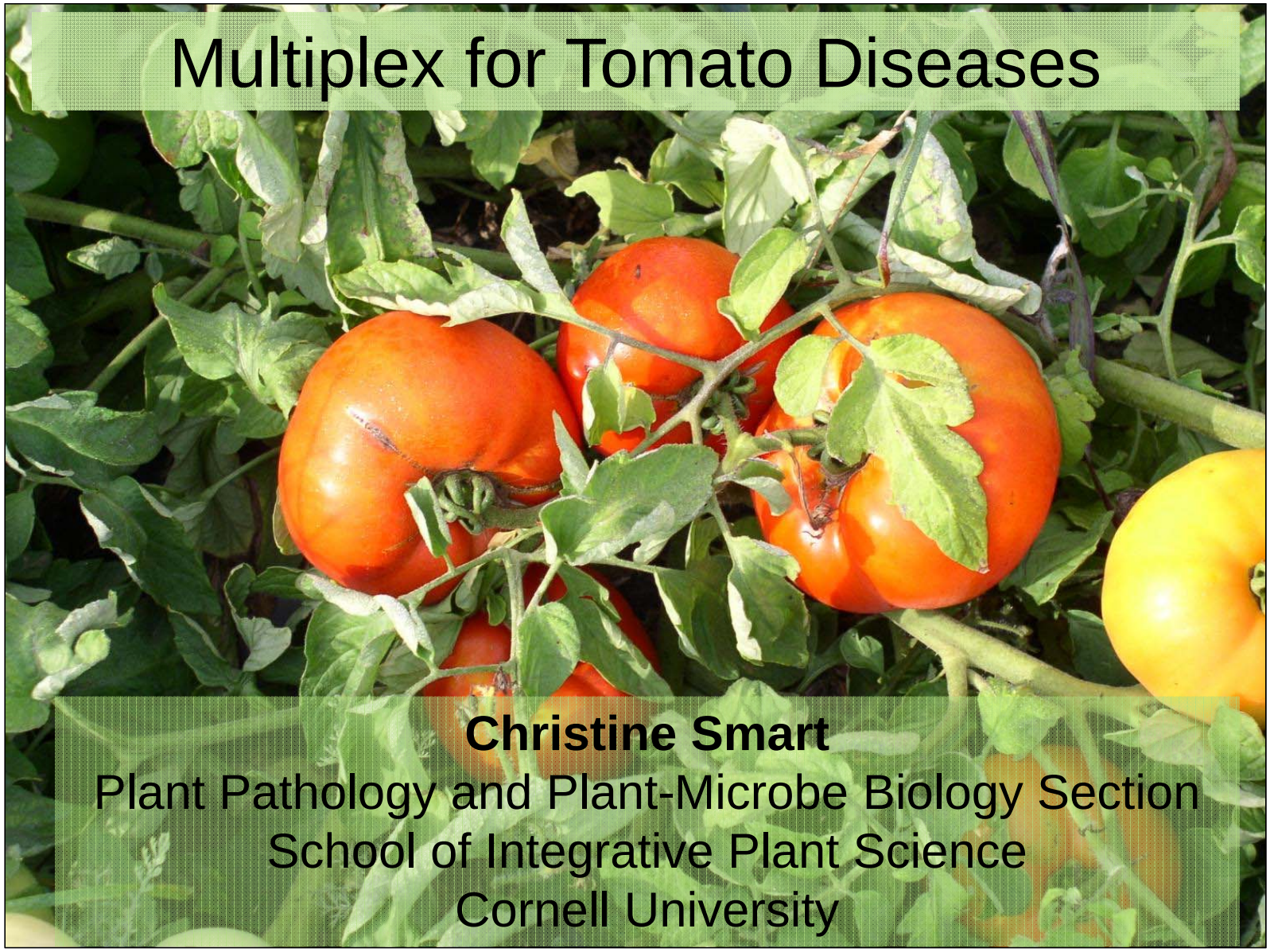
Multiplex for Tomato Diseases



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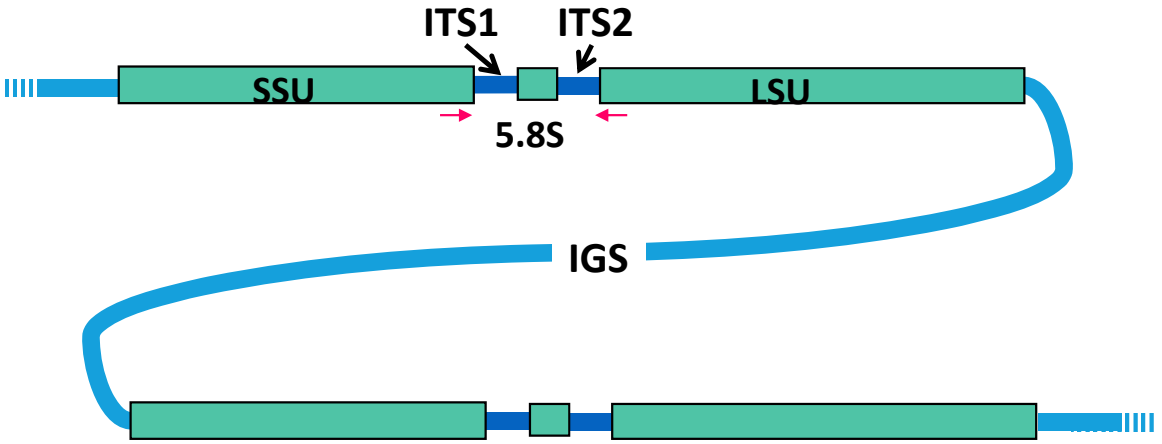
Multi-pathogen Detection

- To produce a rapid method to detect known and emerging pathogens
  - Those that are already present in NY and also pathogens that are present in other parts of the country/world but not yet in NY
- Want to detect multiple pathogens at the same time
  - Fungi and oomycetes (Smart), bacteria (Charkowski), viruses (Perry)
- Started with pathogens of solanaceous crops (potato, tomato, pepper, eggplant)

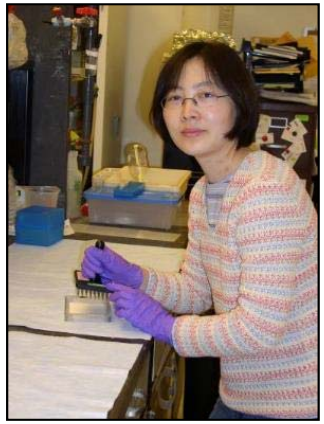
Fungal/oomycete ribosomal RNA gene repeats



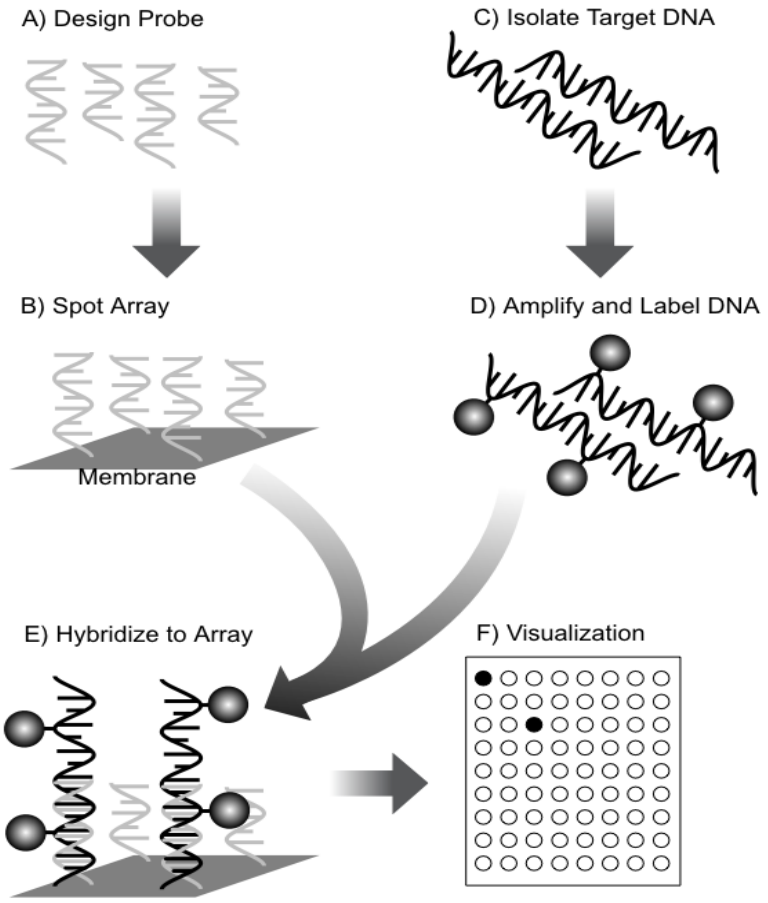
Target Gene: Internal Transcribed Spacer (ITS)

- Variability enables discrimination of closely related species
- Well studied for fungal phylogeny, and therefore a large number of ITS sequences are available
- Universal primers available

Pathogen Detection Macroarray

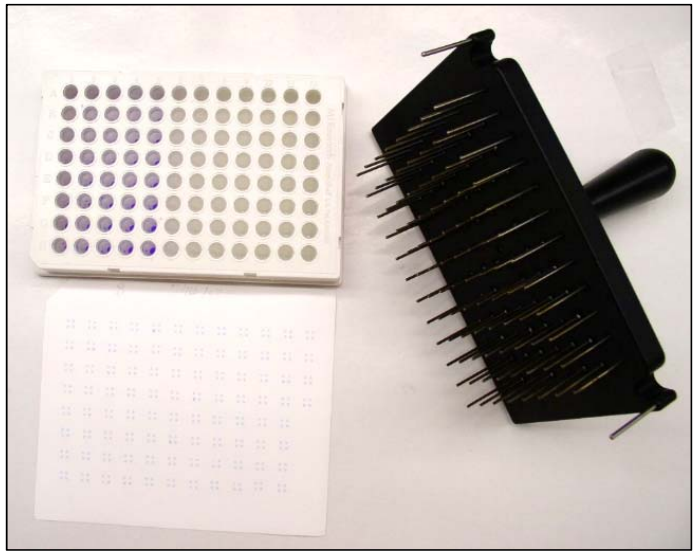


Ning Zhang



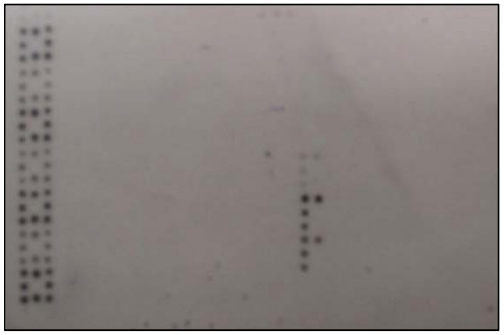
Probes
(Oligonucleotides)

Nylon Memberane
(Hybond N+)



96-pin
replicator

Hybridization

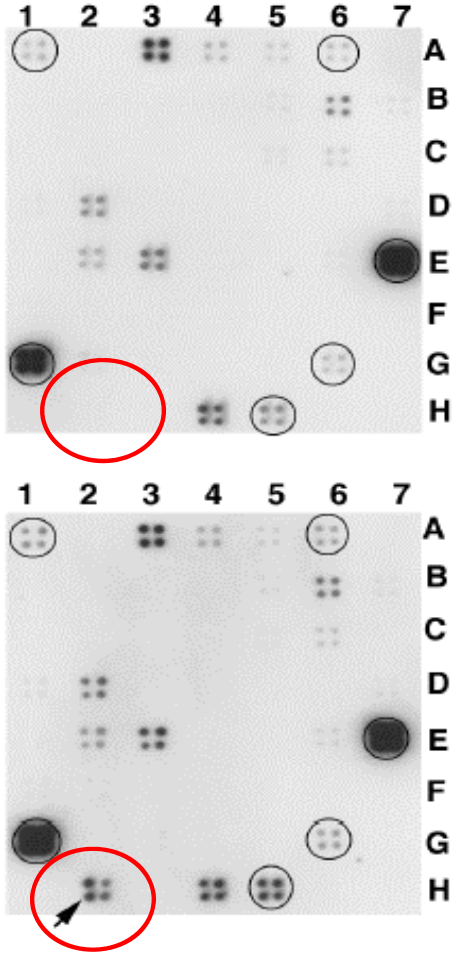


Signal Detection
(Kodak Film)

Keys to Success

- Ensuring all probes have a similar annealing temp can be tricky.
- Hybridization temperature!!
  - 55 C was the temp that worked with our set of probes.
- Probe length and G+C content
  - We found that probe length (22 bp) was more important than GC content in our experiments (50% GC was optimal)
- Location of SNP mismatch in the probe
  - Central mismatch enabled much greater specificity than an end mismatch
- Amount of probe on membrane
- Concentration of PCR amplicon

Two closely related strains of FSSC with one SNP differentiating the strains



Array hybridized with PCR amplicon from strain 1

Single Nucleotide Polymorphisms can be distinguished by probe at bottom of row 2

Array hybridized with PCR amplicon from strain 2

Pathogen Detection Macroarray

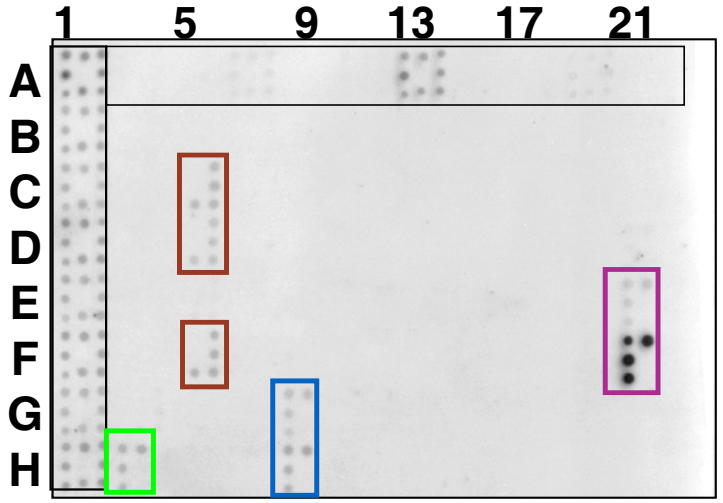
- The current array has 141 oligos (including controls) for the detection of 32 pathogens – plus 12 members of FSSC
  - Minimum of two probes per pathogen
- The array has been tested for cross-hybridization with DNA from 29 of the 32 pathogens (could not obtain DNA from 3) as well as dozens of non-pathogens
- Can detect as little as 0.04pg DNA

Pathogens on Array

Probes designed and tested		Probes designed/not tested
Alternaria alternata	*Phytophthora infestans*	*Pyrenochaeta lycopersici*
Alternaria solani	*Ph. nicotianae*	
Botrytis cinerea	*Ph. erythroseptica*	
Colletotrichum acutatum	*Pythium aphanidermatum*	*Oidiopsis sicula*
Col. coccodes	*Pythium myriotylum*	
Col. gloeosporioides	*Pythium ultimum*	*Synchytrium endobioticum*
Corynespora cassiicola	*Pythium irregulare*	
Fulvia fulva	*Pythium cryptoirregulare*	
Fusarium oxysporum	*Rhizoctonia solani*	
Fusarium sambucinum	*Septoria sp.*	
Fusarium solani **complex (12)**	*Sclerotinia sclerotiorum*	
	Spongospora subterranea	
Oidium neolycopersici	*Stemphylium solani*	
Phoma destructiva	*Verticillium albo-atrum*	
Phytophthora capsici	*Verticillium dahliae*	

Leaf wilting
Vascular discoloration
Unhappy roots
DNA extraction from roots, stem and leaves

Array



Controls

From DNA Extraction to Array Results:

12 hours

Multiplex Detection Power

H3: *Fusarium solani*

C6,D6,F6: *Phoma destructiva*

G9,H9: *F. oxysporum*

E21,F21: *Alternaria alternata*

Culture

***Fusarium* spp.**

Alternaria alternata

Pathogen Detection Macroarray

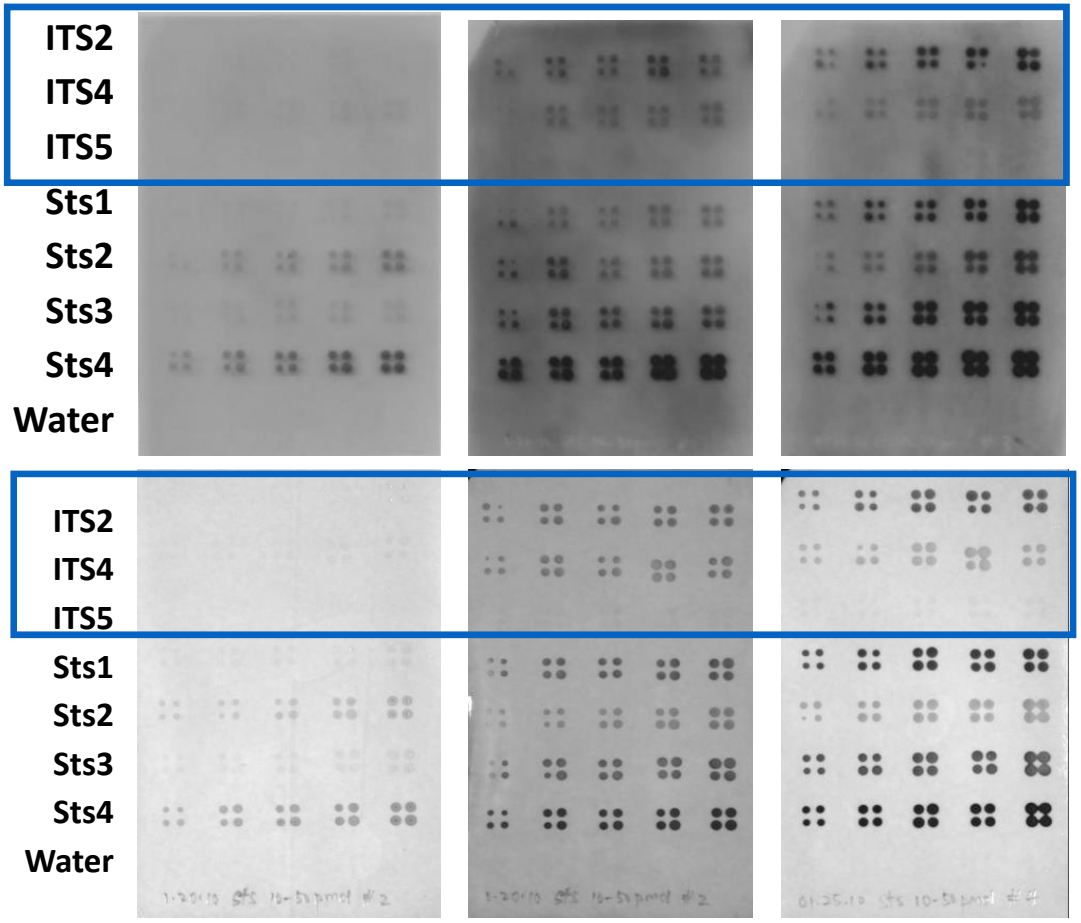
- Current array is working well for diseased plant samples
  - We have switched over to isothermal or multiplex real-time PCR assays
- Used extensively in a cover crop study
- Visiting scientist wanted to develop color-based detection method

Detection Methods (light vs. color)

- Detection
  - Chemiluminescent: CDP-Star (GE Healthcare)
  - Chromogenic: Digoxigenin / NBT-BCIP (Roche)
    - Nitro-blue tetrazolium – 5bromo-4chloro-3indolylphosphate
- Compared concentration of oligos on membrane: 10-50 pmol
- Compared concentration of PCR amplicon (5, 10 & 15 ng/ml)

Mui-Yun Wong
Universiti Putra
Malaysia

Stemphylium solani



Chemiluminescent

Hybridization: o/n
Film exposure: o/n

Chromogenic

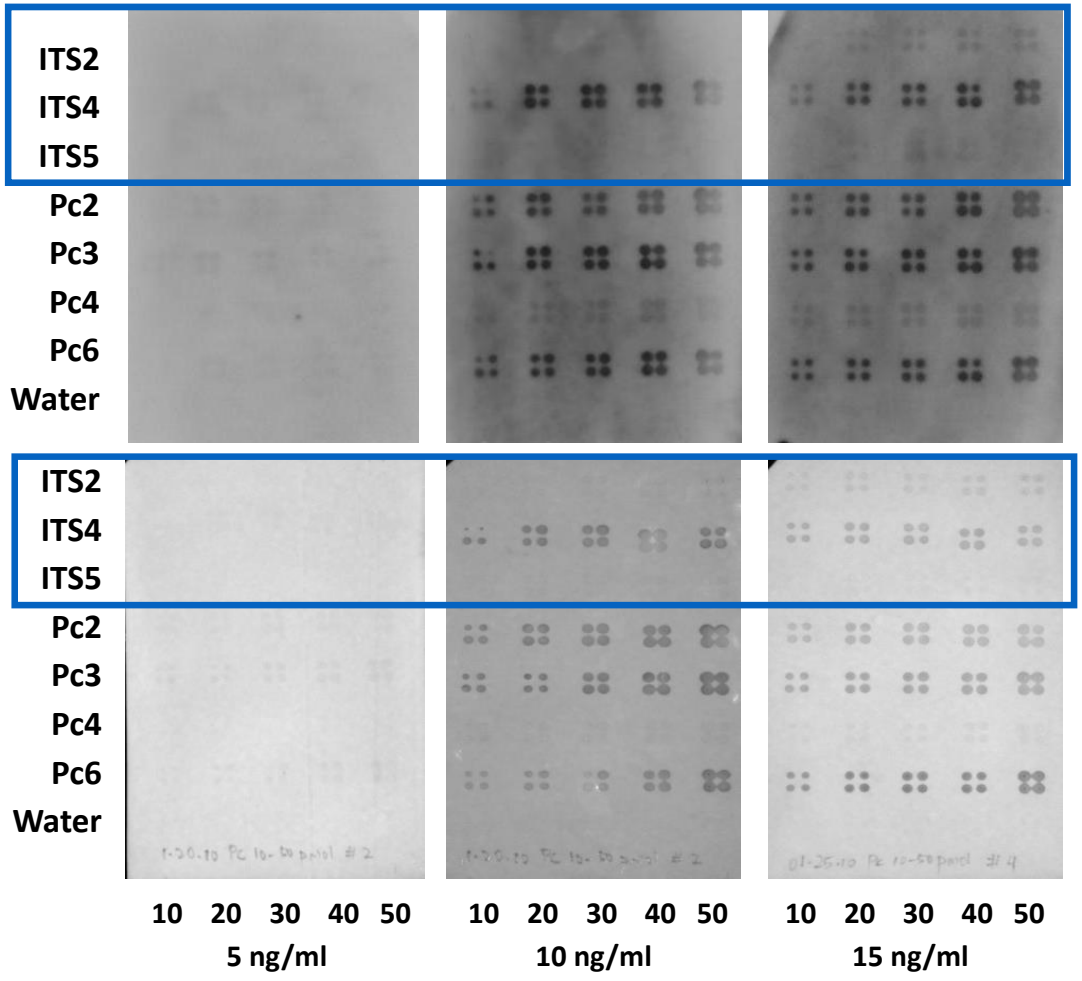
Hybridization: o/n

NBT/BCIP
incubation: 3 h

Oligo concentration (pmol)

PCR product conc.

Phytophthora capsici



Chemiluminescent

Hybridization: o/n
Film exposure: o/n

Chromogenic

Hybridization: o/n

NBT/BCIP
incubation: 3 h

Oligo concentration

PCR product conc.

Light vs Color

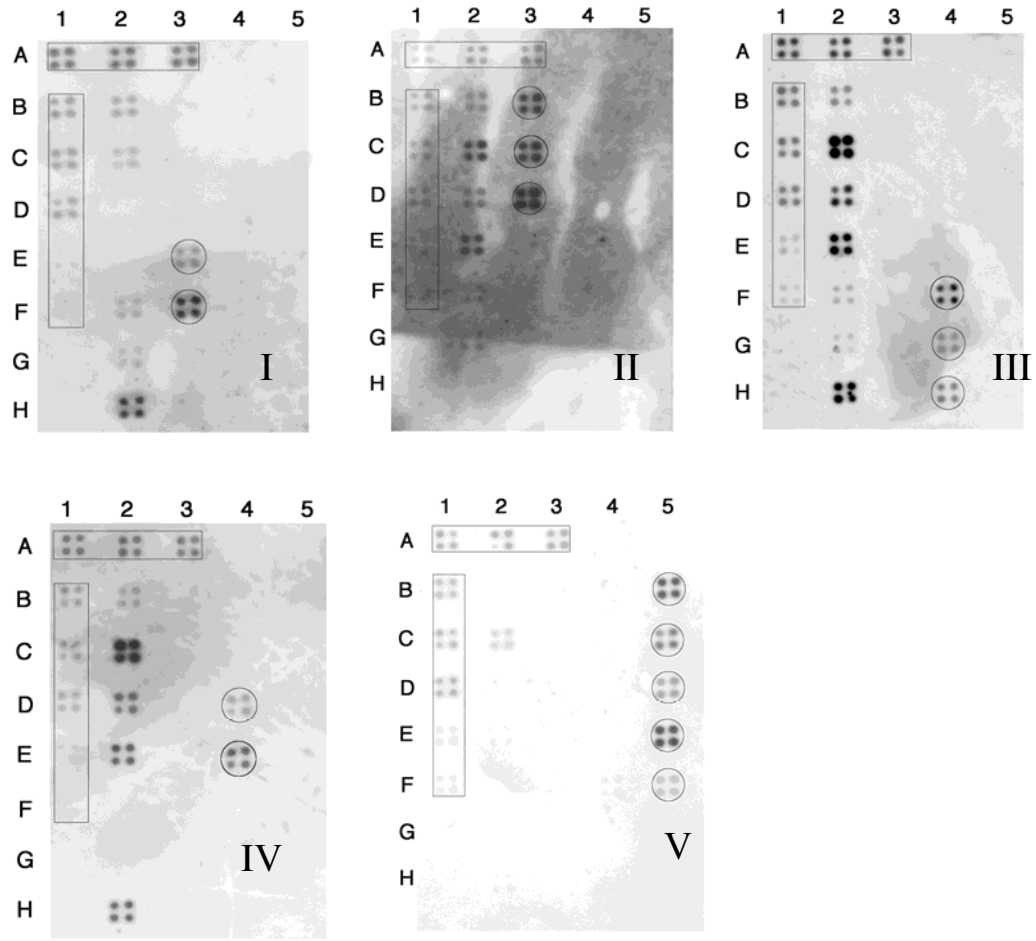
- Increased probe concentration on membrane and target DNA PCR amplicon concentration improved signal
- Chromogenic detection method is comparable to chemiluminescent method in terms of specificity and hybridization duration
- Chromogenic method offers a convenient alternative to chemiluninescent method for researchers who do not have dark room facilities

Detection and differentiation of five lineages of *Fusarium oxysporum* f. sp. *vasinfectum* (Fusarium wilt of cotton)

- First identified in California in 1960
- Widespread in the United States and most cotton-growing areas of the world
- Typical symptoms: wilting, interveinal chlorosis, brown discoloration of vascular tissue
- Eight races had been described – wanted to differentiate 5 lineages

From Mike Davis – UC Davis

Polymorphism (16 SNPs) in the Elongation Factor-1alpha gene (673 bp)

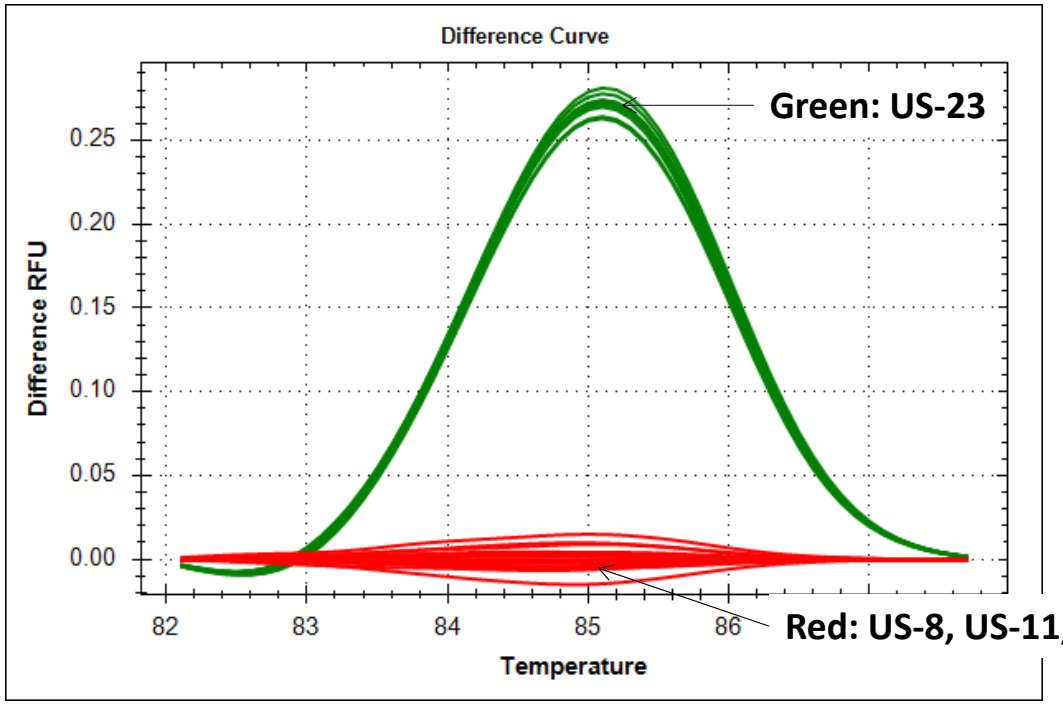


26 Fov-specific probes. Lineage-specific probes in circles.
Positive controls in rectangles.

What's next for multi-pathogen detection?

- If we are looking for 1 particular SNP – high resolution melt analysis (differentiate one lineage from all others)
- If we want to look for a larger number of pathogens, we currently use multiplex real-time PCR with differentially labelled probes
  - Can detect far fewer pathogens than on the array

Phytophthora infestans



Melt curve showing lineage US-23 with a different melt temp than other lineages

Acknowledgements

- Ning Zhang – now at Rutgers
- Mui-Yun Wong – U Putra Malaysia
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- Mike Davis – UC Davis
  - Cotton growers

USDA NIFA
United States Department of Agriculture
National Institute of Food and Agriculture