Detection of *Erysiphe necator* fungicide resistant alleles in environmental samples

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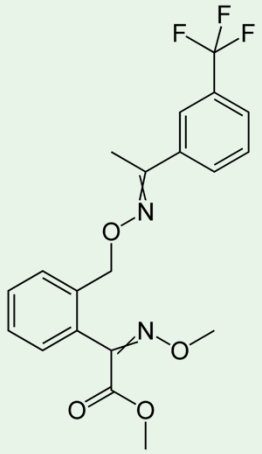
Grapevine powdery mildew control

- Powdery mildew caused by *E. necator* costs >$300 million to manage (Fuller et al., 2014)

- Primarily control methods:
  - DMI and QoI fungicides (FRAC codes 3 and 11, respectively)
  - Alternative fungicides in FRAC codes 13, U8, U6, 7, and 9
  - Elemental sulfur
  - Biological control methods (*Bacillus* spp.)
  - Contact materials (e.g. Stylet oil)

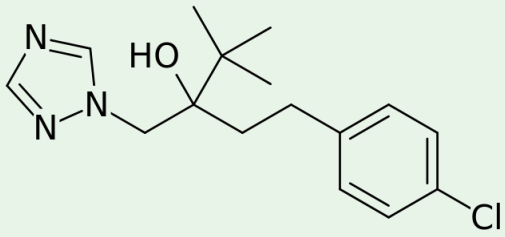
FRAC groups 11 and 3

- FRAC group 11, strobilurins, quinone outside inhibitors, QoIs
  - Inhibit fungal cellular respiration (by blocking the electron transport chain)
  - Inhibit spore germination



Trifloxystrobin

- FRAC group 3, demethylation inhibitors or DMIs
  - Inhibit sterol synthesis in membranes
  - Lack of sterols causes abnormal fungal growth, ultimately leading to death



Tebuconazole

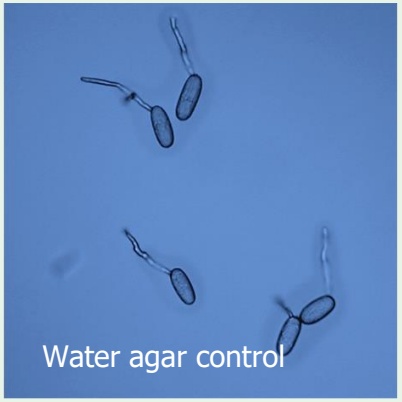
Fungicide resistance for FRAC 11

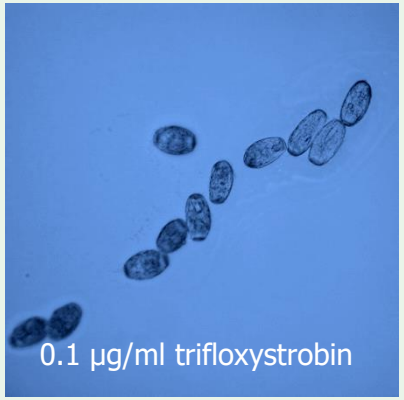
- Resistance to strobilurins
  - New York (Wilcox *et al*., 2003)
  - Virginia (Baudoin *et al*., 2008)
  - Michigan (Miles *et al*., 2012)
  - Oregon and CA (Miles and Mahaffee, unpublished)

- In total, 11 point mutations have been identified in the cytochrome b gene, conferring different levels of resistance to Qo inhibitors. They are spread in two regions: aa 127–147 and aa 275–296.

- Mutation G143A has been related to high resistant levels in plant pathogenic fungi.

Susceptible isolate



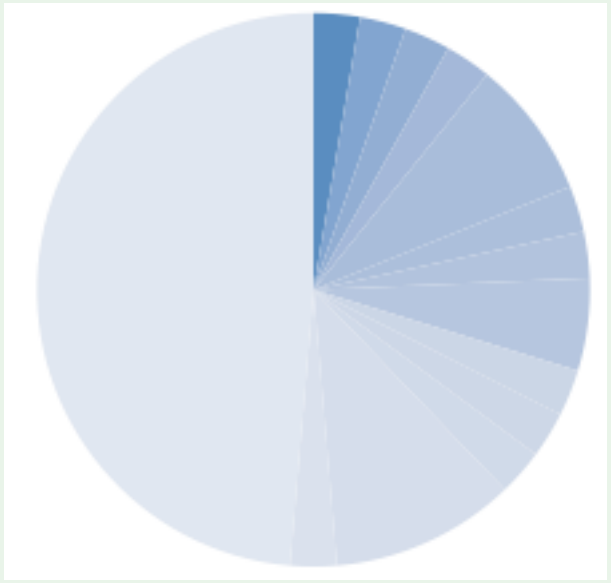


Resistant isolate

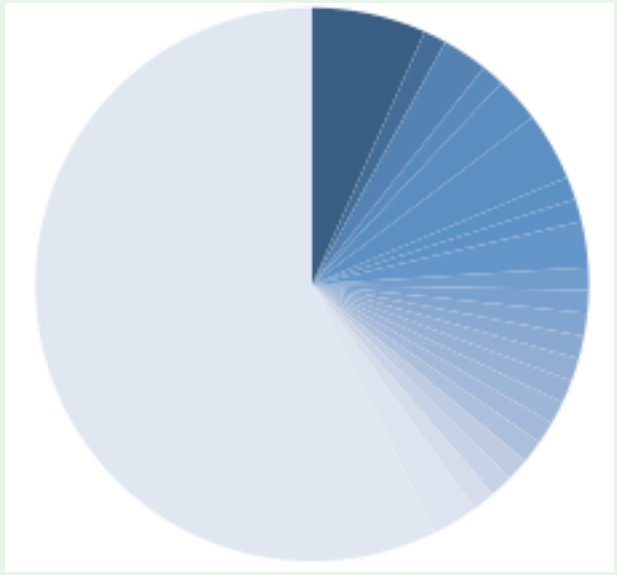
L. Miles et al., 2012, Plant Dis.

Fungicide resistance survey in Michigan during 2009

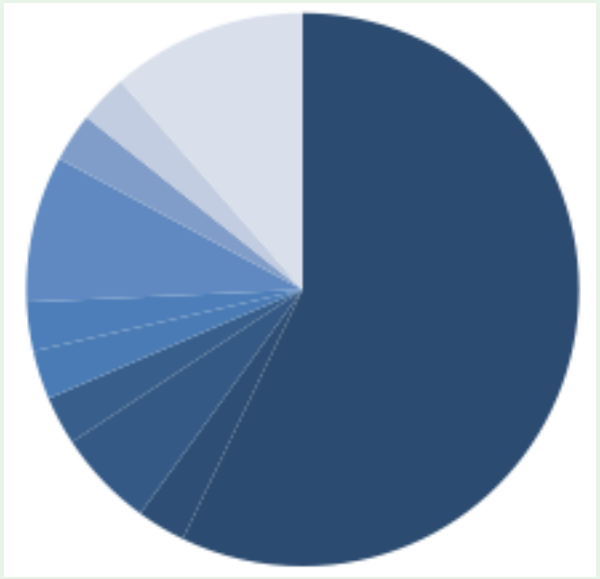
The effective dose, required to inhibit the isolate by 50% (EC50)



Baseline vineyards
n=37



Commercial vineyards
n=75



MSU research vineyards
n=35

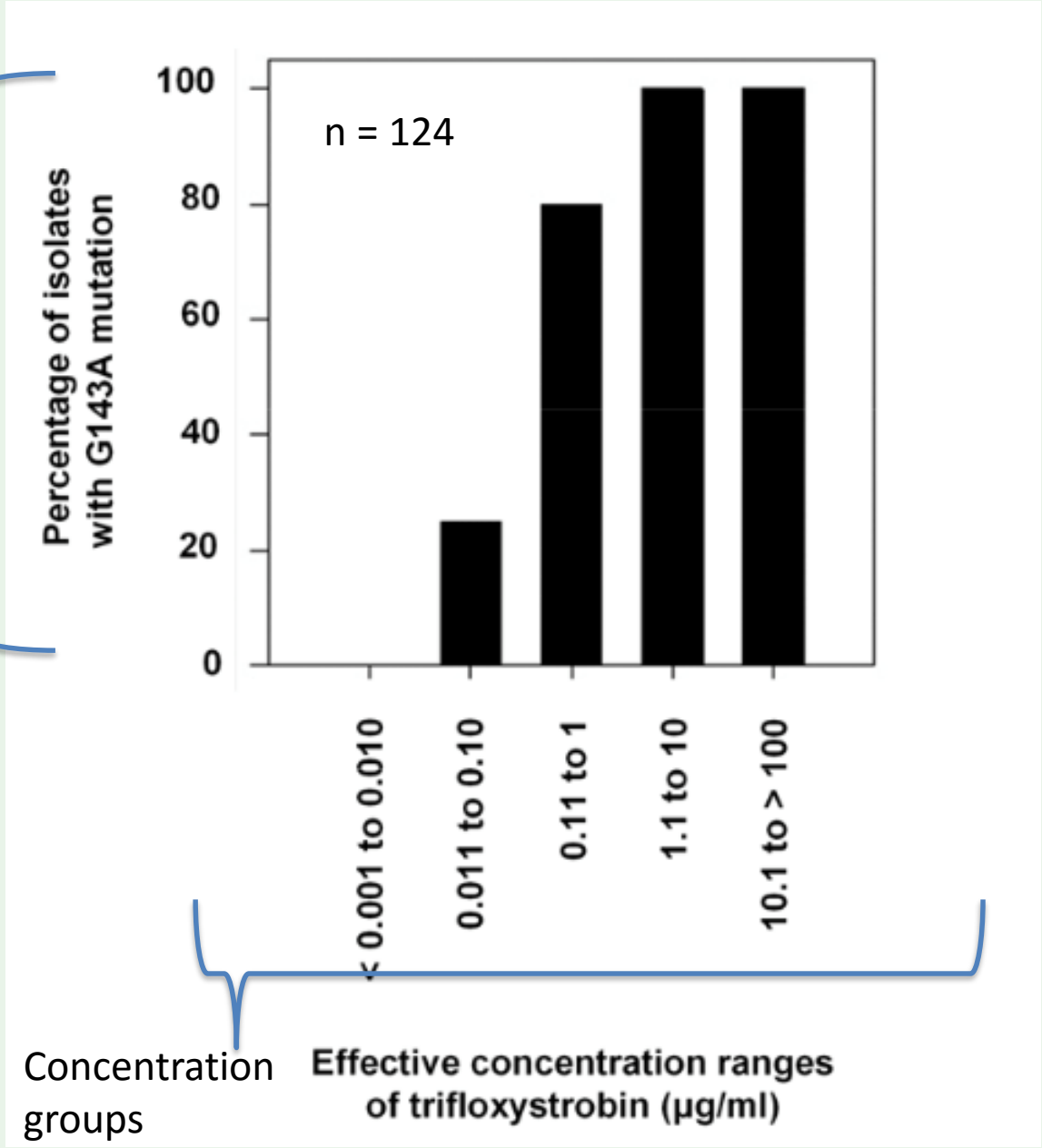
≤0.001 µg/ml

≥100 µg/ml

L. Miles et al., 2012, Plant Dis.

Correlation of G143A to resistance

SNP presence



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Research outline

- Survey Oregon and CA for the presence of fungicide resistance
- Develop molecular assays to detect the QoI alleles without the need for culturing
- Making assays field portable

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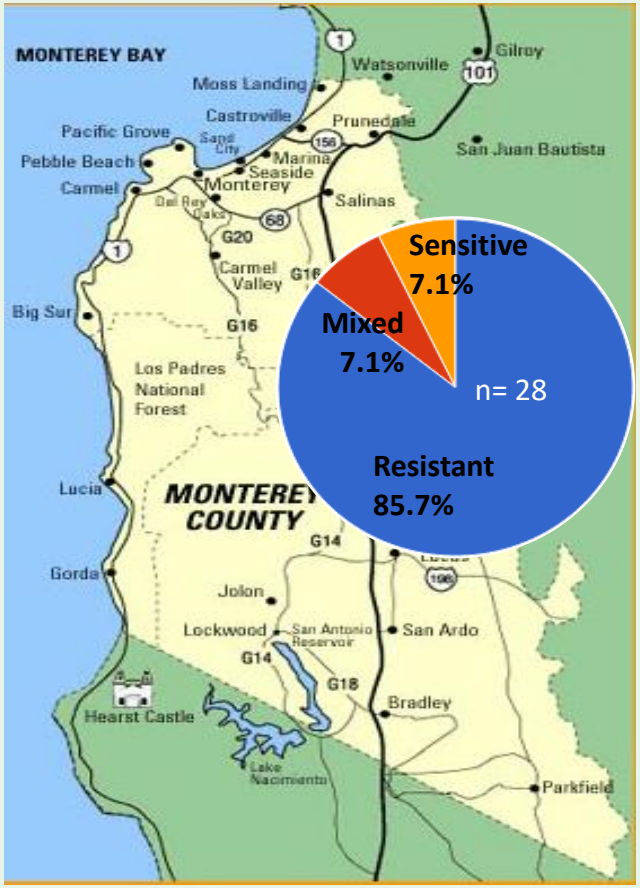
Survey methods

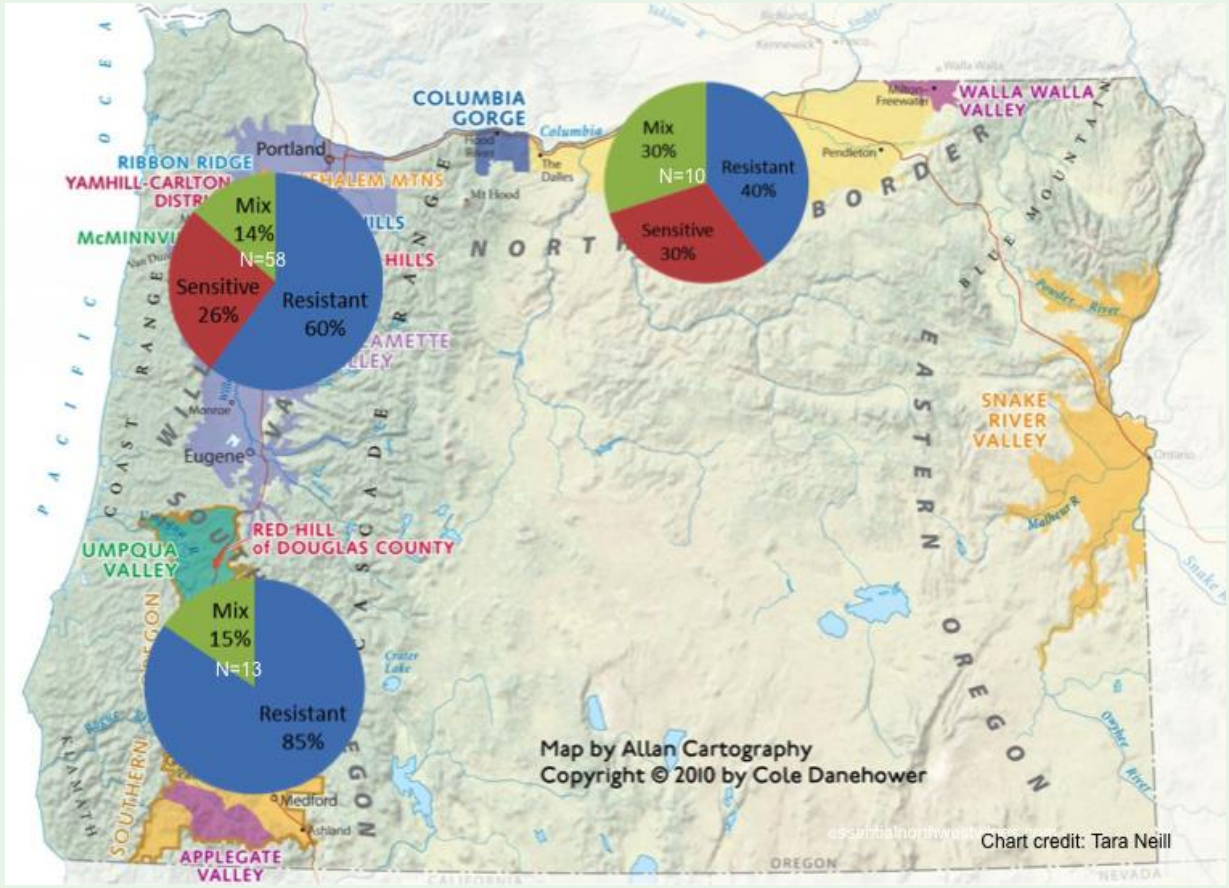
- Surveyed vineyards in CA and Oregon, plants with obvious symptoms were stored and returned the the laboratory for DNA extraction
- Often adhesive tape was used to excise colonies from leaves
- Later in the collection single spore isolates were obtained in Oregon locations by propagating isolates. These single conidium isolates were also used for DNA extraction
- Additionally a collection of air samples were also used to validate markers that was collected from impaction traps (provided by Mahaffee's laboratory)

2015 isolate collection and QoI resistance testing (confirmed by sequencing)

California – Santa Lucia Highlands Vineyards



Oregon – Various regions in collaboration with Walt Mahaffee's laboratory



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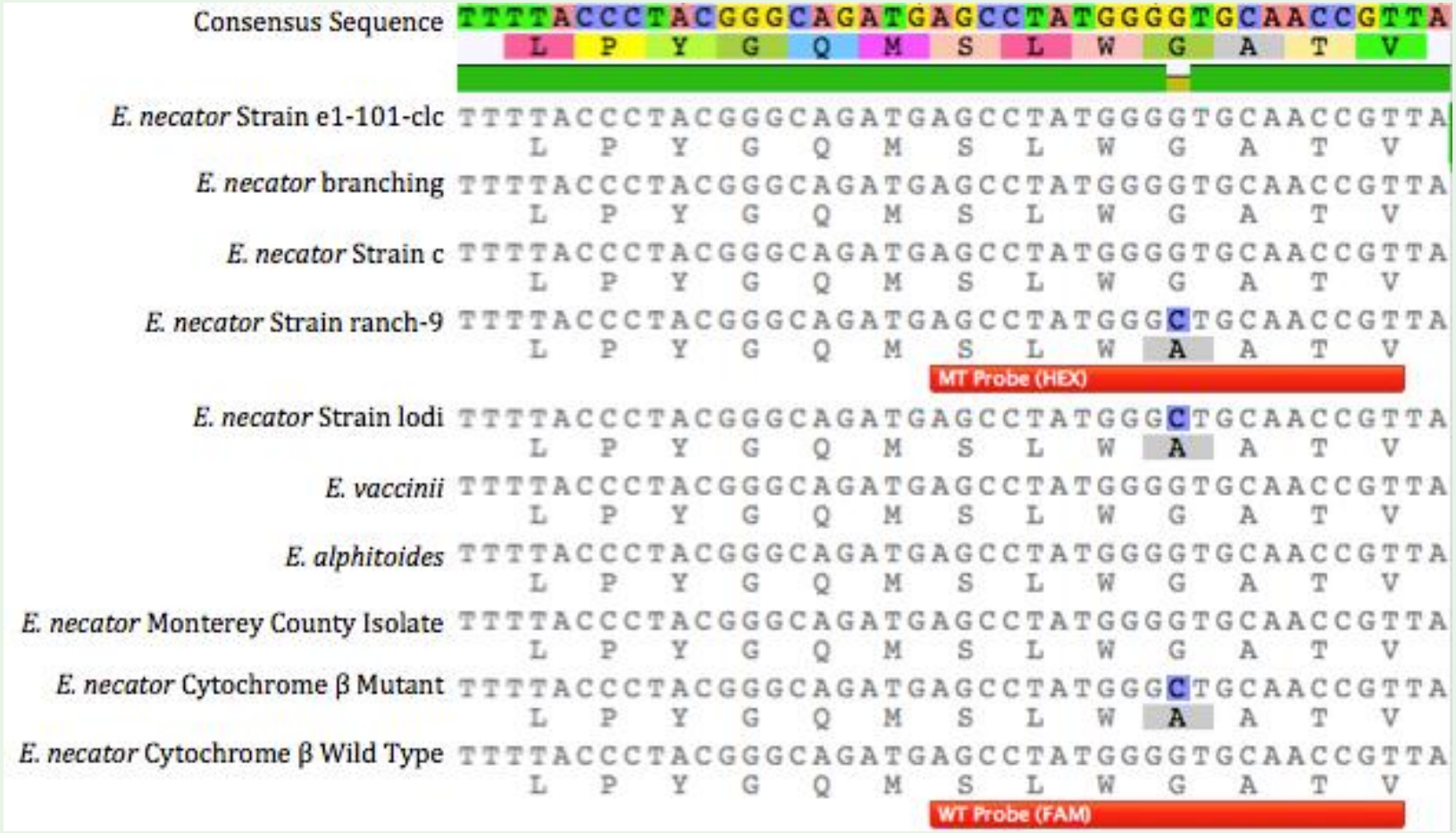
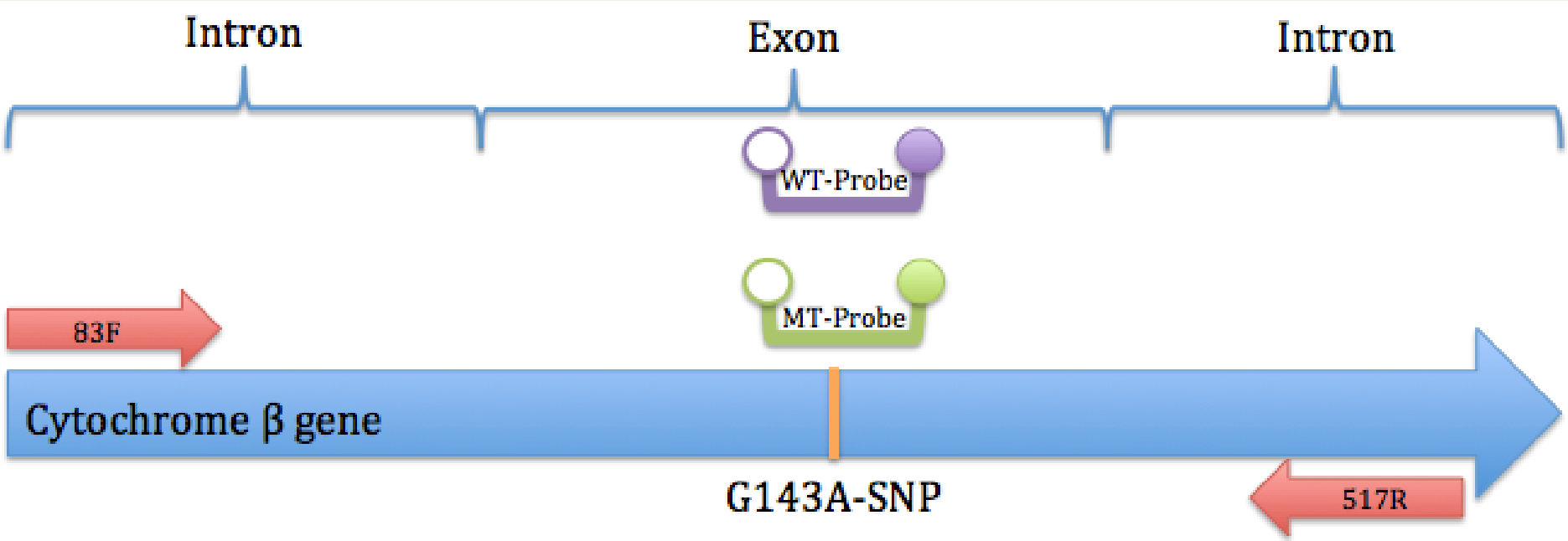
QoI assay development and comparative genomics



TaqMan assay for G143A



Tested for specificity against a wide variety of PM pathogens including (PM of pea, oak, blueberry, strawberry, cucumber and rose)

Assay cross reacts with PM of oak and blueberry

Assay validation using Michigan samples

Conventional germination assay

Wild type detection

Mutant detection

	Sample size (n)	EC_{50} (µg of trifloxystrobin per sample)	TaqMan FAM C_t (**G**143A)	SYBR Green WT C_t (**G**143A)	TaqMan HEX C_t (G143**A**)	SYBR Green MT C_t (G143**A**)
Sensitive Isolates[1]	59					
Baseline	36	0.3 ± 0.0	27.2 ± 0.8	21.9 ± 0.63	-	33.6 ± 1.0
Research	5	-	28.6 ± 7.2	21.4 ± 5.34	-	31.1 ± 7.9
Commercial	18	0.1 ± 0.0	28.3 ± 1.7	22.7 ± 1.33	-	33.2 ± 2.0
Resistant Isolates[2]	47					
Baseline	0	-	-	-	-	-
Research	26	> 1000	0	29.6 ± 1.2	27.0 ± 1.1	18.1 ± 0.8
Commercial	21	> 1000	0	30.3 ± 1.5	27.0 ± 1.4	18.9 ± 0.9

Validating these assays with leaf and air samples from Mahaffee's laboratory

	Sample size (n)	FAM C_t (**G**143A)	HEX C_t (G143**A**)
Leaf samples	82		
Sensitive	18	25.9 ± 1.5	-
Resistant	50	0	23.9 ± 0.5
Mixed	14	28.2 ± 2.2	27.0 ± 2.1
Air samples	66		
Sensitive	57	33.6 ± 0.6	-
Resistant	1	-	40.2
Mixed	8	32.1 ± 4.6	34.1 ± 4.9

- The majority of isolates obtained from leaves in the validation were resistant but the majority of air samples were sensitive, but this could be due to the time samples were collected.
- Mixed samples were observed in both leafs and air samples

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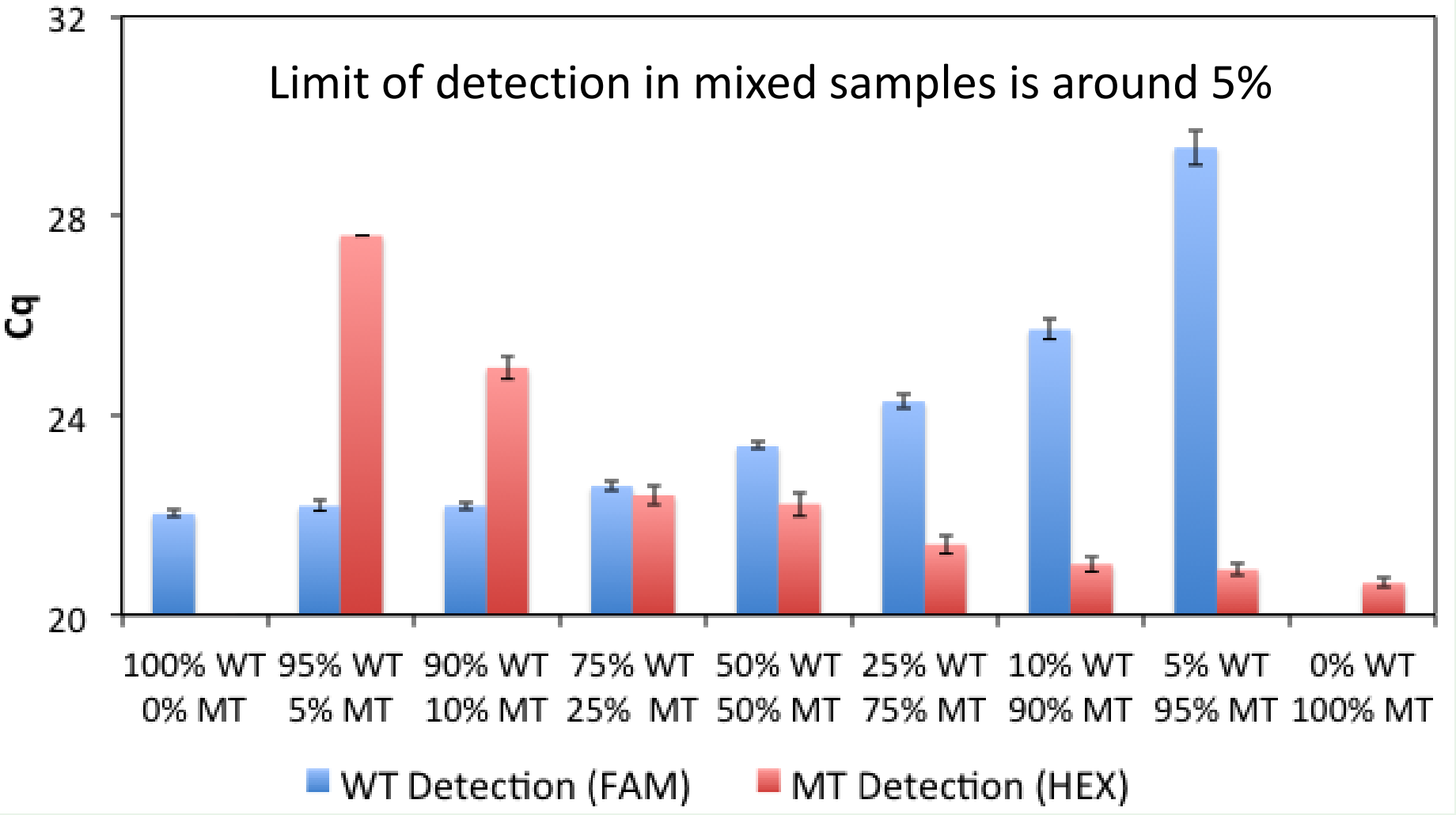
The problem of mixed samples when using environmental samples

- Taking samples directly from leaves can have multiple isolates, some which are resistant to a fungicide, some which are sensitive
- The same problem can be seen in air samples when using spore trap detection technologies
- Tools that allow us to molecularly identify mixed alleles would have utility when dealing with these samples

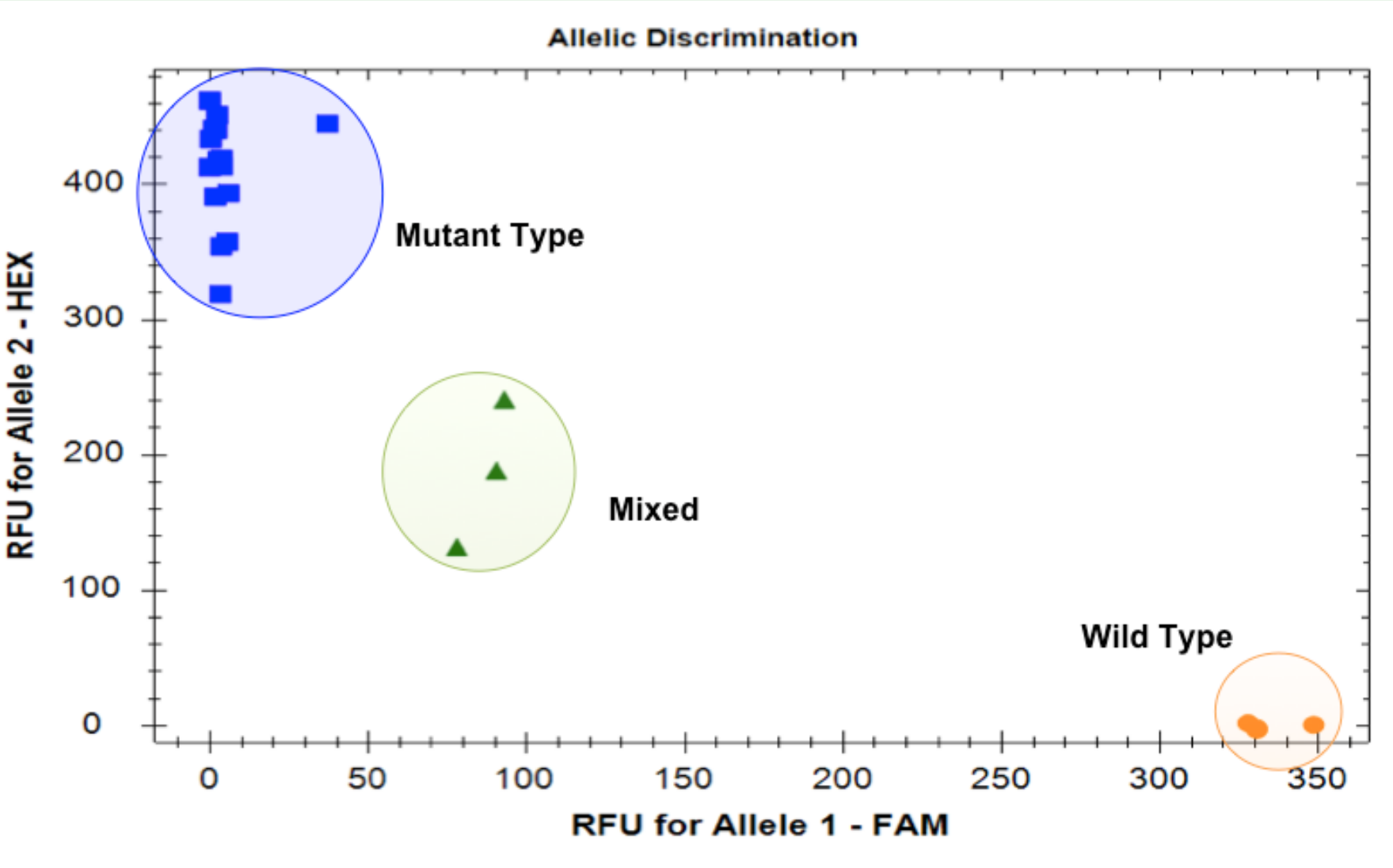
Grape leaf likely to have many isolates

Impaction trap as designed by Mahaffee

Investigating mixed samples with this assay

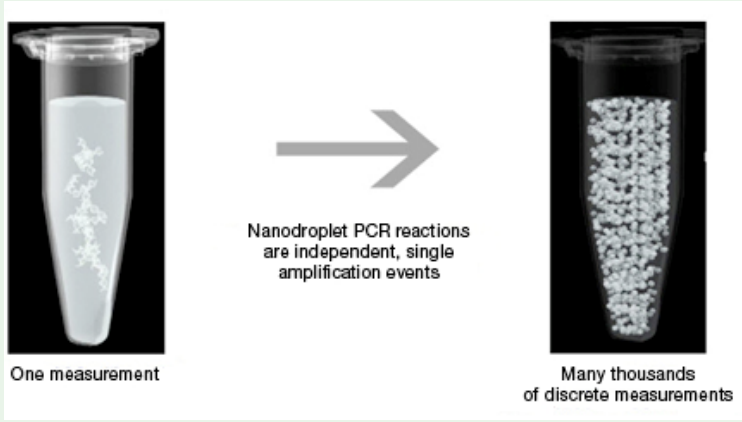


Investigating leaf samples from Oregon



New technologies such as Digital Droplet PCR

- Digital Droplet PCR allows one reaction to be replicated into thousands



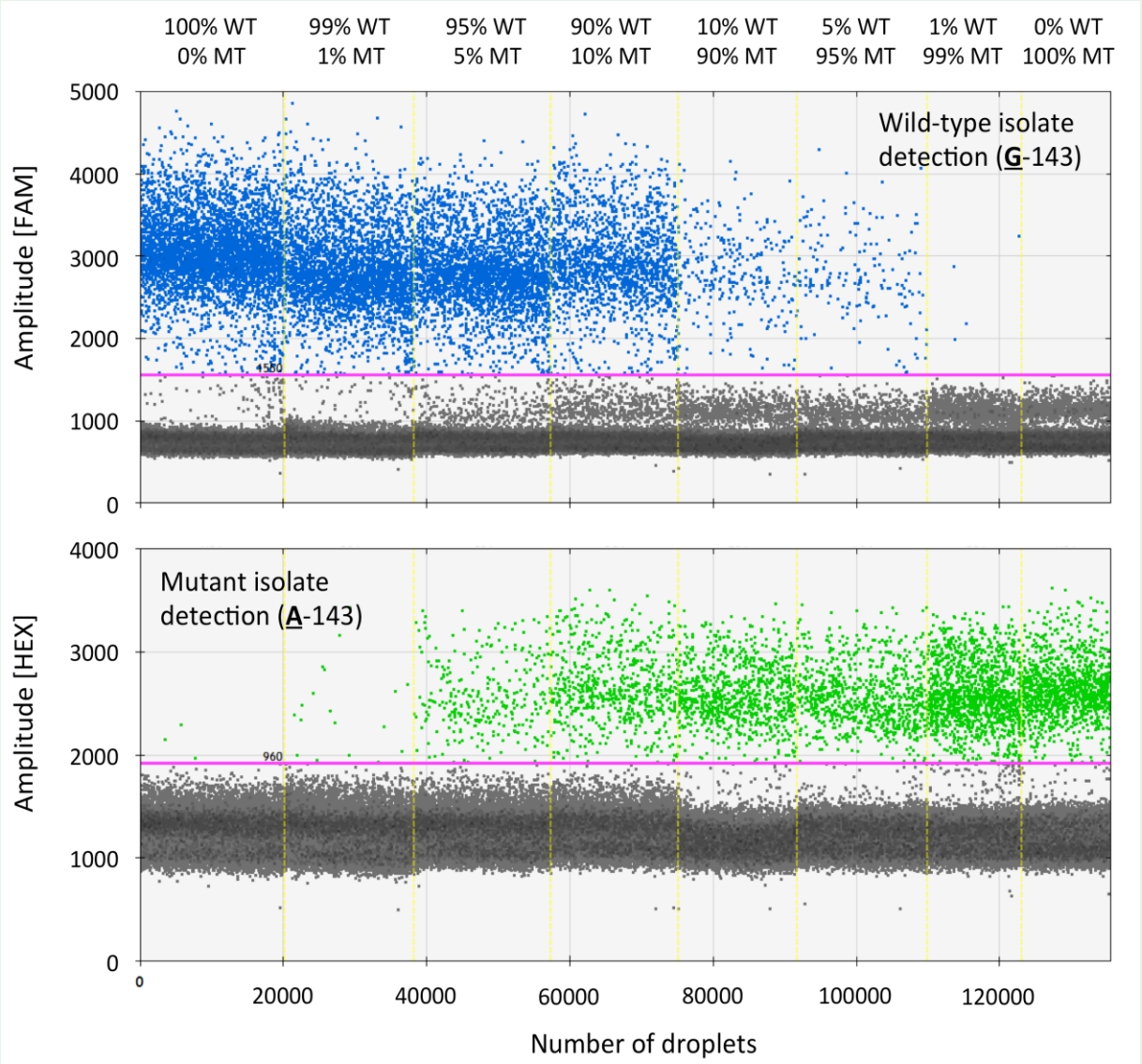
- Significant advantages when dealing with mixed samples

QX200 Droplet Digital PCR System

Observing mixed samples with Digital Droplet PCR

Wild type detection

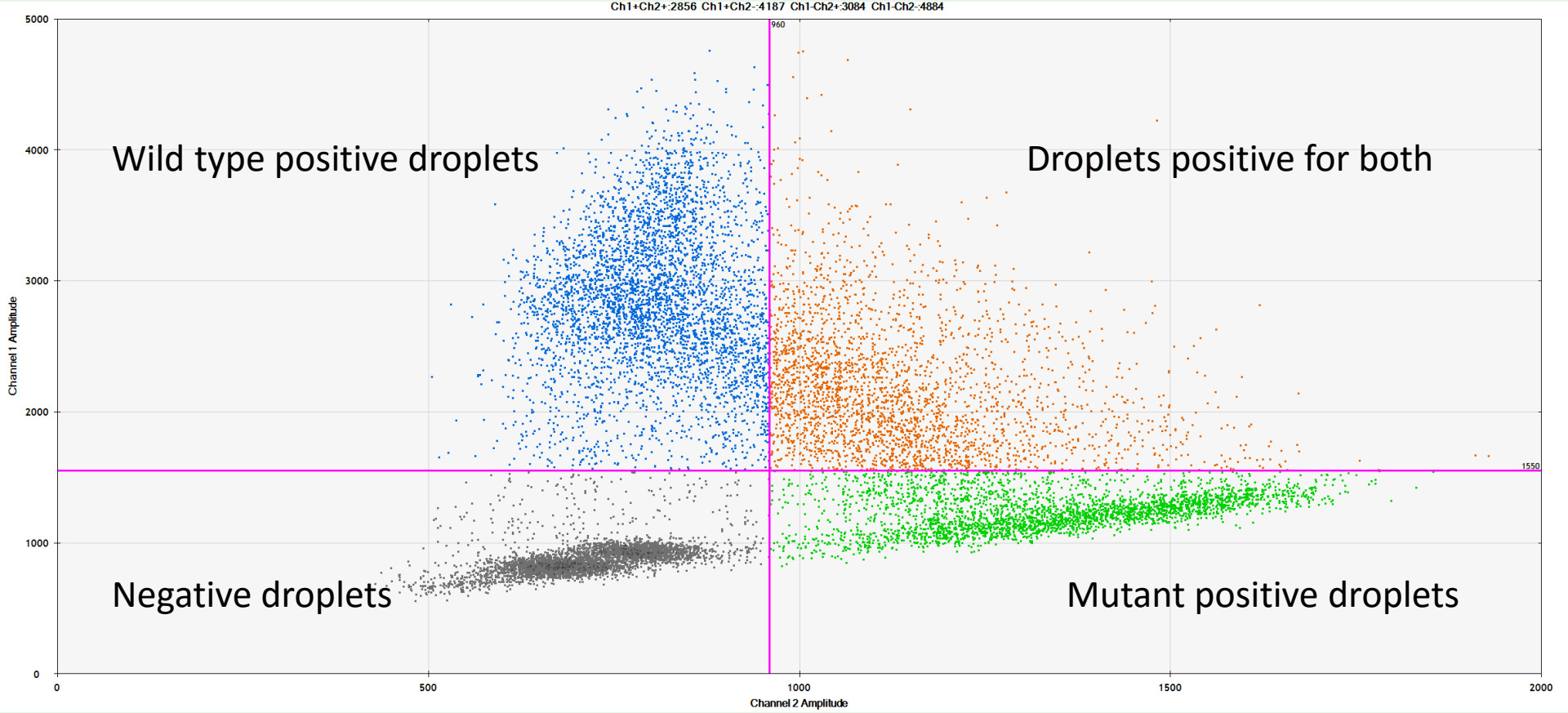
Mutant type detection



Limit of detection in mixed samples is around <1%

A single leaf sample infected with powdery mildew using Digital Droplet PCR

G143A detection



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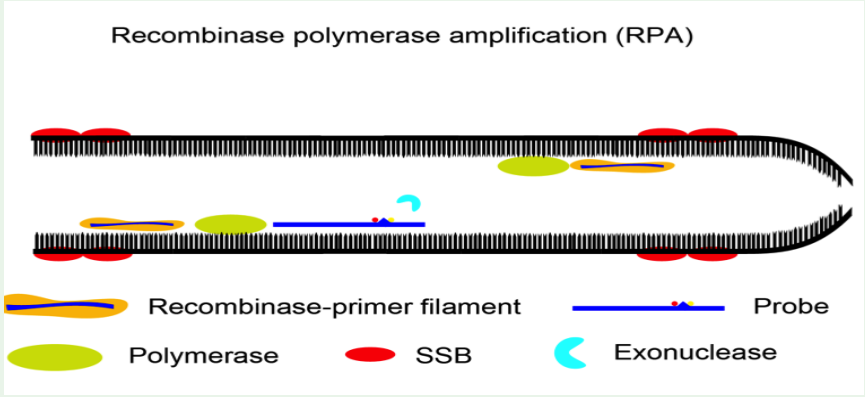
Making these assays portable

- Recombinase polymerase amplification
  - ***Very easy*** sample prep
  - ***Fast results*** (within 5-25 min)
  - Utilizes 3-4 enzymes to produce an amplicon
  - Can be multiplexed, similar to TaqMan
  - Not quite as sensitive as TaqMan PCR



Typical RPA setp enzymatically

Can be read using a variety of platforms

Advantages of RPA over other technologies

- **Fast!** (Did I mentioned this before?)
- No traditional DNA extraction is required and can use very crude samples (see right)
- Tolerant of PCR inhibitors
  - Multiple ways to read results (fluorometric or lateral flow), some are portable
  - Can perform nested PCR to confirm a product

Crude root sample

RPA conclusions so far

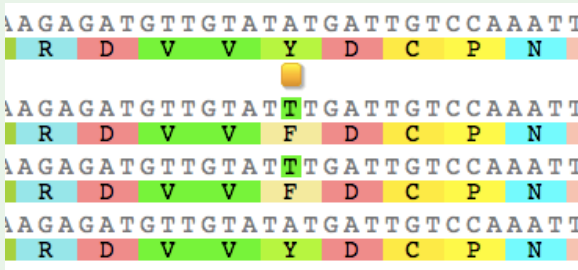
- Great method for increasing sensitivity on crude samples
- Using crude samples: sensitivity can be increased by 100 to 1,000 times
- Some issues with specificity with RPA assays compared to laboratory assays like TaqMan and Digital droplet PCR
- Will require additional optimization before it can be used in the field to determine small differences such as those related to fungicide resistance

Conclusions

- Accurate and consistent detection of the G143A mutation was obtained.
- The Digital Droplet PCR is suitable for detecting a 1% concentration of target in a mixed DNA sample.
- The qPCR and Digital Droplet PCR assay eliminated the need for a gel electrophoresis.
- QoI resistance was found in air-borne spore samples from 2013 indicating the resistant *E. necator* populations were present well before field control failures.
- There is possible probe cross-specificity with both *E. vaccinii* (blueberry) and *E. alphitoides* (oak)
- RPA detection may allow us to increase sensitivity and work with crude samples

Future directions – FRAC code 3

- Collections are ongoing currently in CA and Oregon to detection DMI resistant isolates
- Currently we have detected the common Y136F mutation in the cyp51 gene in over 90% of isolates
- Work was slowed significantly by being unable to find a sensitive isolate
- We haven't found any other mutations
- Very little correlation observed currently between QoI and DMI resistance in isolates



Y136F mutation in *E. necator* cyp51 gene

Acknowledgements

- Miles laboratory personnel:
  Juan Cerda and Philip Engelgau
- Laura Miles for guidance with working with *E. necator*
- Mahaffee laboratory:
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