding of Life database. **ThermaGenix's FASTFISH-ID™ Test** uses **FASTDNA-ID™** technology to enable rapid identification of fish species CO1 sequences without DNA sequencing. **FASTFISH-ID™** uses fish-specific CO1 primers and asymmetric PCR to amplify excess single-stranded products comprising the entire CO1 DNA barcode segment. After amplification, the temperature is dropped to allow binding of two sets of ON/OFF probes of two specific colors (Cal Red 610 and Quasar 670) to two separate regions of the CO1 DNA barcode single-stranded product (Barcoding Segment 1 and Barcoding Segment 2). Subsequent melting of these probe sets generates unique fluorescent signature patterns corresponding to the sequence variants for each fish species.





Each species generates a unique combination of Barcoding Segment 1 and Barcoding Segment 2 fluorescent signatures. Species are readily identified upon comparison of these sets of fluorescent signatures to a reference library of species-specific signatures. *In silico* analysis reveals that a single-set of reagents can identify over 1000 different fish species/sub-species.

APPLICATION: A Portable, Accurate, and Cost-Effective Strategy and Platform for On-Site Authentication and Characterization of Commercially Important Species in Many Industries

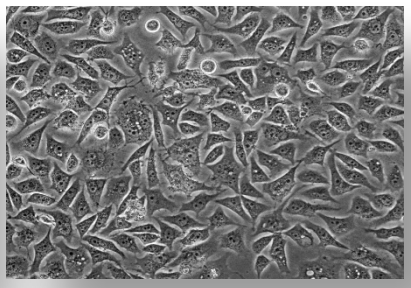
MEAT TESTING

FISH PRODUCT TESTING

PATHOGENIC BACTERIAL/ FUNGI TESTING

INVASIVE PEST TESTING

DISEASE VECTOR TESTING



CELL LINE SPECIES TESTING

INTRODUCTION

- **ThermaGenix** is developing accurate and cost-effective tests for rapid on-site identification of commercially important species for the food, agricultural pest management, environmental assessment and other industries.

- Our first product, **FASTFISH-ID™**, enables authentication of >700 species/subspecies of commercial fish products anywhere along the supply chain using a universal two-hour single-tube test.

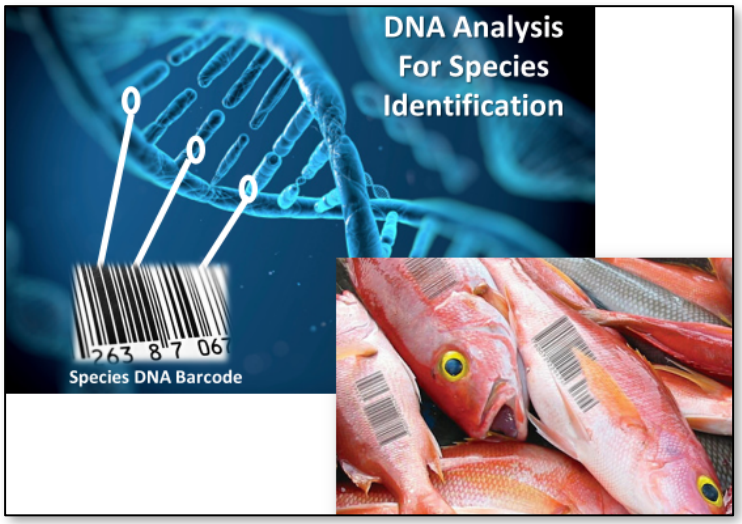
WHY TEST FOR SPECIES?

In the food industry, mislabeled products disguising lesser-value/lower-quality species unfairly compete for profits, harm brands/consumer trust, and risk consumer safety by preventing proper testing for species-specific hazards (toxins, pathogens, chemicals). The agricultural pest

management industry relies in rapid identification of invasive/destructive species, particularly at ports of entry. Health entities requires identification of disease vector species for proper intervention. Biopharmaceutical companies demand species certification of production cultured cell lines.

TECHNOLOGY OPPORTUNITY

- DNA species identification relies on the sequence of standardized DNA segment as a molecular identification tag for most species on Earth (mitochondria COI DNA barcode for animals, 16SrDNA for bacteria). **But reading the DNA barcode sequence is costly, time-consuming, and requires sending each sample out for sequencing**.



- Species-specific DNA tests are simpler and faster to do **but require separate tests for each species**.

THERMAGENIX'S SOLUTION

- Specimens collected on-site are treated in a single step to release DNA
- DNA is then added directly to the PCR reaction.
- High-Precision PCR (HP-PCR) including Therma**Stop**™ amplify the DNA molecular identity error free
- Sets of ON/OFF probes strategically located along the length of the PCR products convert species-specific highly variable sequences into characteristic "fluorescent signatures" that are automatically compared to a reference library for immediate species identification in less than two hours.

- These technologies are combined on a highly-affordable portable instrument (MIC PCR Cycler) from Bio Molecular Systems (Queensland, Australia) for rapid on-site species identification.

Universal Single-Tube Test for Identification of >700 Species/Subspecies Using the Same Set of Reagents

Strategies for DNA Species Authentication & Characterization

	One Test for All Species	Single-Tube Test	Rapid Low-Cost	Portable On-Site
DNA Sequencing	YES	NO	NO	NO
Species Specific PCR	NO	YES	YES	YES
THERMAGENIX FAST-ID TESTS	YES	YES	YES	YES

For more information about applications specific to identification of commercial fish species please double-click on the following embedded video links.

What is FASTFISH-ID™?

What is FASTFISH-ID Video Presentation.mp4

What does FASTFISH-ID™ do?

VIDEO 1 What does FASTFISH-ID do.mp4

How does FASTFISH-ID™ identify fish species?

VIDEO 2 How does FASFISH-ID identify fish species.mp4