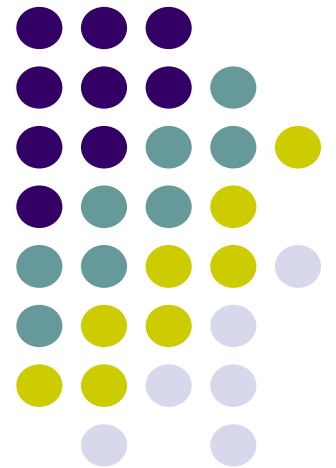
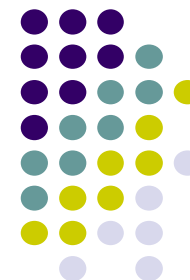


# Neuraminidase inhibitor resistance testing

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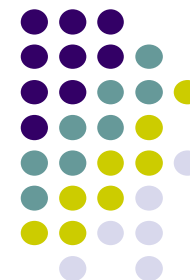
Dr. Bin Wang  
Centre for Virus Research  
Westmead Millennium Institute





# The impact of Influenza

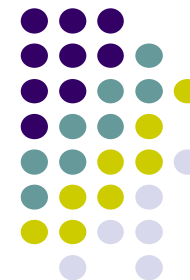
- Influenza A (H1N1, H1N2, and H3N2) and B strains cause seasonal outbreaks
- Influenza A can also cause pandemics
  - 3 influenza A viruses associated with 20<sup>th</sup> century human pandemics all have genetic components originally housed in viruses in birds
  - 1918 H1N1 virus killed 20 to 40 million people worldwide
  - 1957 H2N2 and 1968 H3N2 viruses each responsible for more than 1 million deaths
  - Now, H5N1; “bird flu” is a major concern



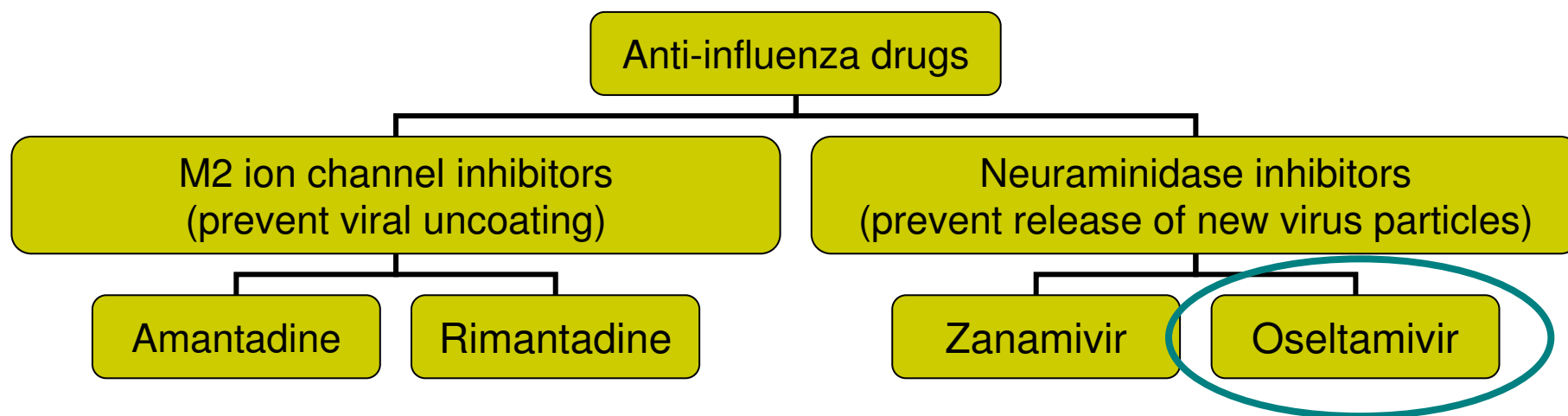
# Managing an Influenza Pandemic

There are 3 stages of managing a pandemic:

1. Vaccination
2. Antiviral drugs
3. Infection control



# Anti-viral drugs to treat Influenza



Toxicity

Resistant strains rapidly emerge

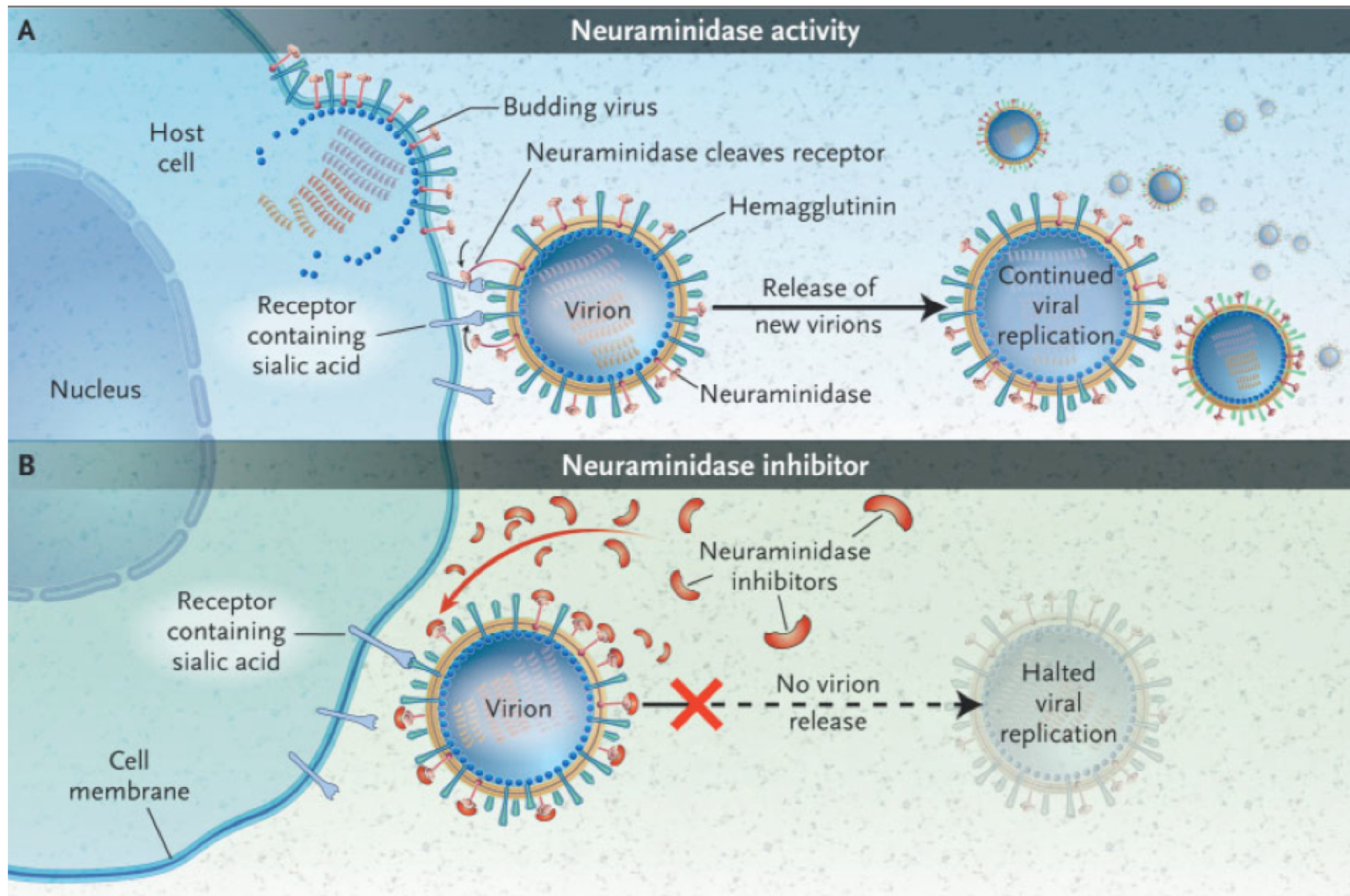
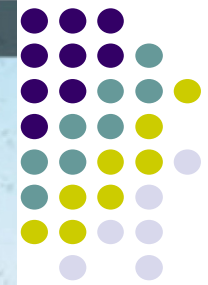
Recent H5N1 strains were highly resistant to these drugs

Given by inhalation, may not be effective against H5N1 which leads to systemic infections

New drug

Drug of choice in the event of an H5N1 pandemic

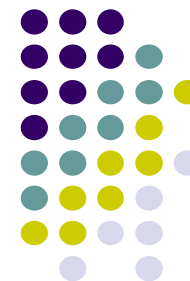
Stockpiles are being created



Mechanism of Action of Neuraminidase Inhibitors (Anne Moscona, 2005 NEJM)



- Due to the high mutation rate of influenza, the emergence of resistance against any specific and potent anti-influenza drug is virtually inevitable.
- Drug resistance is costly to the health service, the patient who fails to gain the maximum therapeutic benefit, and for the community in which resistant viruses may be widely spread.



# Neuraminidase inhibitors resistance mutations

- Mutations in NA and HA which result in resistance to oseltamivir have been identified in challenge studies and in patients with naturally acquired infections
- One study showed a frequency of up to 18% in a group of treated children.
- Mutations which lead to resistance:
  - N1: His274Tyr = CAY → TAY
  - H5N1: His274Tyr = CAY → TAY
  - N2: Glu119Val = GAR → GTN
  - N2: Arg292Lys = AGR → AAR

# Neuraminidase inhibitor resistance testing



## Phenotypic assays

plaque reduction assay: PRA

NA inhibition (NAI) assay

In vivo assay using ferrets.

cumbersome, time-consuming, and subjective

Genotyping assays (sequence analyses of the neuraminidase gene)

## PCR and Sequencing based genotypic resistant testing

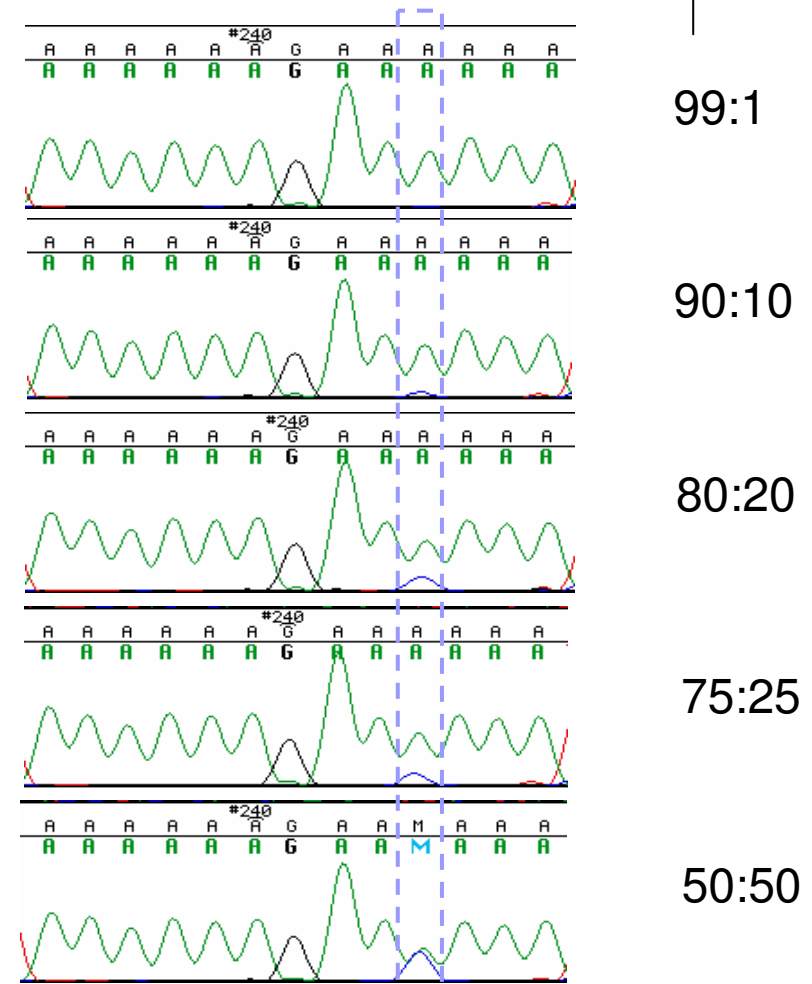


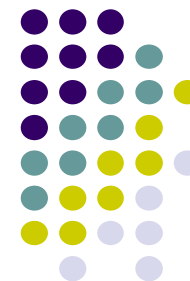
- Less complex test
  - Less expensive
  - More widely available
  - More rapid results
- 
- Links between mutations and resistance not fully understood
  - May need expert interpretation
  - Most cannot detect minor species
  - Lab quality varies



# The problem with sequencing

- Sequencing is currently the standard method used to screen for drug resistance mutations.
- Low level variants are not detected when they constitute less than 20% of the total population.



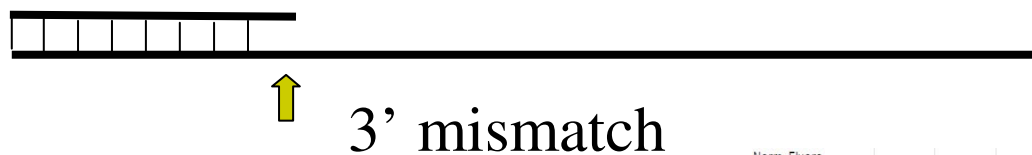


# Alternatives to sequencing

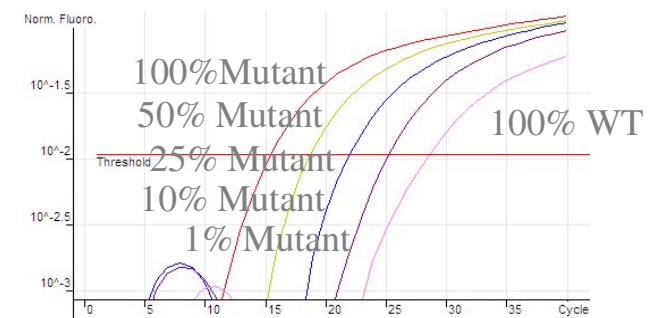
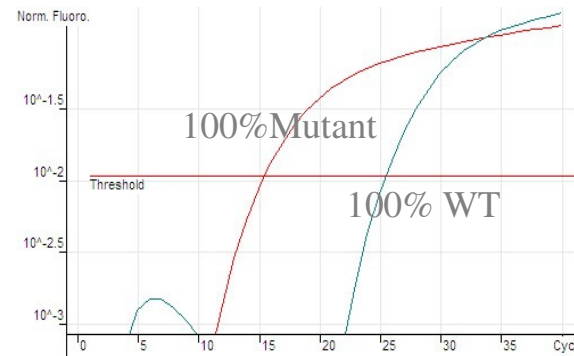
- Allele specific real time PCR
- Ligase chain reaction (LCR)
- Rolling circle amplification (RCA)



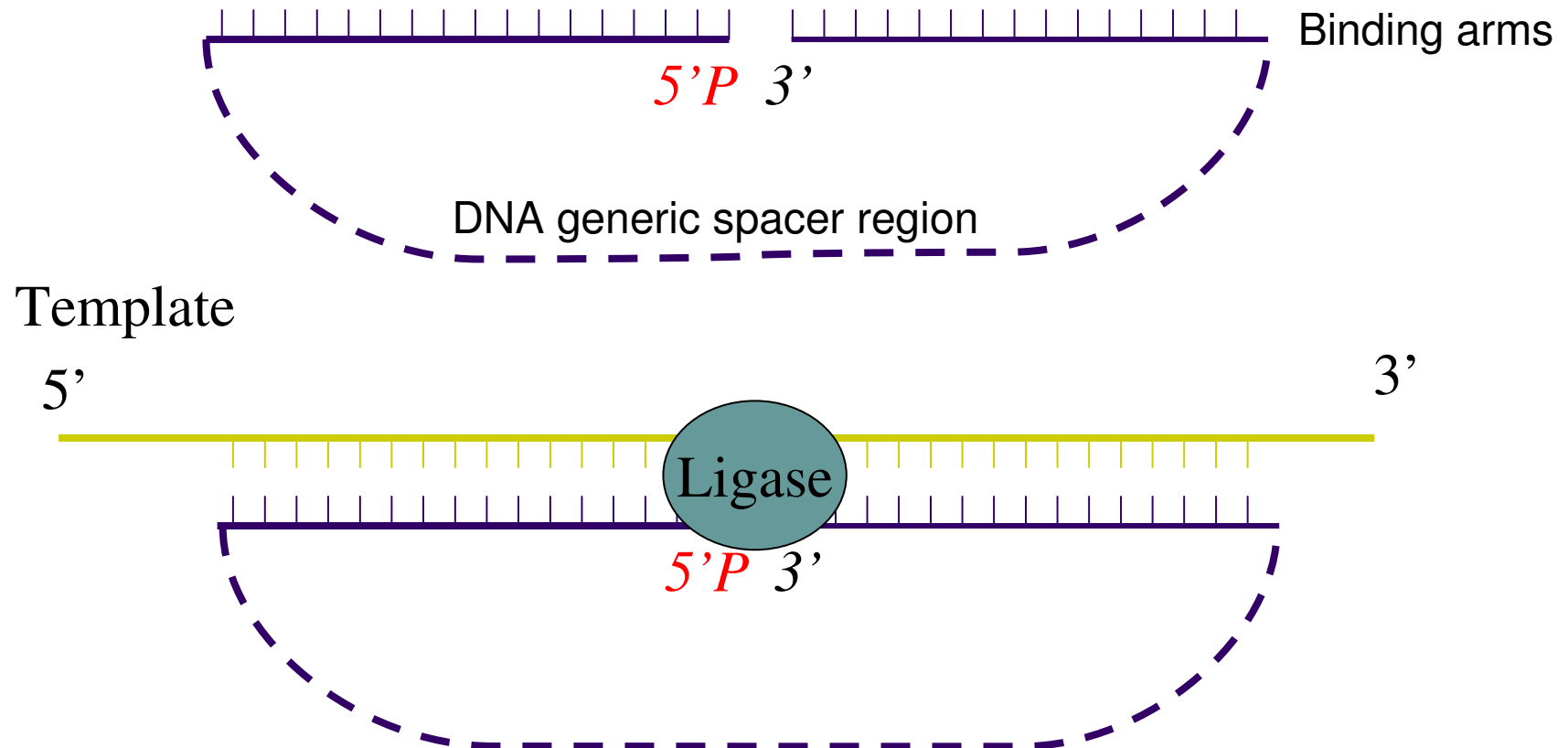
- Allele specific real time PCR
- Sensitivity of  $<1\%$
- Large-scale studies
- Machine dependant



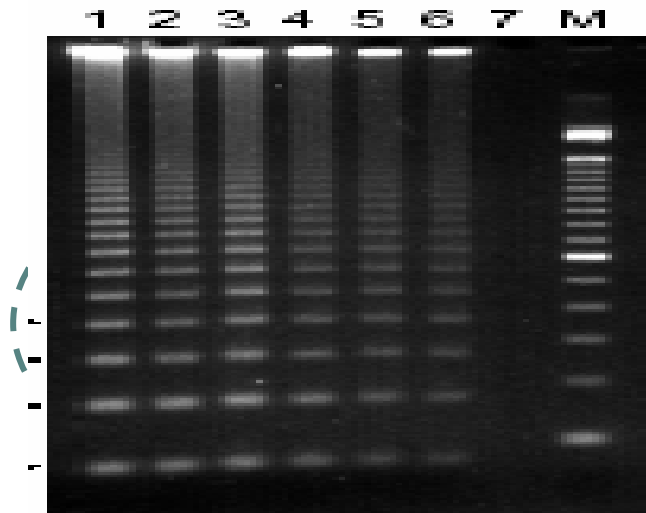
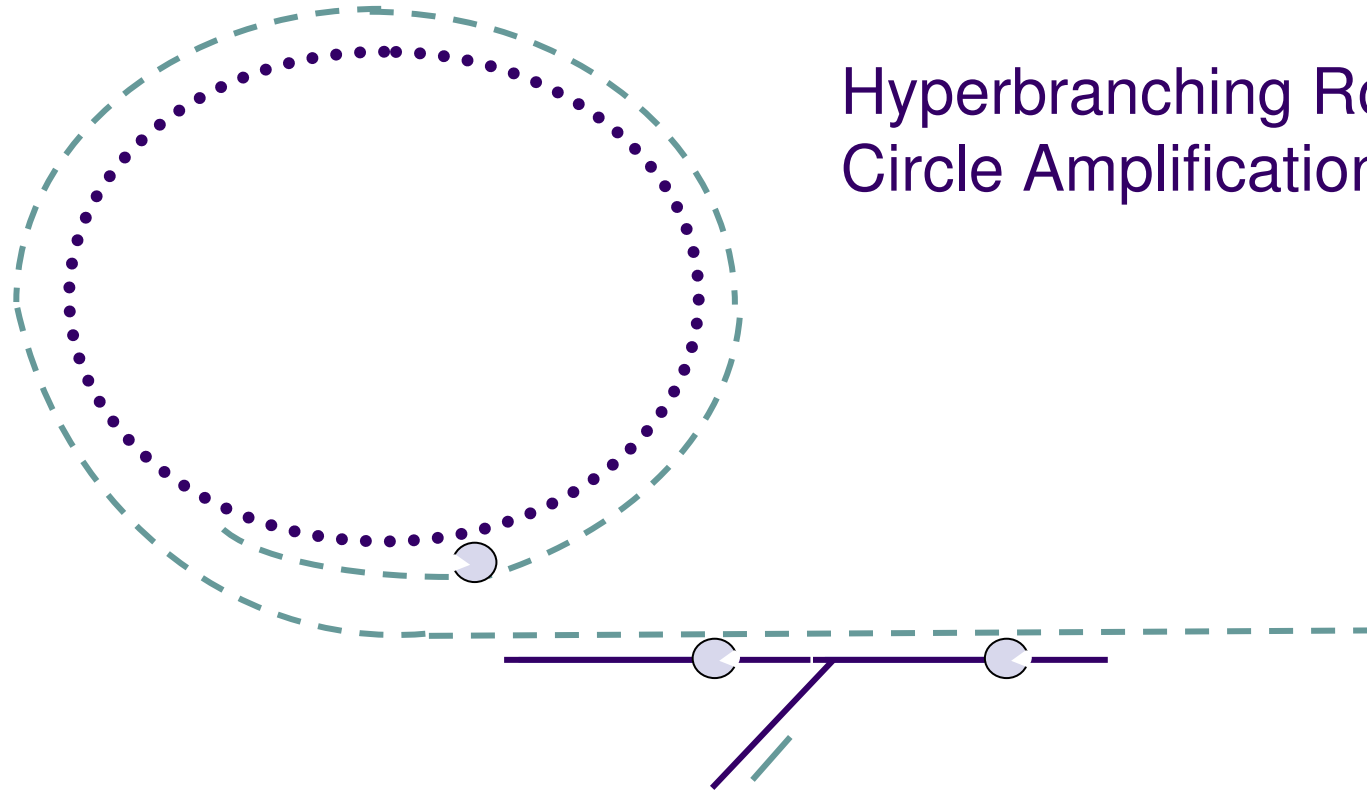
>10 cycle different (1000 times less efficiency)



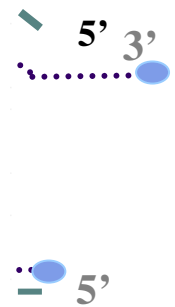
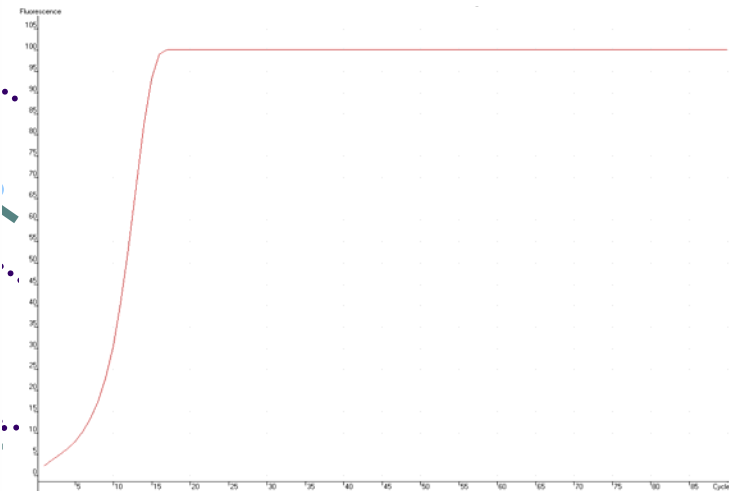
# Detection of NA resistance using Padlock Probes and Rolling Circle amplification



# Hyperbranching Rolling Circle Amplification



- 400
- 300
- 200
- 100





# Advantages of RCA

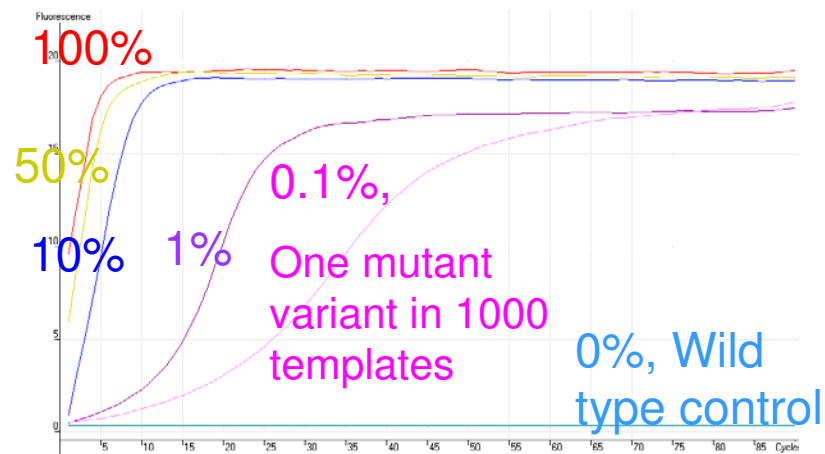
**Properties of various nucleic acid amplification technologies.**

Property	PCR	LCR	SDA	NASBA	bDNA	Invader	RCA
DNA target amplification	✓	✓	✓	✗	✗	✗	✓
RNA target amplification	✓	✗	✓	✓	✗	✗	✓
DNA signal amplification	✗	✗	✗	✗	✓	✓	✓
RNA signal amplification	✗	✗	✗	✗	✓	✓	✓
Protein signal amplification	✗	✗	✗	✗	✗	✗	✓
Multiplexing	Little	✗	Little	Little	✗	✗	✓
Mesothermal	✗	✗	✓	✓	✓	✓	✓
Amplification within cells	✓	✗	✗	✗	✗	✗	✓
Amplification on microarrays	✗	✗	✓	✗	✗	✗	✓
Sensitivity (copies)	<10	100	500	100	500	600	1
Range (logs)	5	3	4	5	3	4	7
Specificity (allele discrimination factor)	50	5000	50	50	10	3000	100,000

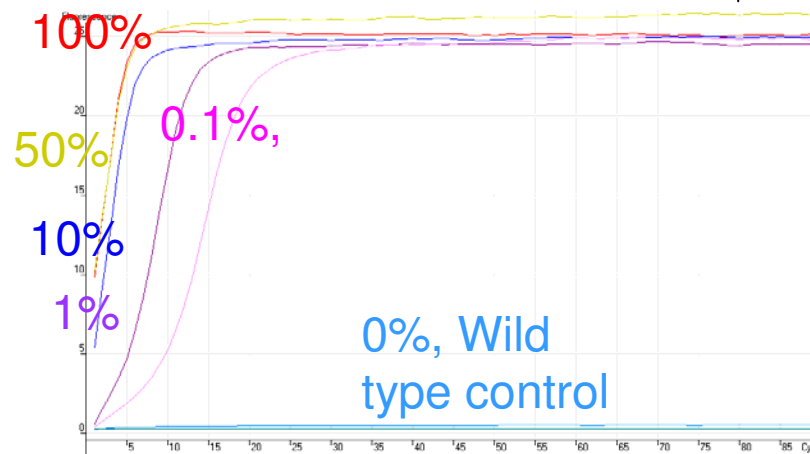


# RCA detection of resistance mutations

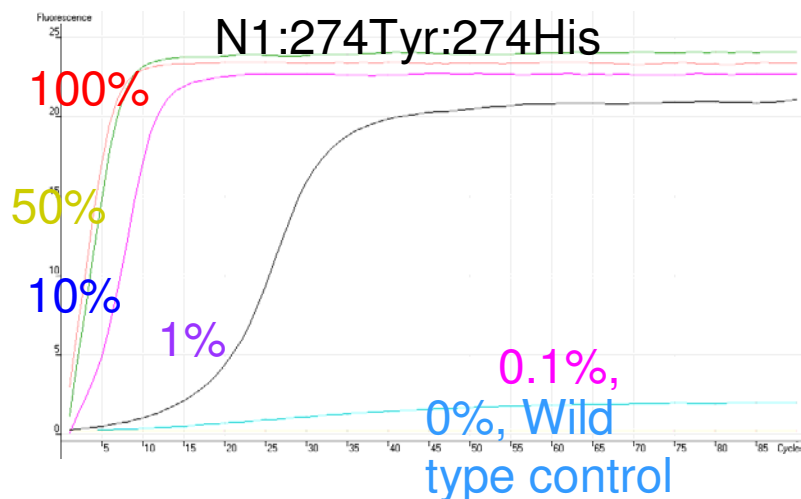
N2:119Val:119Glu



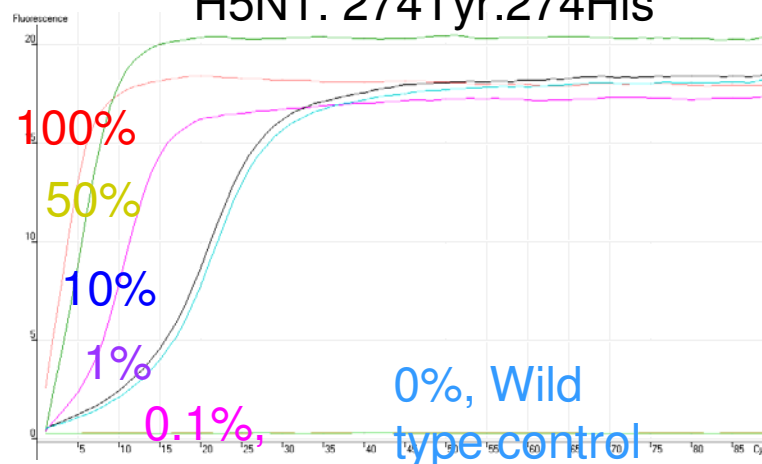
N2:292Lys:292Arg

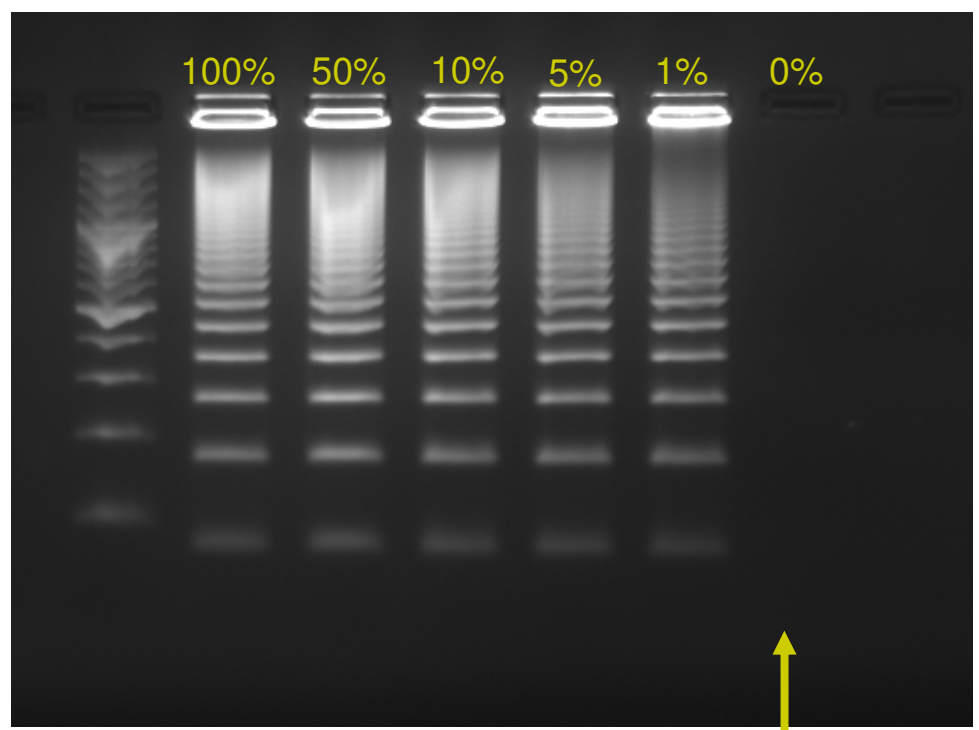
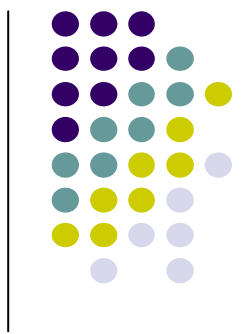


N1:274Tyr:274His



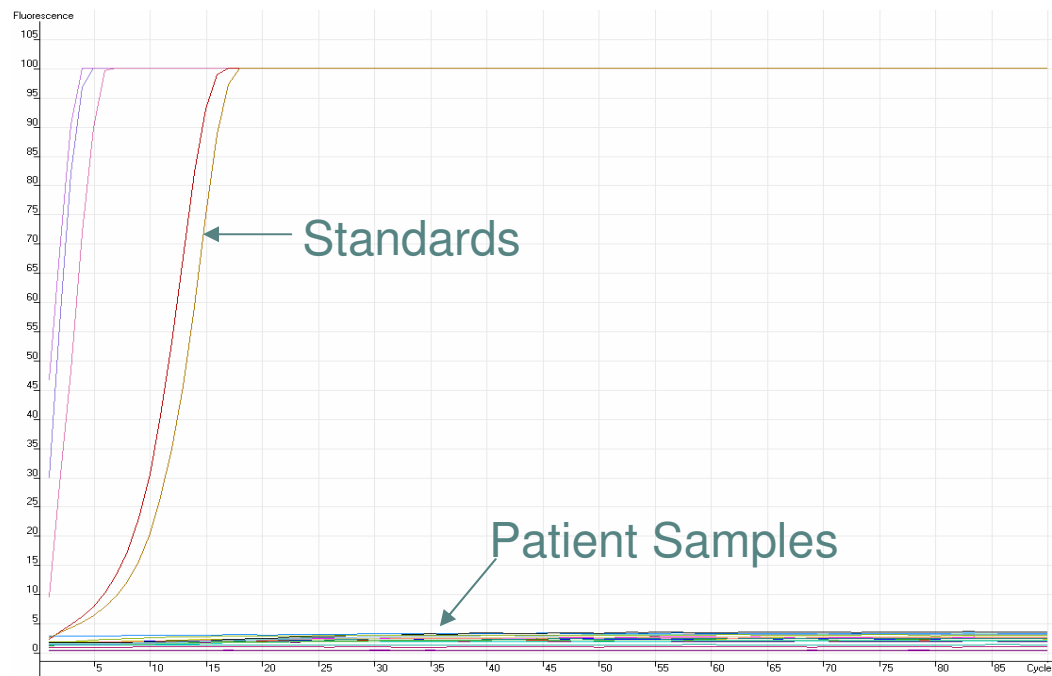
H5N1: 274Tyr:274His





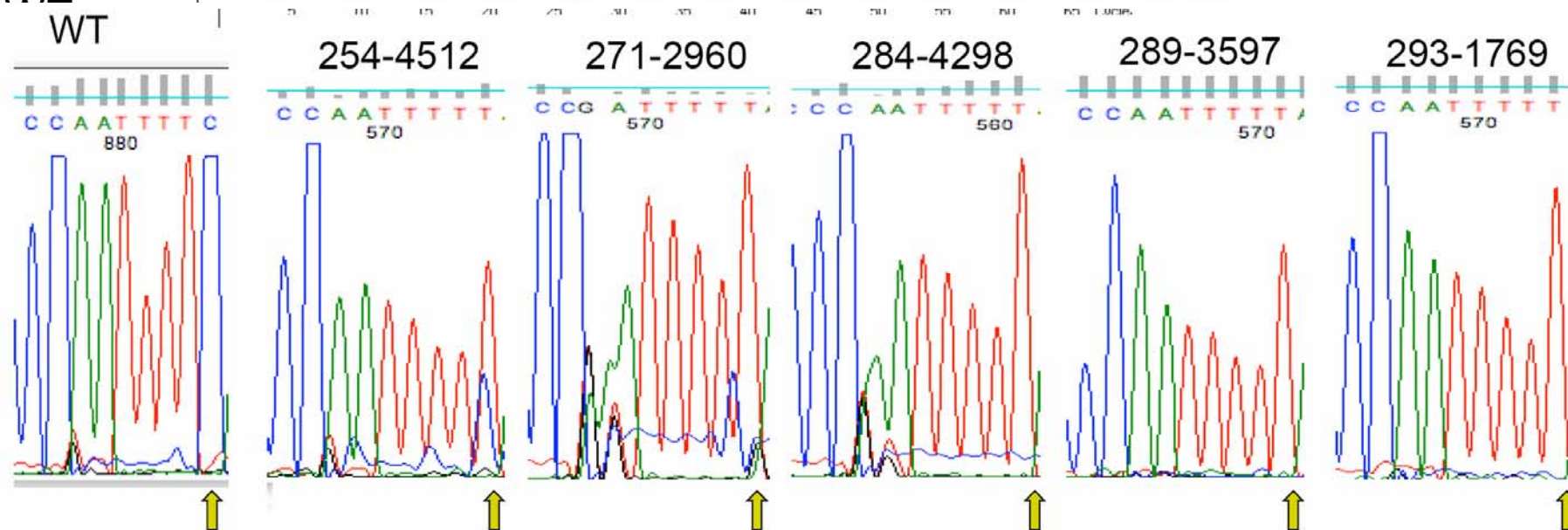
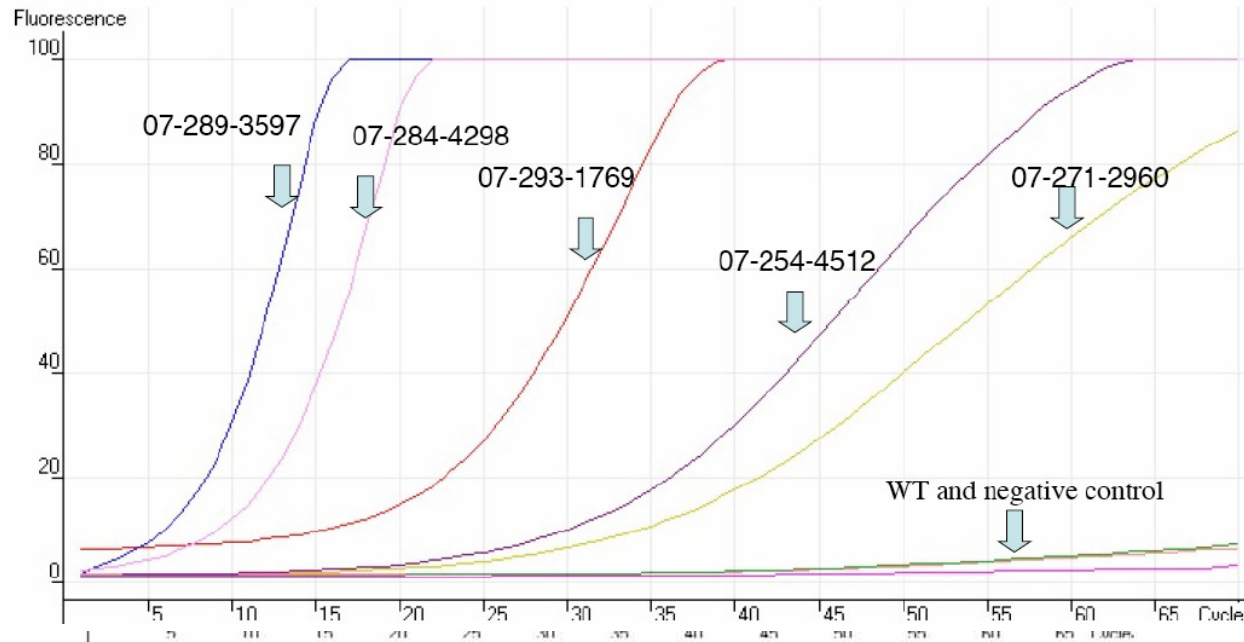
Wild type control  
Contains  $10^{12}$  copies of template

Advantages on RCA based NA resistance testing:  
Rapid test, High accuracy, Cost effective,

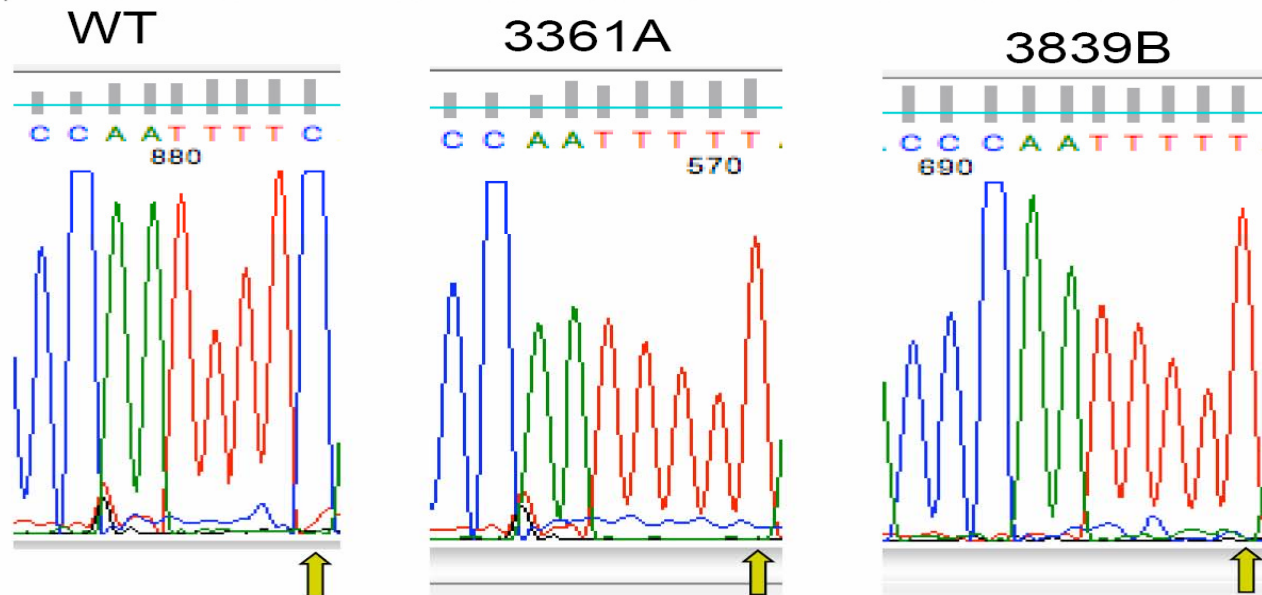
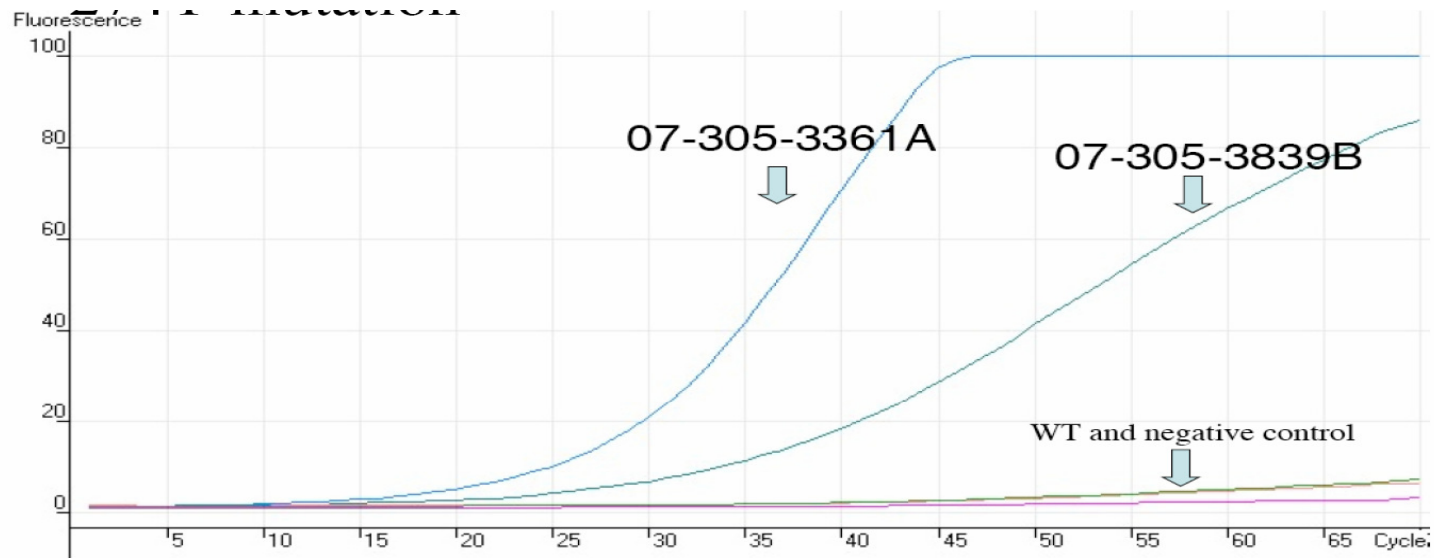


50 N2 and 17 N1 samples from treatment naïve patients have been tested using RCA, and no drug resistance mutations were detected

# RCA detection of the emergence of the 274Y mutation in a immunosuppressed bone marrow transplant recipient infected with influenza A H1N1



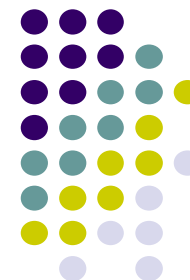
Two individuals were infected with influenza A H1N1 bearing the 274Y mutation



# Clinical significance in NA resistance testing



- In the event of an H5N1 pandemic, drug resistance, and its transmission, will be clinically highly significant.
- Using rolling circle amplification, we have developed specific tests to detect influenza A drug resistance variants, to a level of sensitivity of <1%.
- During a pandemic these assays can be made readily available for monitoring the emergence of drug resistance and will improve patient care.
- The technology is also suitable for poor resource settings.
- Early testing from treatment naïve patients showed no drug resistant variants
- Recently data demonstrated first time in real-time detection of emergence of OST resistance mutation in a immunosuppressed individual in response to treatment.
- We also demonstrated the transmission of OST resistance H1N1 strain.
- With increasing usage of OST, we would expect increasing incident of resistance strains.



# Acknowledgements

- Megan Steain
- Chenda Kol
- Nitin Saksena
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