ENVIROLOGIX™ Putting Science to the Test

DNABLE Rapid On-Site DNA Detection

RNABLE Rapid On-Site Gene Testing

Technology Development
Molecular Diagnostics

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Technology Development
ENVIROLOGIX INC.

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Overview

- Competing Technology – qPCR
- DNAble v1.0
- DNAble v2.0 (Patents Pending)
- Detection Methods
- Detection Instrumentation
- High-Throughput Assays – Douglas Scientific

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Competing Technology – qPCR

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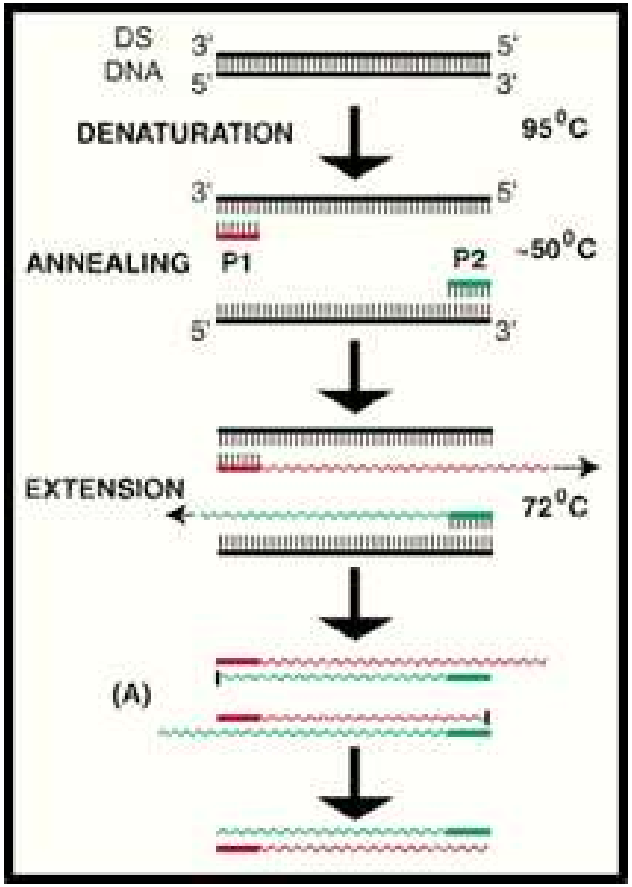
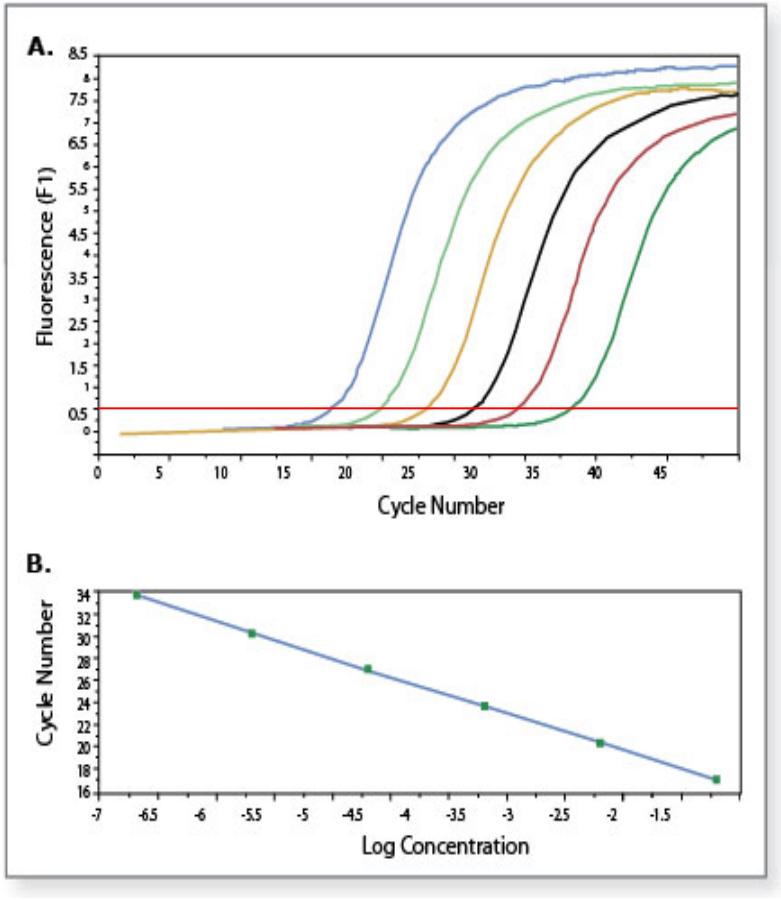
Competing Technology - qPCR





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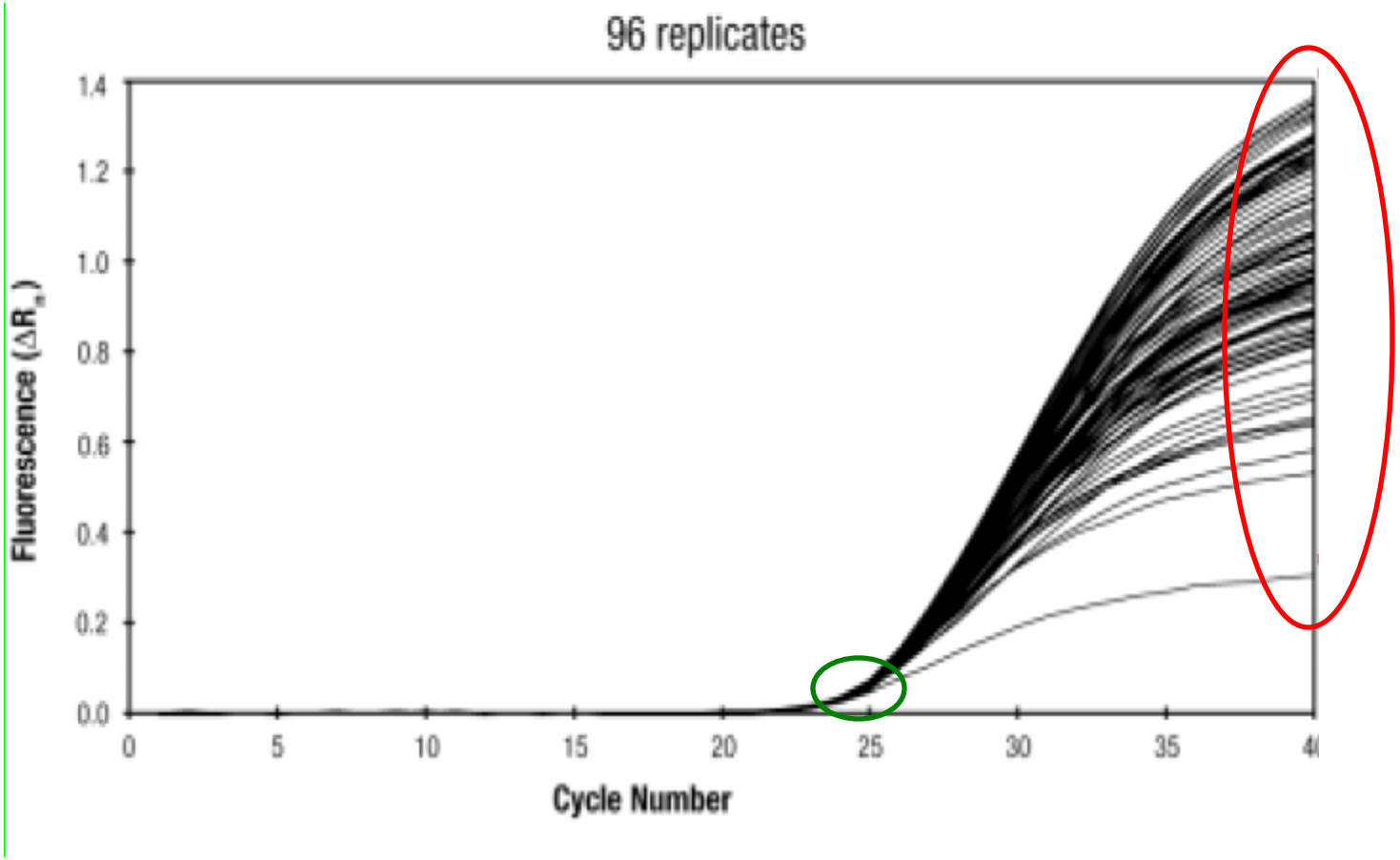
Competing Technology - qPCR



History

•Isothermal amplification technologies in general are notorious for high levels of non-specific amplification products.

•DNAble™ v1.0 is based on the **N**icking and **E**xtension **A**mplification **R**eaction (Ionian Technologies, Inc). A license for its use in Ag, Hort, and Vet applications was acquired by EnviroLogix, Inc in 2009. This initial technology called DNAble™ (v1.0) is also known to generate high levels of background, non-specific amplification products.

•Non-Specific reaction products can consume the reactants before the specific product is amplified.

•Assays have been developed leveraging the use of a background product as an endogenous, internal, positive control.

History

•Background products can vary depending on sample complexity creating a challenge for assay development that depends on them.

•Limits the utility of DNAble™ v1.0 (multiplexing).

•Assays are typically set up at the reaction temperature as a mock “hot-start”.

(Spoiler Alert : *We Have Changed History*)

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DNAble™ Rxn Components

✓Familiar qPCR Reaction Components….

- DNA or RNA target
- 2 target specific primers
- dNTPs
- DNA Polymerase

✓Not So Familiar DNAble Reaction Components….

- Nicking Enzyme
- Amplification in 10 min reaction time, or less
- Target (DNA or RNA) does not need to be purified
- Isothermal

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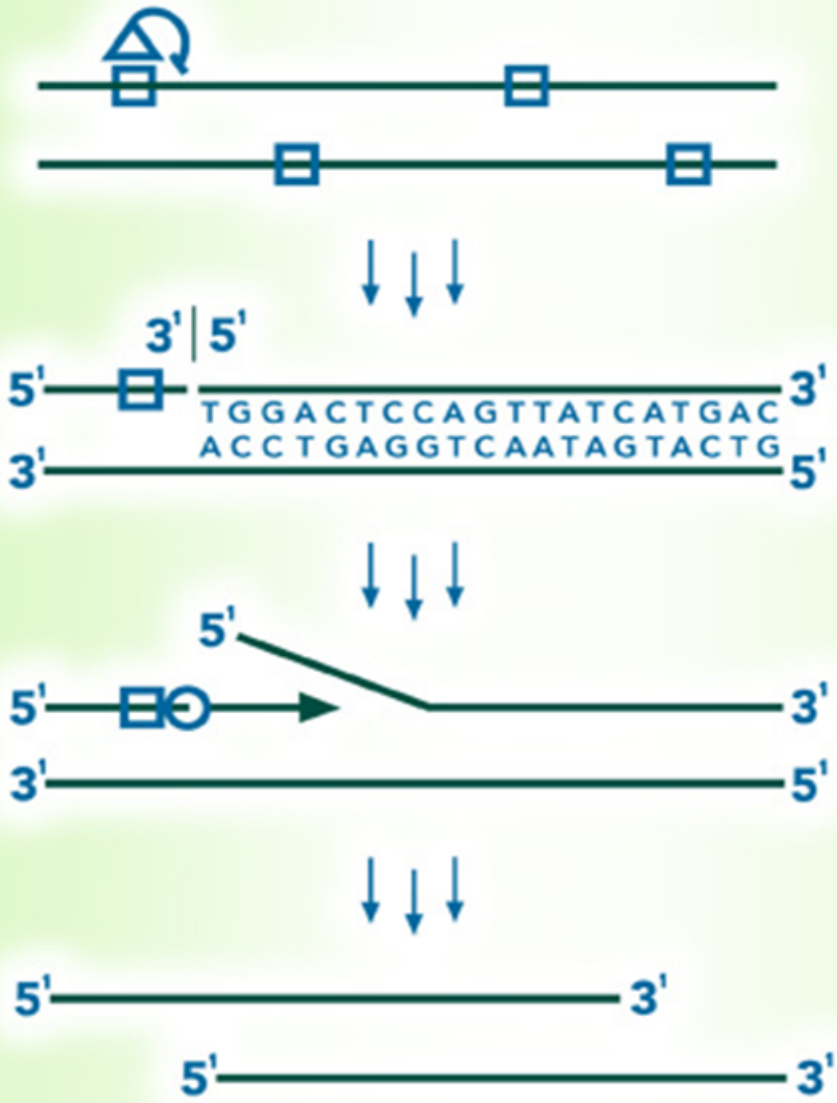
DNAble™ Schematic

DNABLE Rapid On-Site DNA Detection

△ Nicking Enzyme
□ Nicking Enzyme Binding Site
○ Strand Displacing Polymerase

- ✓ The nicking enzyme nicks the target DNA.
- ✓ Polymerase attaches to open 3'-end of nicked strand.
- ✓ Polymerase extends and displaces DNA strand.



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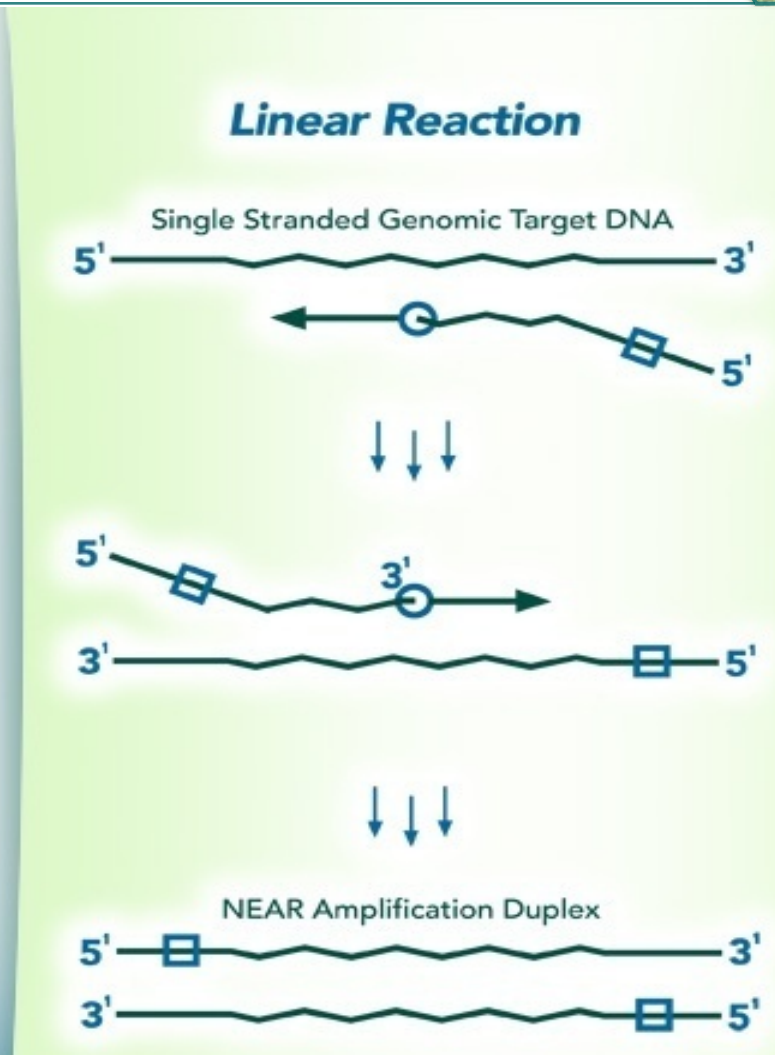
DNAble™ Schematic

DNABLE Rapid On-Site DNA Detection

- Nicking Enzyme
- Nicking Enzyme Binding Site
- Strand Displacing Polymerase

- ✓ Reverse primer anneals and polymerase extends.
- ✓ Forward primer anneals and polymerase extends beyond Nicking Enzyme Recognition site.
- ✓ Nicking enzyme nicks.
- ✓ Polymerase attaches and extends.



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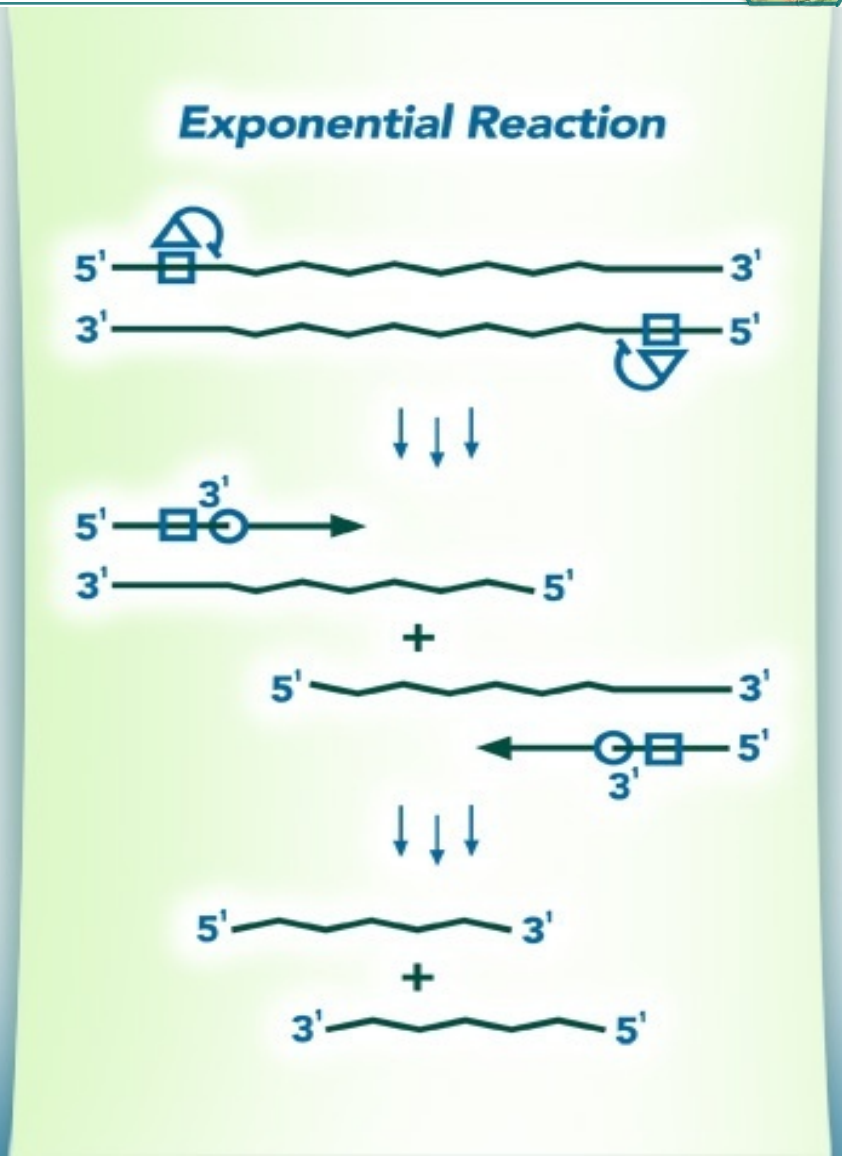
DNAble™ Schematic

DNABLE Rapid On-Site DNA Detection

△ Nicking Enzyme
□ Nicking Enzyme Binding Site
○ Strand Displacing Polymerase

- ✓ Formation of DNAble™ Duplex.
- ✓ Exponential Amplification.
  - Nick
  - Displace
  - Extend

  } "Nick & Kick"
- ✓ Formation of amplification products.
- ✓ Detection.



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DNAble v2.0

(Patents Pending)

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New Proprietary Second Generation DNAble™ v2.0

- Determine the DNAble™ reaction conditions that:
  - ✓Do not generate background products.
  - ✓Perform better than standard reaction (LOD/POD and S/N).
  - ✓Provide a means to predictably modify the reaction to yield conditions conducive to duplexing, enabling amplification and detection of a secondary and specific target as an endogenous, internal, positive control.
  - ✓Provide a means to predictably modify the reactions to yield conditions conducive to multiplexing, enabling amplification and detection of multiple, specific targets in addition to an endogenous, internal, positive control.
  - ✓Enable a “cold” start for convenient pipetting.

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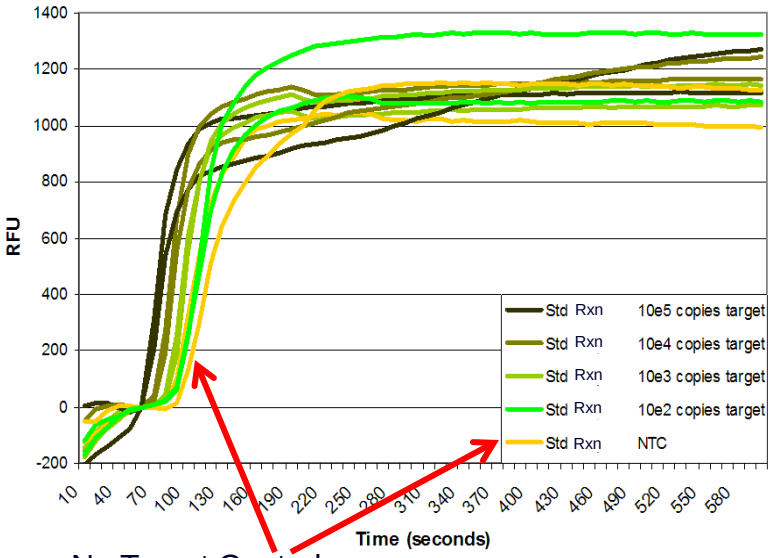
DNAble™ v1.0 vs DNAble™ v2.0

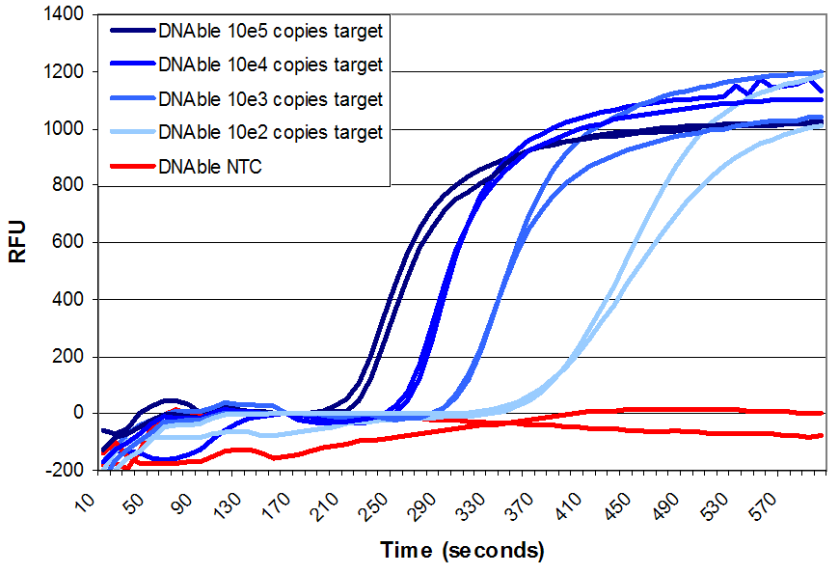
SYBR Green I Detection

DNAble™ v1.0



No Target Control

DNAble™ v2.0



Assay Conversion

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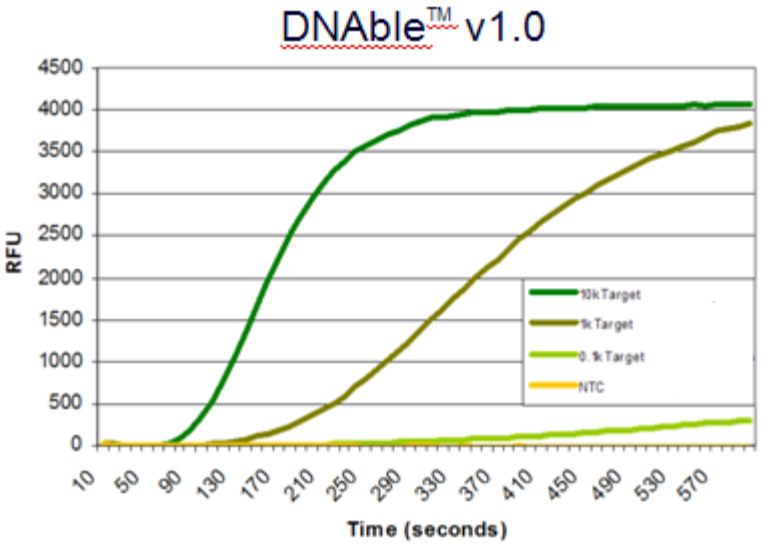
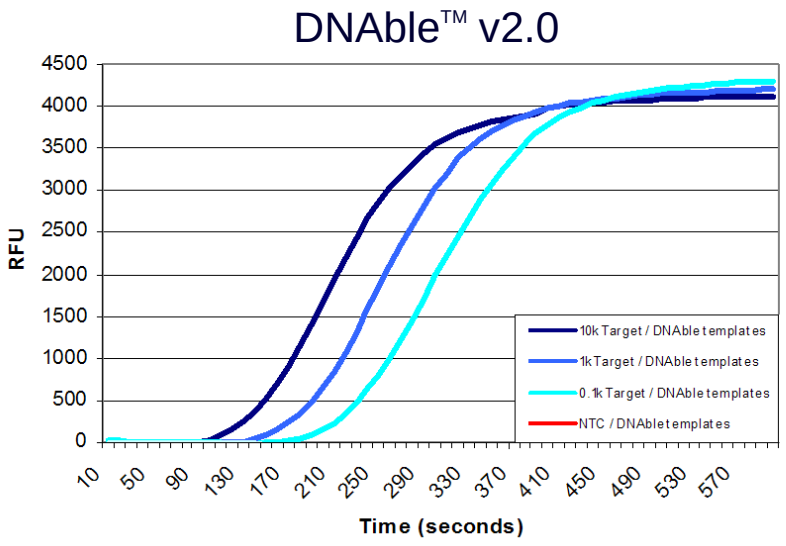
DNAble™ v1.0 vs DNAble™ v2.0

Molecular Beacon Detection





Assay Conversion

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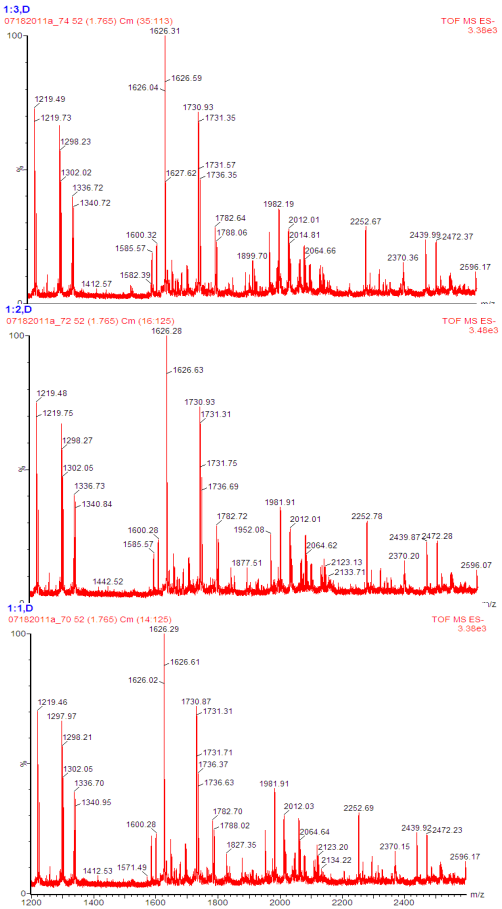
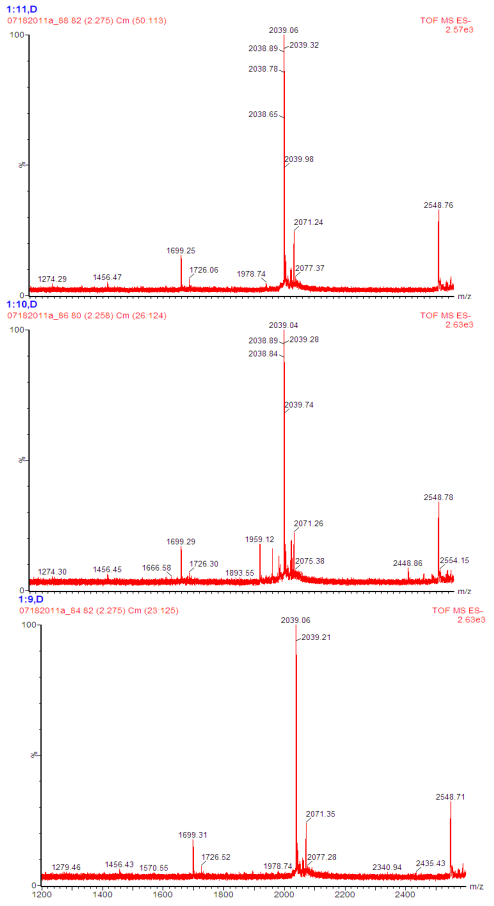
DNAble™ v1.0 vs 2nd Gen. DNAble™ v2.0 Mass Spec (NTCs)

DNAble™ v1.0



DNAble™ v2.0



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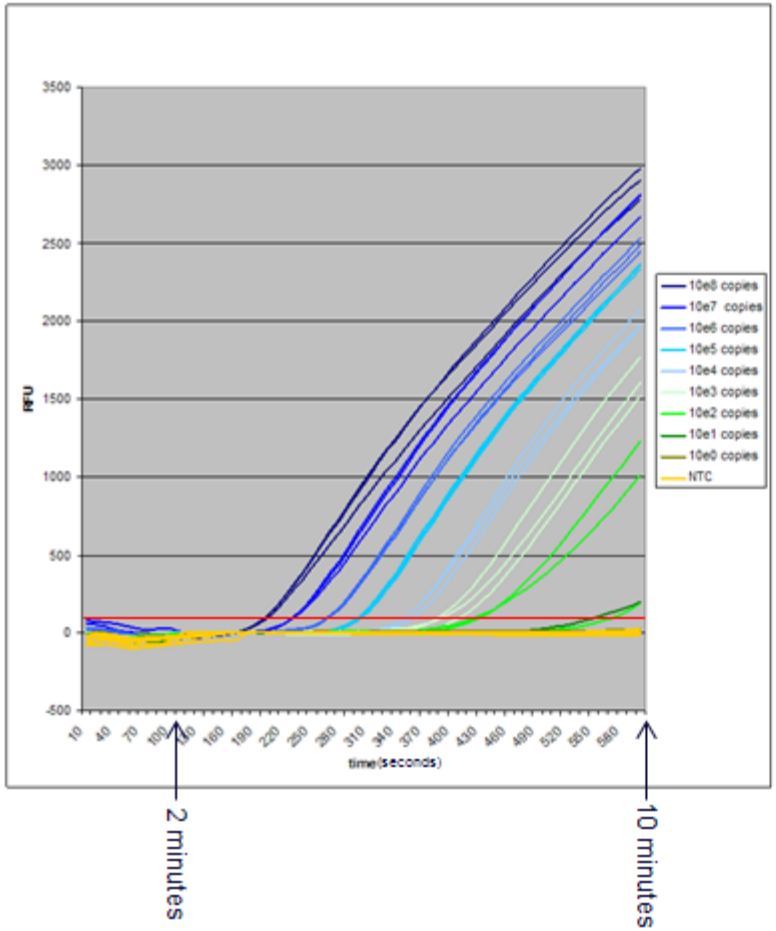
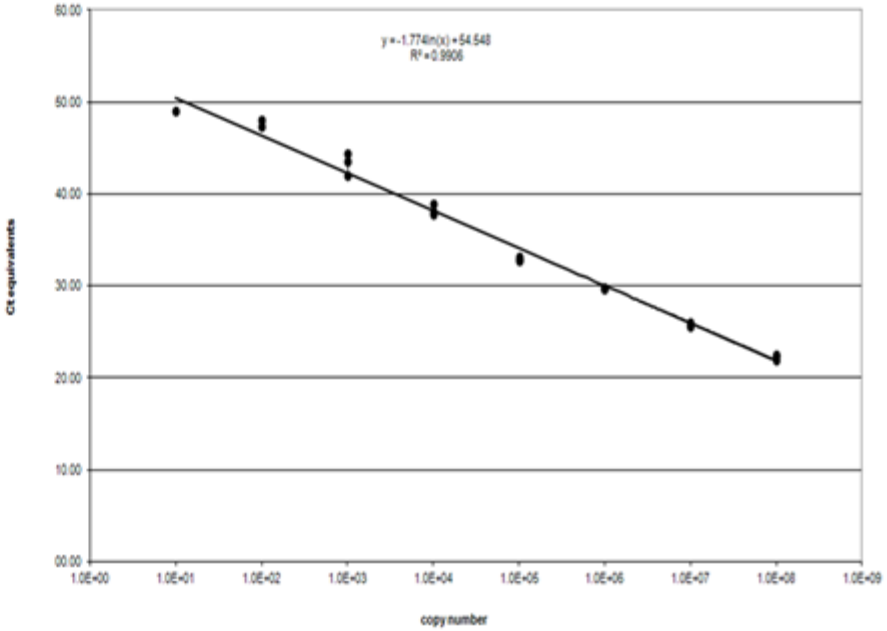
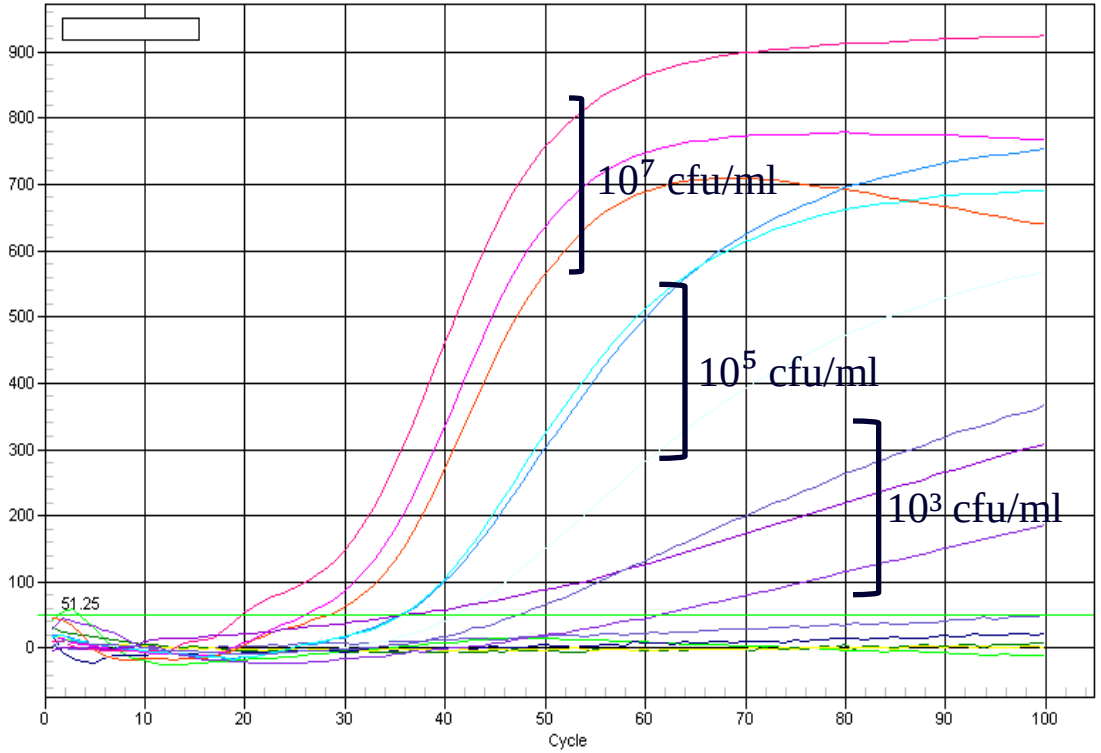
DNAble™ v2.0 Dynamic Range







Detection of Cmm bacteria in Citrus leaf sample prep, using 30mg of Citrus leaf tissue from Harris punch sampling. 25ul total reaction volume, 2.5ul sample per reaction. BioRad iQ5

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Crude Sample Prep

Protocol:

-Using Harris punch take 2 discs of tissue along the mid-line of the leaf.

-Crush with pestle for 30 seconds (or bead-beater).

-Add 200ul of extraction buffer (MB6c) and crush with pestle for addition 30 seconds (or quick 1 second bead-beater pulse).

-Heat for 5 minutes at 98^{o}C.

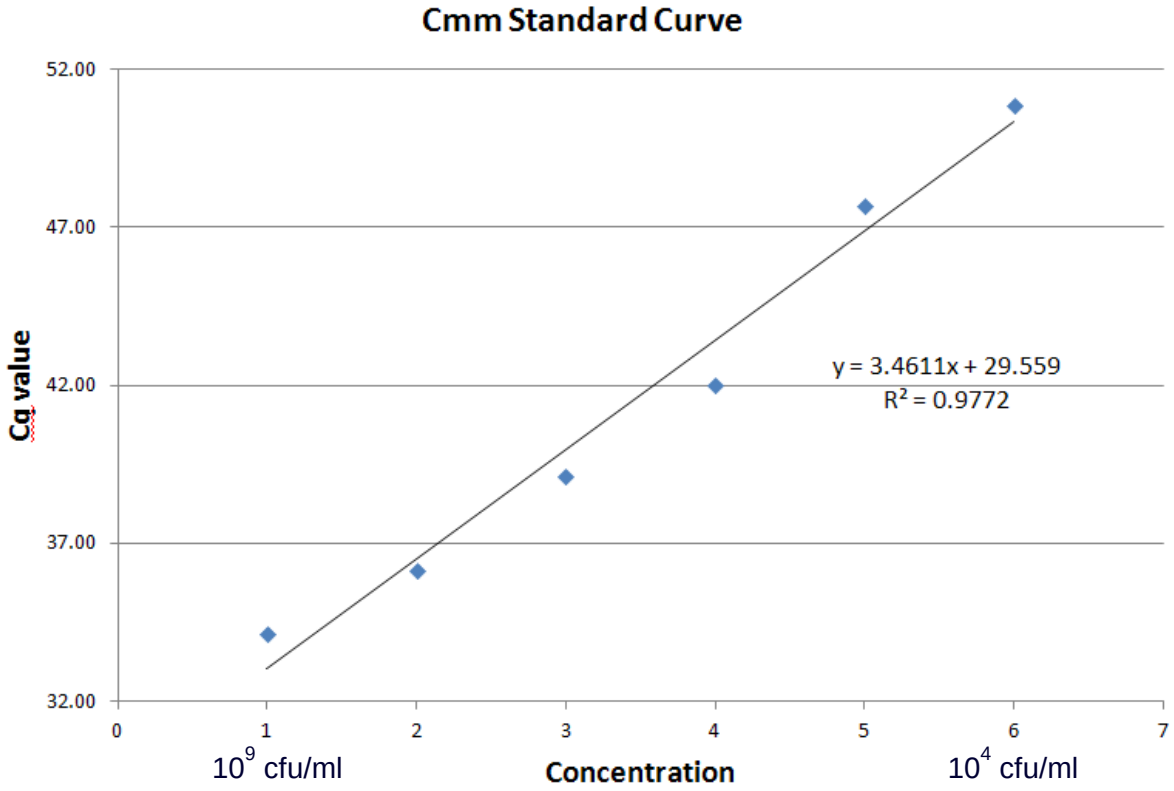
-Add 400ul of dilution buffer.

-Run Amplification for 10-15 minutes.

Short Sample-to-Data Processing Time.

Missing Something? Where is the DNA purification Step?

DNAble™ v2.0 Dynamic Range



Cmm bacteria were spiked in to seed wash at **known** concentrations and lysed using the standard seed wash sample prep protocol. Samples were assessed using the Cmm DNable assay

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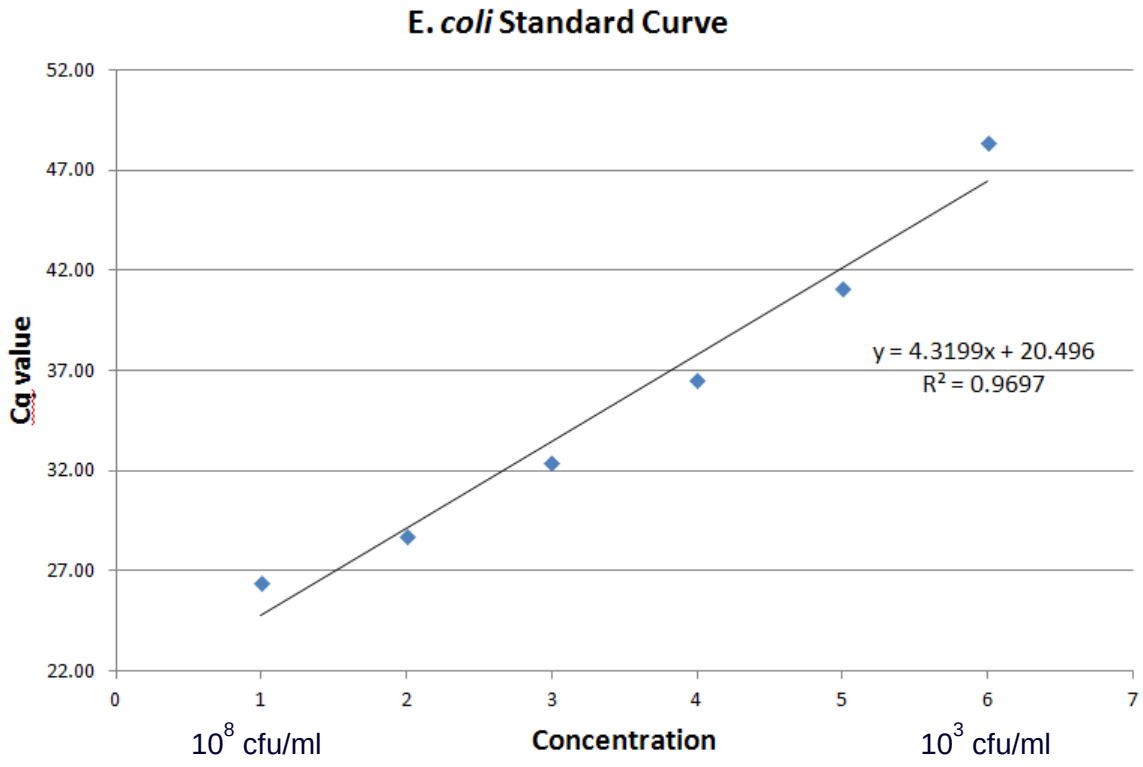
DNAble™ v2.0 Dynamic Range



E.coli bacteria were spiked in to cow feces at known concentrations and lysed using the standard fecal sample prep protocol. Samples were assessed using the *E.coli* DNable assay.

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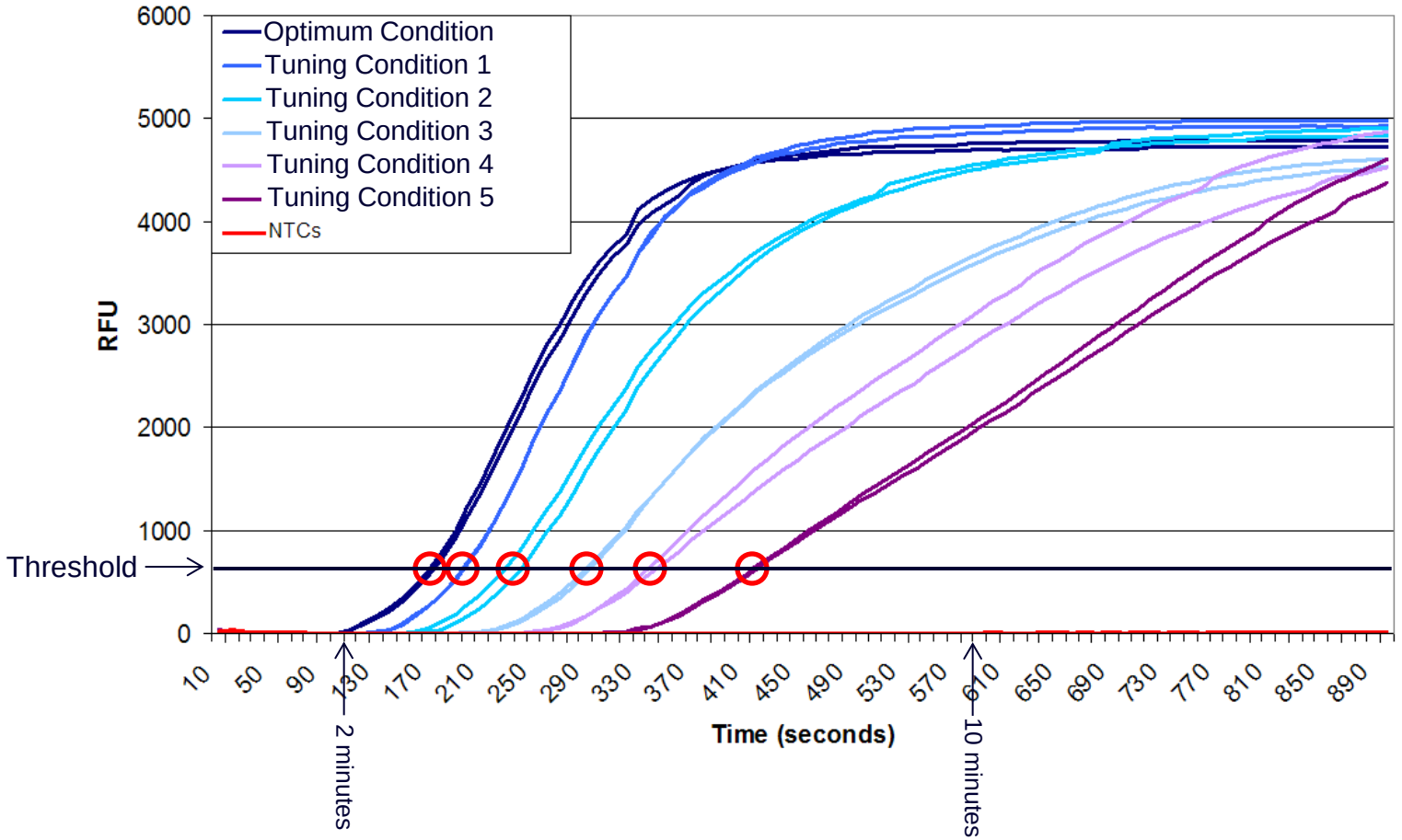
DNAble™ v2.0 Tunable Rxn



Duplicate 50ul reactions, 10K Genomic DNA target as genome equivalents, FAM Beacon, iQ5

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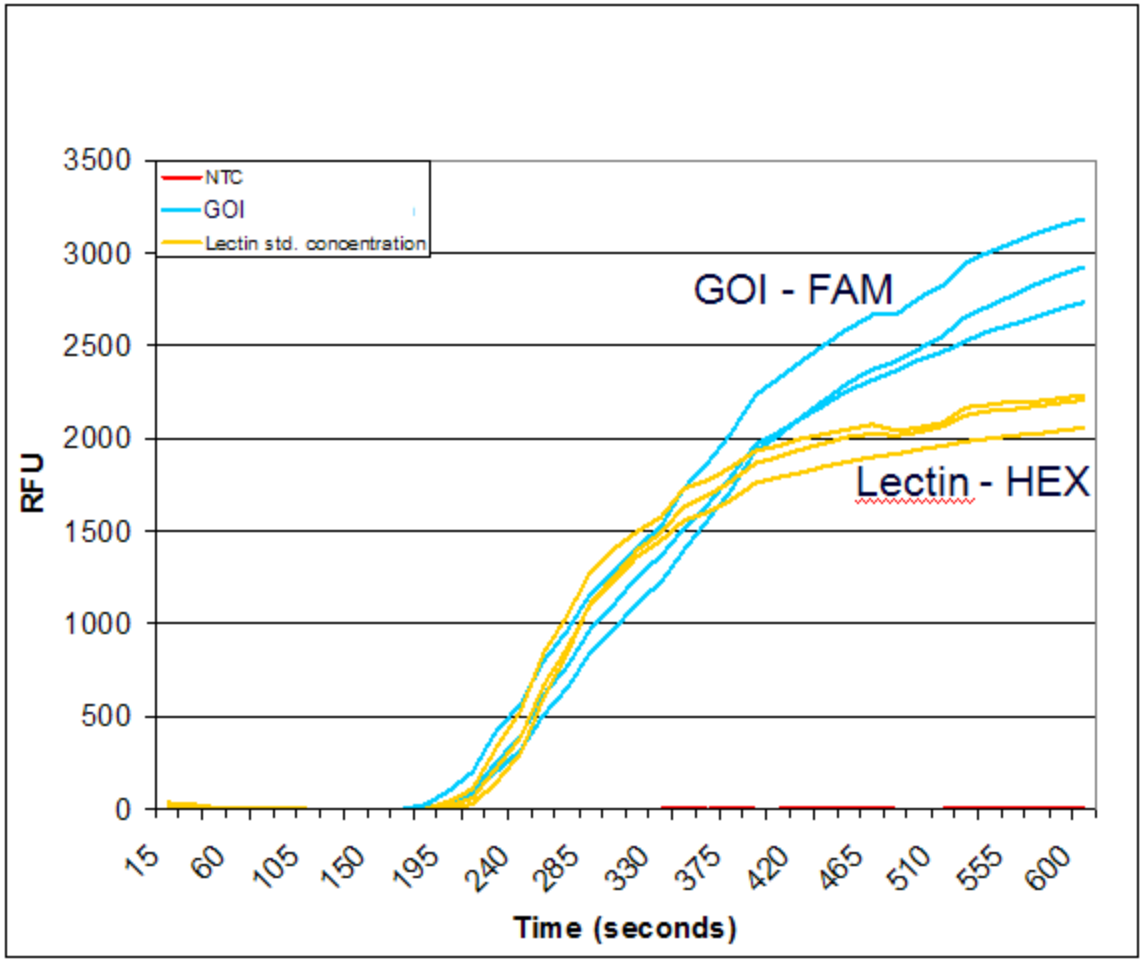
Duplexing Example – GMO, Soy Internal, Endogenous Control System



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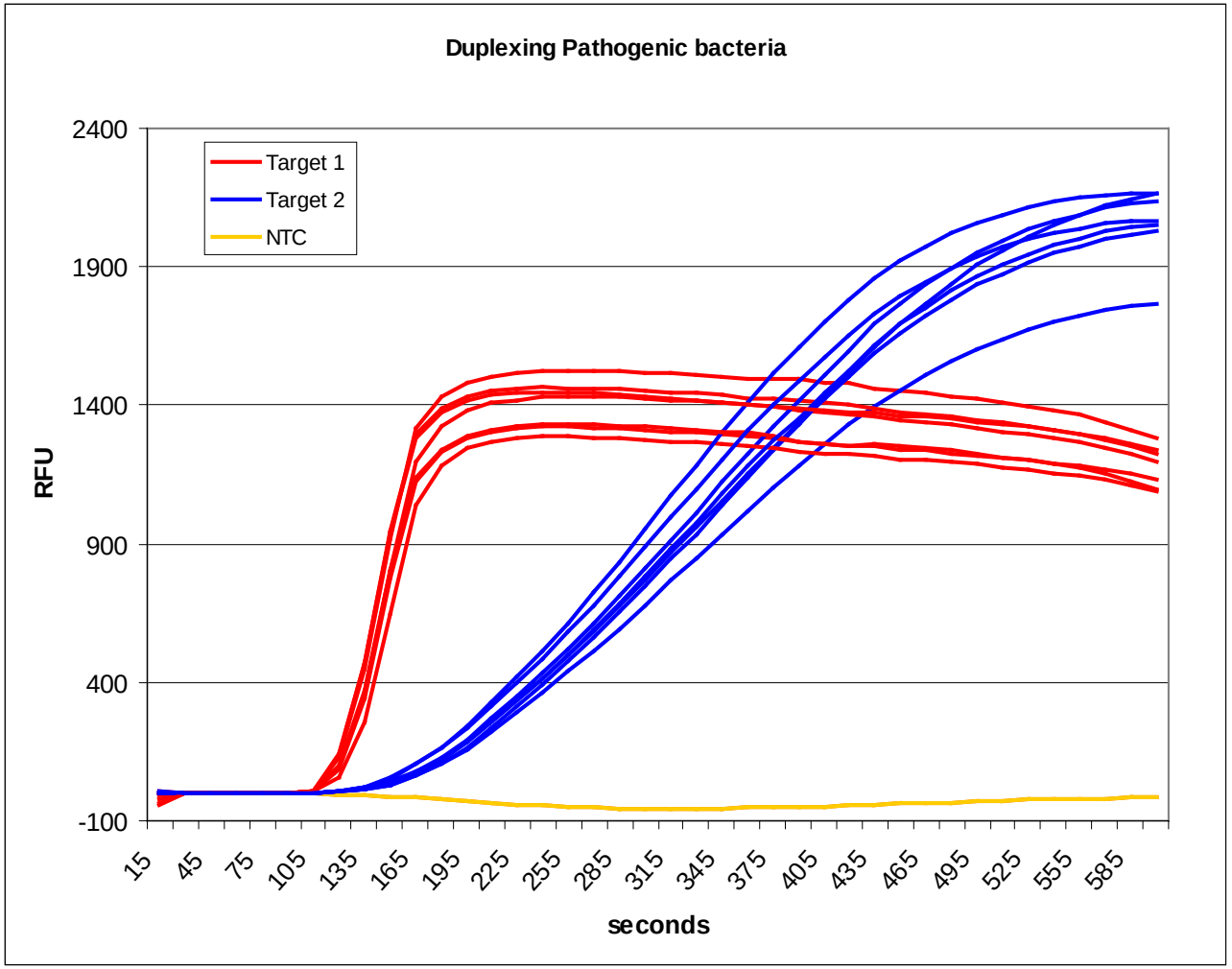
Duplexing – Simultaneous Detection of 2 Targets within One Bug



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DNAble™ v2.0 Reaction

- New assay control/tuning mechanism beyond the usual “primer limiting” conditions.
- Provides defined reaction conditions allowing duplexing/multiplexing.
  - Efficiency matching.
  - Decrease competitive reaction conditions.
- Allows “cold start” reaction enabling use in robotic liquid handling.
- Generates classic sigmoidal amplification curve with high end-point.
- Allows rapid discovery/development of new assays.
- Absolute and Relative quantification.
- Compatible with all levels of throughput capacity.
  - oRanging from POC to Douglas Scientific UHT.

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Detection Methods

- Lateral Flow Device (LFD)
  - Presence/Absence – DNA
  - Presence/Absence/Quantitative – Immuno
- Fluorescence
  - End-Point
    - Presence/Absence
  - Real-Time
    - Presence/Absence/Quantitative

Detection Instruments

- Lateral Flow Device

Closed reaction-containing tube is transferred into the LFD, snapped closed, and read in seconds by visual inspection.

LFD

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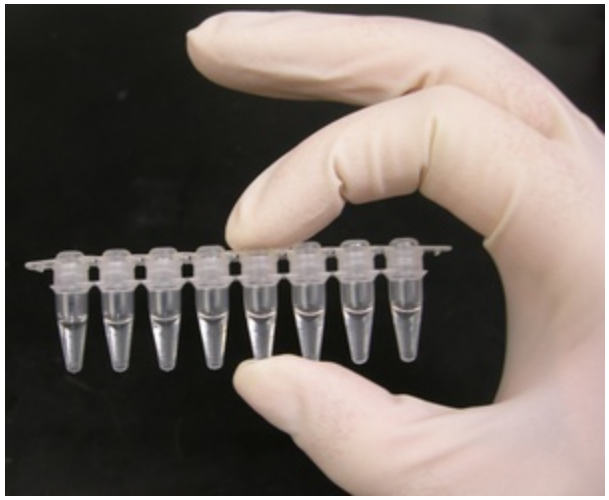
Detection Instruments

- Fluorescence



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Axxin Detection System

Axxin 16 Tube Detection System

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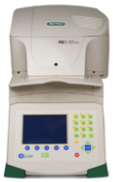
Other Detection Systems

ABI 7900
28 x 33 x 25

ABI 7500
13 x 18 x 19

StepOne
10 x 20 x 17

CFX96
13 x 18 x 14



iQ5
11x 23 x 15

MiniOpticon
7 x 13 x 13

LightCycler 480
24 x 24 x 22

LightCycler 2.0
11 x 20 x 15

Mx4000
30 x 18 x 20

Mx3005P
13 x 18 x 17

SmartCycler
12 x 12 x 10

RotorGene Q
15 x 17 x 11

RealPlex
10 x 16 x 16

PIXO
12.3 x 13.6 x 12.3

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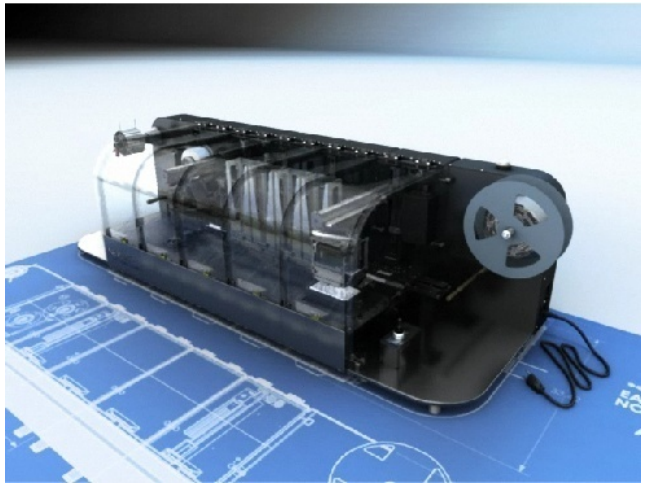
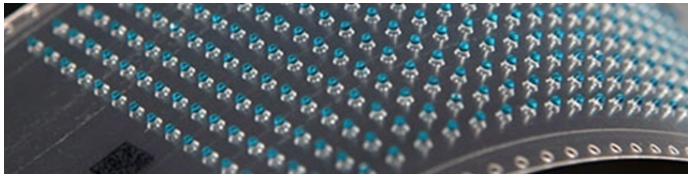
Throughput Quantum Leap

Douglas SCIENTIFIC



Nexar®



Array Tape™

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EI Nexar – End-Point/Isothermal

FELIX

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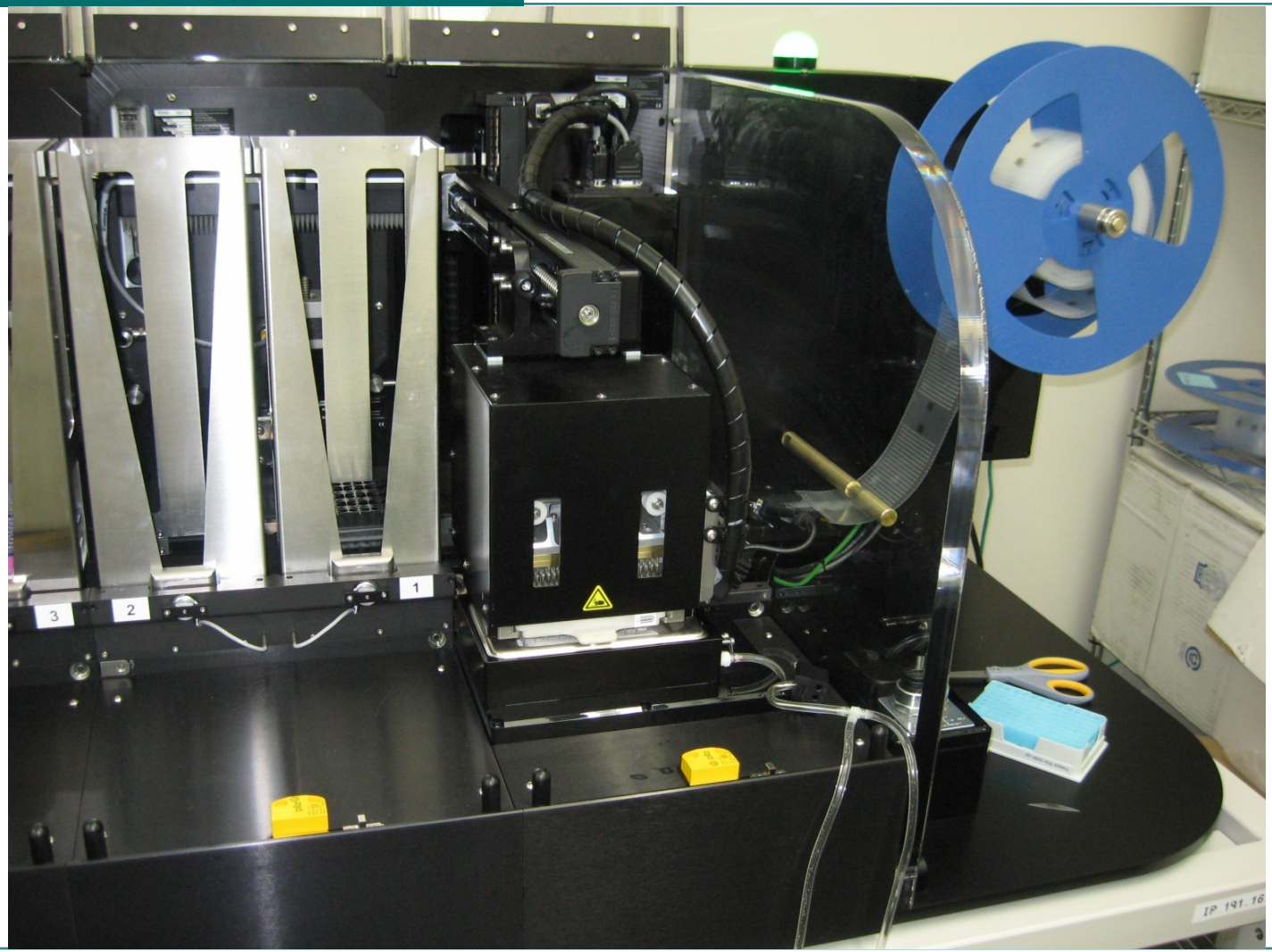
EI Nexar – End-Point/Isothermal



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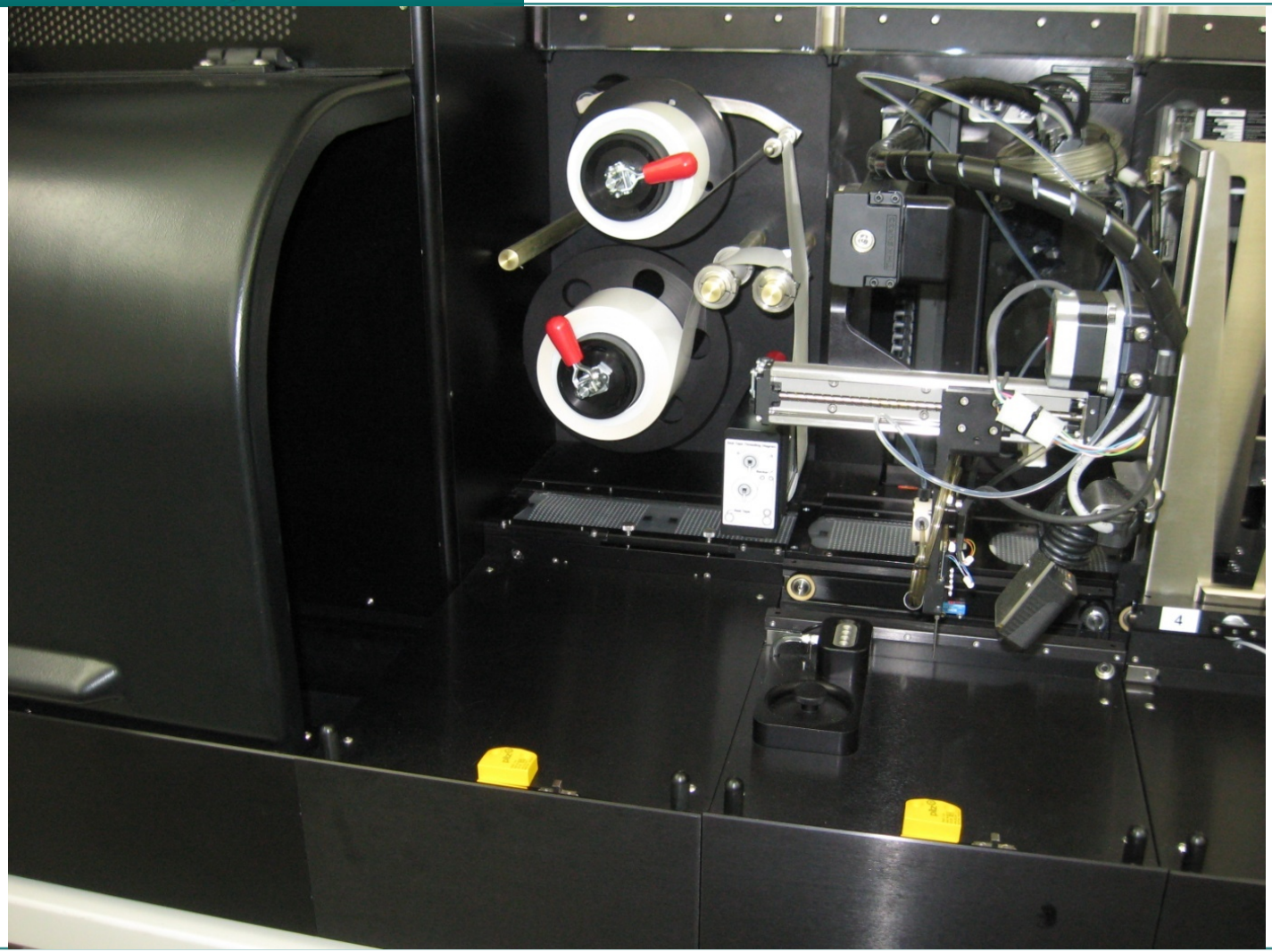
EI Nexar – End-Point/Isothermal



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EI Nexar – End-Point/Isothermal

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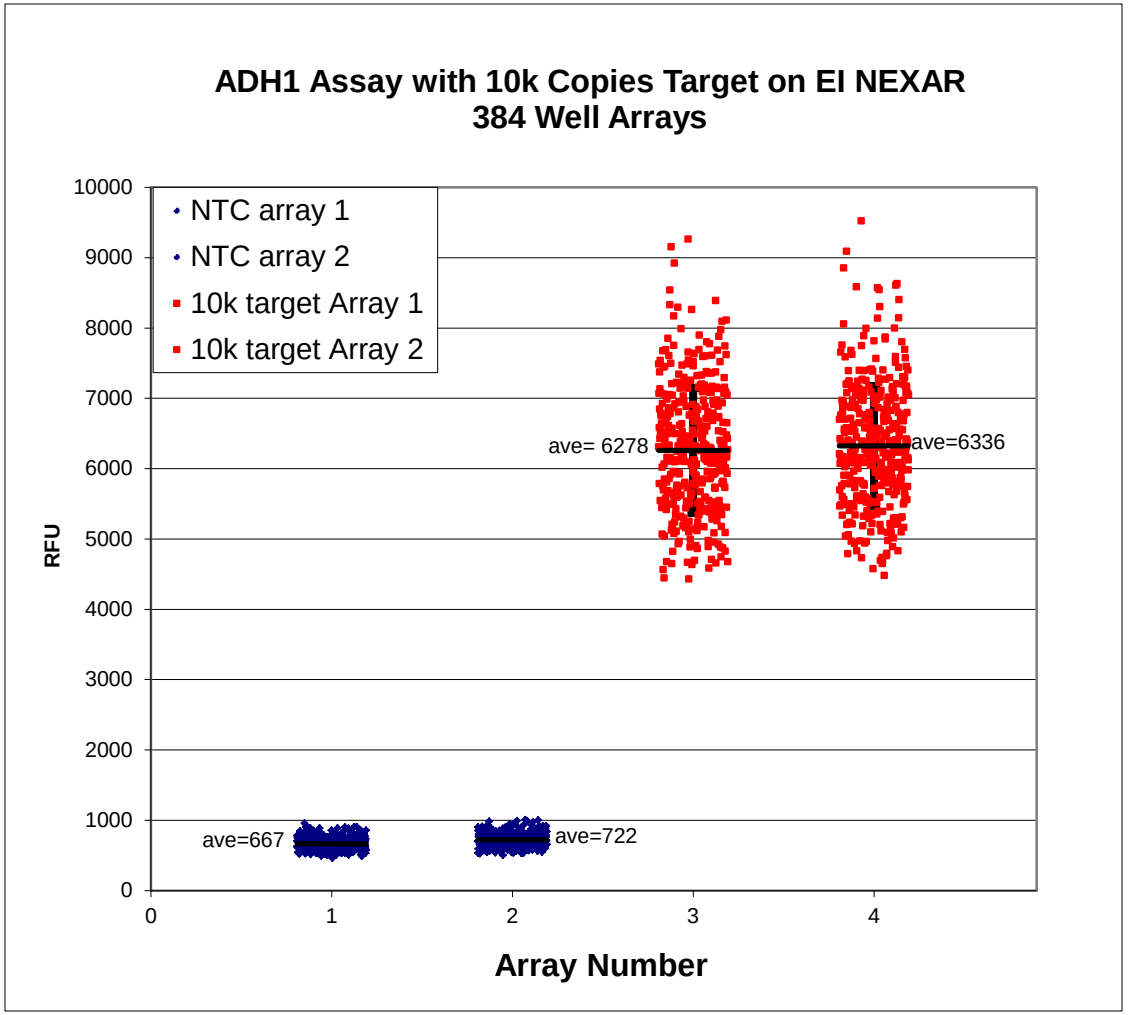

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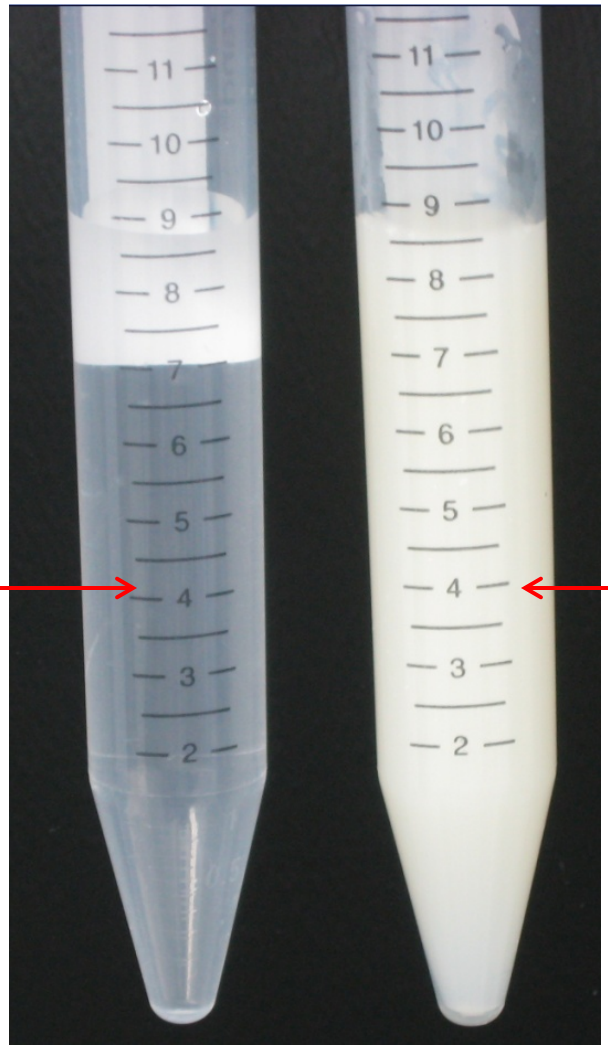
Crude Soybean Sample



Water

Soy

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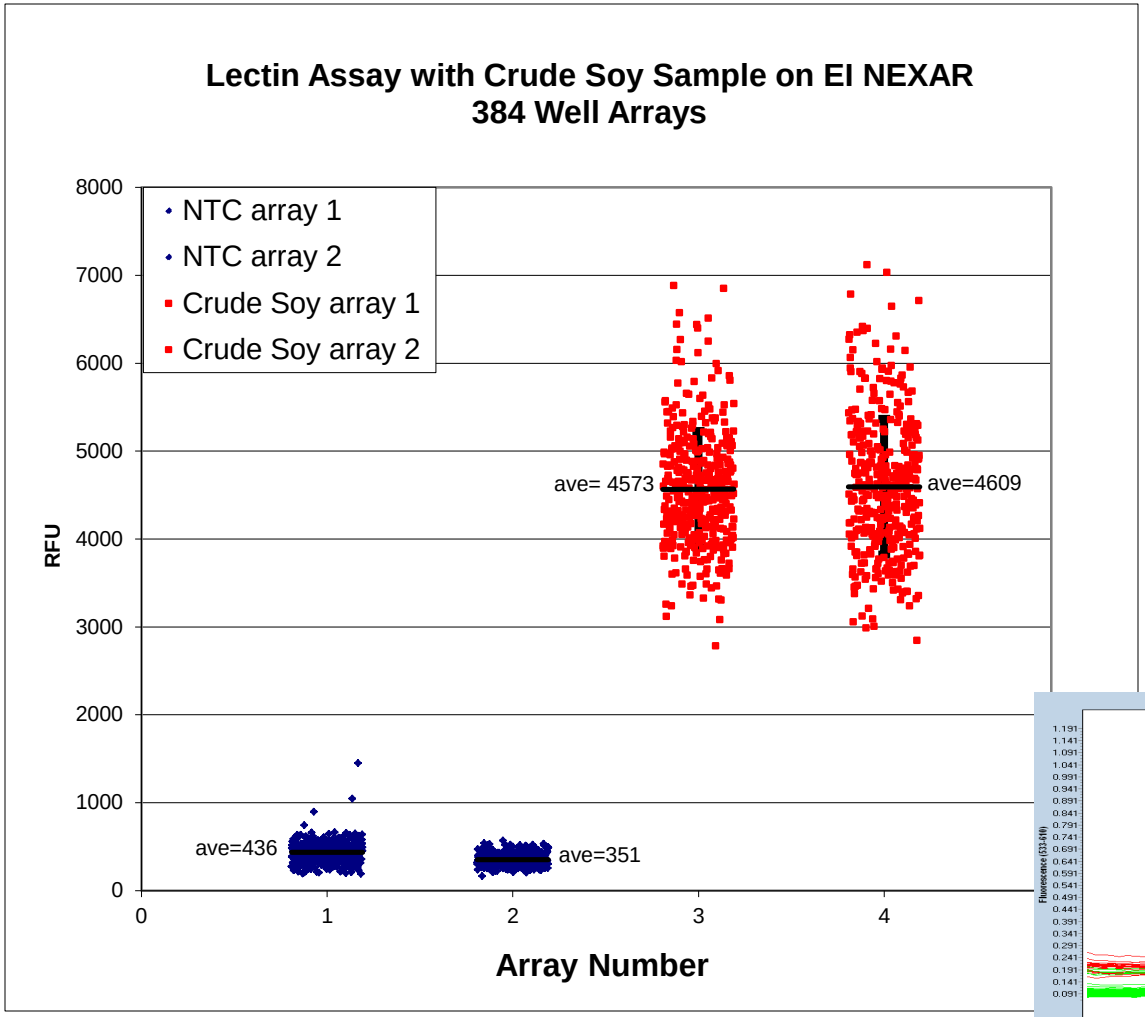
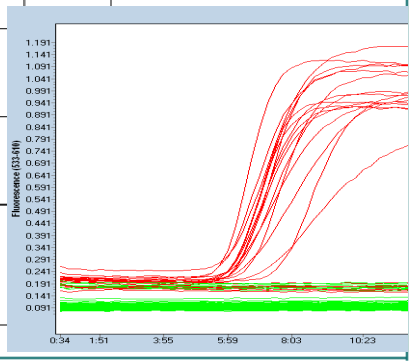
EI Nexar – End-Point/Isothermal





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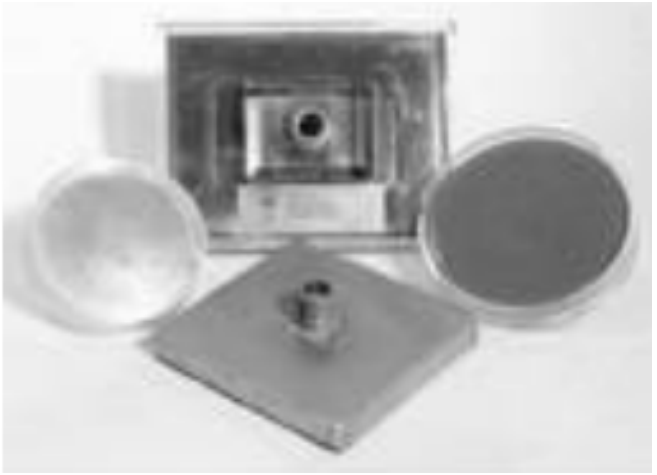
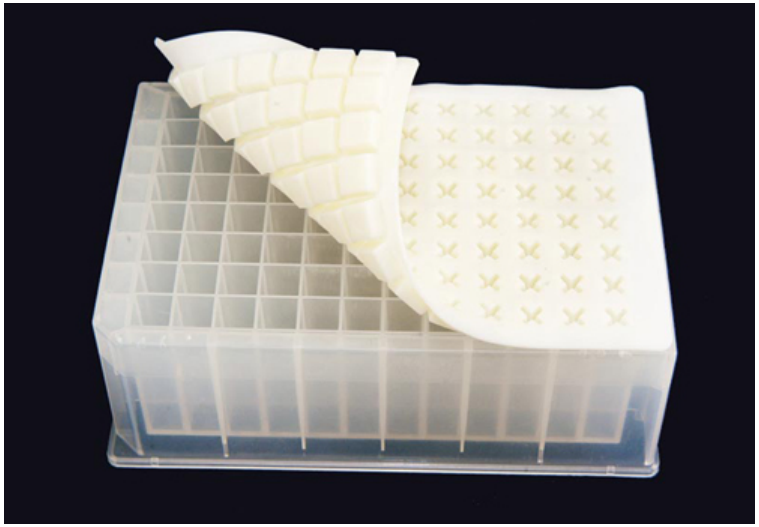
Single Tomato Seed HT Sampling



Recommended Hole Sizes for Vacuum Seed Counting Heads

0000 - Petunia, Agrostis, Tobacco
000 - Blue Grass
00 - Fescue
0 - Rye Grass
1 - Timothy, Rye Grass
2 - Clover, Alfalfa, Conifers
3 - Cereals, Wheat, Barley
4 - Squash, Sunflower, Pine
5 - As Required
6 - As Required
7 - Soybeans, Peas
8 - As Required
9 - As Required
10 - Corn

Single Tomato Seed HT Sampling



One seed per well.
SS ball.
3 X 10 second GenoGrind.
Add MB7.
Cook for 20 minutes.
Add MB7.1.
Mix by hand.
Use 2.5ul sample + 2.5ul 2X MM…..

…Or transfer to sample plate and run on EI Nexar (0.8ul sample + 0.8ul 2X MM).

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Single Tomato Seed HT Endogenous Control Assay

Proof of Concept

NTC	-0	0	1.2	1.2	1.3	1.4	1.4	1.4	1.4	1.5	1.2	1.2	1.3	1.4	0.5	0.6	1.4	1.4	1.4	1.3	1.2	1.3	-0	-0	NTC
	0	0.1	1.5	1.3	1.4	1.4	1.4	1.5	1.4	1.3	1.3	1.2	1.3	1.3	0.7	0.6	1.4	1.4	1.4	1.4	1.3	1.3	0.1	-0	
	1.2	1.3	1.3	1.2	0.4	0.5	1.2	1.1	1.4	1.4	0.2	0.2	1.3	1.3	1.4	1.3	0.9	0.9	1.2	1.2	0.5	0.5	1.3	1.3	
	1.2	1.4	1.3	1.3	0.4	0.5	1.3	1.2	1.5	1.4	0.2	0.2	1.5	1.3	1.5	1.4	1	1	1.1	1.2	0.5	0.5	1.4	1.2	
	1.3	1.4	1.3	1.3	1	1.2	1.3	1.4	1.6	1.5	0.3	0.3	1.6	1.6	1.5	1.5	1.7	1.5	1.5	1.4	1.4	1.3	1.4	1.2	
	1.2	1.3	1.2	1.3	1.2	1.1	1.4	1.4	1.6	1.6	0.3	0.3	1.6	1.7	1.6	1.5	1.7	1.6	1.5	1.5	1.4	1.2	1.4	1.3	
	0.9	1.1	1.3	1.2	1.3	1.4	1.1	1.1	0	0.1	1.5	1.5	1.3	1.4	1.3	1.4	1.4	1.5	1.3	1.3	1.4	1.4	1.4	1.3	
	1.1	1.2	1.1	1	1.4	1.5	1.2	1.2	0.1	0.1	1.5	1.4	1.3	1.5	1.2	1.3	1.3	1.4	1.3	1.2	1.3	1.4	1.4	1.4	
	1	1	1.2	1.3	1.5	1.4	1	1.2	1.6	1.5	1.7	1.5	1.6	1.5	1.4	1.4	1.7	1.6	0.2	0.2	1.1	1.2	1.4	1.3	
	1	1.1	1.2	1.3	1.3	1.5	1.2	1.3	1.5	1.6	1.7	1.7	1.5	1.7	1.4	1.4	1.7	1.7	0.2	0.2	1.3	1.1	1.4	1.3	
	1.2	1.1	0.1	0.1	1.1	1.4	0.2	0.2	1.3	1.4	1.6	1.7	1.3	1.5	1.6	1.5	1.3	1.3	0.2	0.2	1.3	1.2	0.9	0.9	
	1.1	1.2	0.1	0.1	1.2	1.2	0.2	0.2	1.3	1.4	1.7	1.7	1.4	1.5	1.7	1.6	1.4	1.3	0.2	0.1	1.3	1.4	0.9	0.8	
	0.2	0.2	0.7	0.7	1.2	1.4	1.1	1.4	1.7	1.7	1.7	1.7	1.6	1.6	0.3	0.3	1.2	1.1	1.1	1	1.3	1.5	1.2	1.2	
	0.2	0.2	0.6	0.7	1.2	1.2	1.3	1.1	1.6	1.5	1.5	1.6	1.5	1.5	0.2	0.3	1.2	1.2	1.1	0.9	1.4	1.2	1.2	1.2	
NTC	0	0	1	0.9	0.1	0.1	1.3	1.4	1.5	1.3	1.5	1.5	1.3	1.3	1.3	1	1.1	1.1	0.4	0.2	0.8	0.8	0.1	0	NTC
	-0	-0	0.9	0.8	0.1	0.1	1.3	1.4	1.2	1.3	1.4	1.5	1.3	1.3	1.1	1.2	1.1	1	0.2	0.3	0.8	0.9	-0	-0	

Tomato Endogenous Control gDNA DNable reaction. Corners are NTC's. Single tomato seeds from a crude reaction mix. Colors are determined by 2 std deviations from mean of the NTCs. First trial. Project ongoing. Red ovals are drop-outs. 96.875% correct calls. 3 out 92 drop-outs (3.261%).

Single Tomato Seed HT Endogenous Control Assay

Proof of Concept

Advantages:

- Rapid Sampling –
  - Minimal potential for missed targets.
  - Endogenous internal control target.
  - Proprietary culture method for increased sensitivity.
  - No organic solvents.
- Scalable -
  - Standard qPCR instruments, 96-well or 384-well.
  - EI Nexar HT
- Statistically Significant Data –
  - True counts of detectably infected samples (seed).
  - Not biased by handling procedures.
  - Technical replicates for added confidence.

Throughput Potential:

Assume a continuous run of samples and Array Tape (samples and Tape non-limiting)….

then in **ONE** 8 hour day it is possible to acquire 200-300K data points using 1.6ul DNAble reactions…. or about 500 plates….upwards of 31,000 reactions per hour.

To put this in perspective……

ENVIROLOGIX™ Putting Science to the Test

Previous “high-throughput” qPCR world

300 384-well plates run on Roche LightCycler 480 in **~60 days** with 10ul reactions

DNAble™ v2.0 Features Summary

Sequence-driven (primer) amplification specificity – First Layer of Specificity

Detection via Fluorescent Probe/Beacon – third point of contact – Second Layer
- Beacons – High Specificity, SNP discrimination
- Probes - High Specificity, SNP discrimination

Wide dynamic range

Theoretical single molecule detection

Sample Prep –
- QPCR typically needs cleaner purified DNA
- **DNAble™ tolerates "crude" sample types**

Very High S/N ratio -
- DNAble™ compatible with small volume reactions

Faster Time-to-Results

- QPCR typically 1-2 hours cycling time
- **DNAble™ < 10 minutes**

ENVIROLOGIX™ Putting Science to the Test

Summary

✓Fundamental understanding of the reaction enables better/faster/easier assay development.

✓Predictable assay performance – DNAbler Software, Tunable reactions.

✓Improved assay performance – Higher specific reaction efficiency.

✓Expanded assay performance – Endogenous or Exogenous Internal Controls.

✓Applications: Anything that has historically been "owned" by PCR – SNP's, +/-, Absolute Quantification (Std Curve), Relative Quantification (Internal Normalization).

✓High- and Ultra High-Throughput end-point detection using the Douglas Scientific EI Nexar system.

✓High- and Ultra High-Throughput real-time detection using the Douglas Scientific EI Nexar system moving forward.

Acknowledgements

The Douglas Scientific Team – Too many great people to mention them all but…
Visit the booth to meet them and see the EI Nexar system.

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Dr. Stephen Judice – Senior Scientist
Dr. Jonathan Rud – Senior Scientist

Lars-Erik Peters – Director, Mol Dx
Dr. Breck Parker – VP, Product Development

ENVIROLOGIX™
Putting Science to the Test

Thank You For Your Time

Questions?