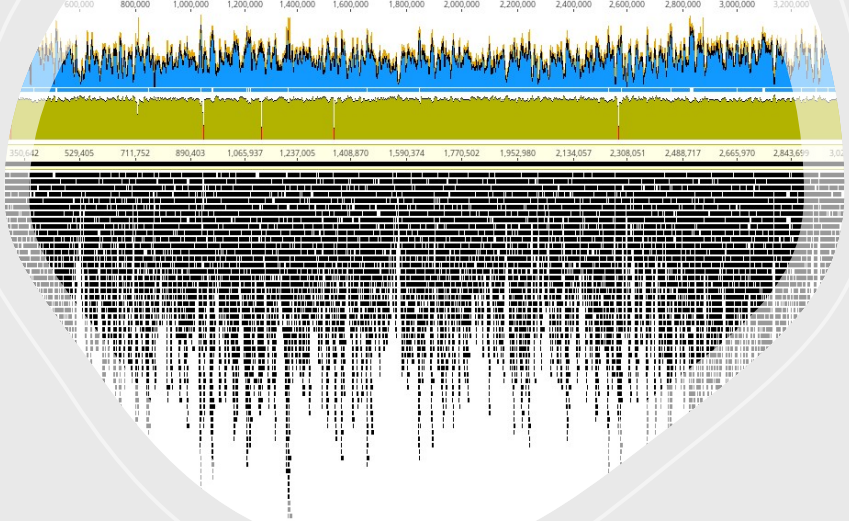

CRISPR technologies for improved point-of-care diagnostics

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Science and Technology
Plant Protection and Quarantine
Animal Plant Health Inspection Service
United States Department of Agriculture

Plant Pathogen Confirmatory Diagnostics Laboratory

Our mission is to develop, adapt, validate, and implement advanced biochemical and molecular methods for the detection of high consequence plant pathogens, including the USDA Select Agents and plant pathogens in foreign germplasm.

USDA APHIS PPQ, ST PPCDL Facility in Laurel, Maryland

Plant Pathogen Confirmatory Diagnostics Laboratory

CONFIRMATORY DIAGNOSTICS

DEVELOPMENT OF MOLECULAR DIAGNOSTICS METHODS

VALIDATION OF DIAGNOSTIC METHODS

TRAINING

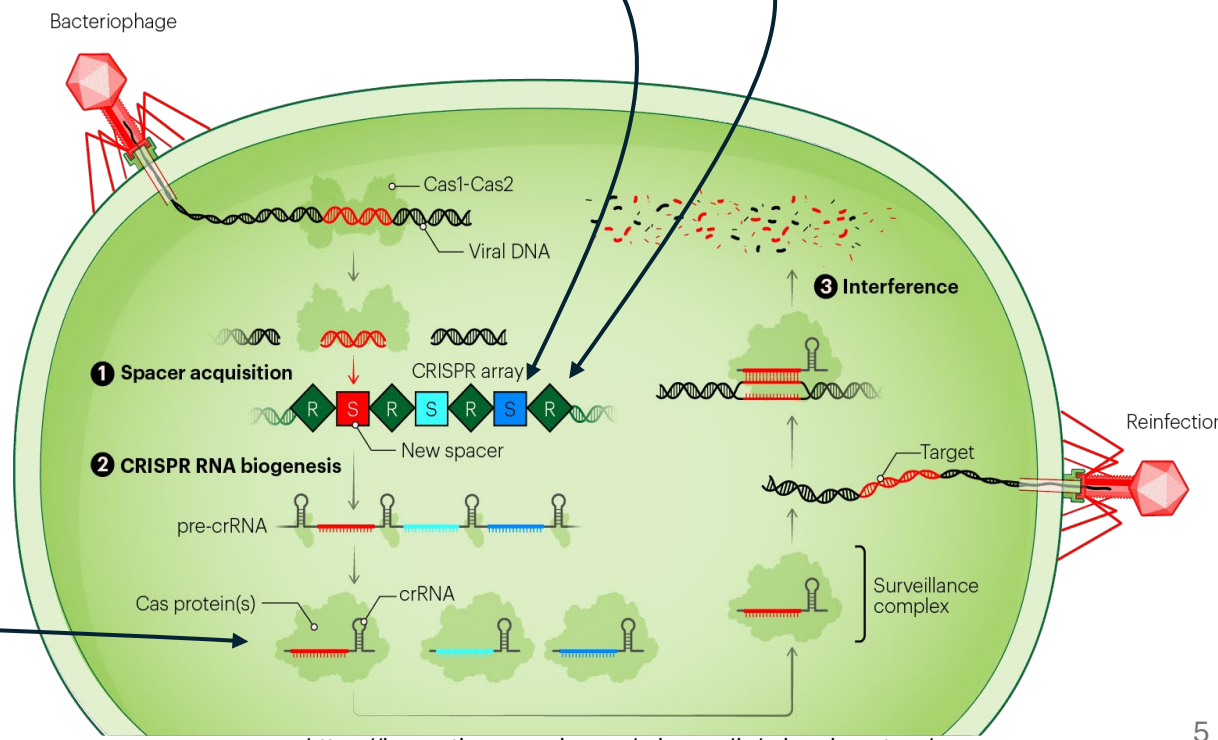
DIAGNOSTIC CERTIFICATION PROGRAM

Outline

- Introduction to CRISPR
- CRISPR-based diagnostics
- USDA APHIS PPQ work on CRISPR-based detection methods
    1. Tomato brown rugose fruit virus (*Tobamovirus fructirugosum*)
    2. *Ralstonia solanacearum* Phylotype IIB Seq. 1 and 2 (R3B2)
    3. '*Candidatus* Liberibacter asiaticus'
- Challenges
- Future and opportunities

Introduction to CRISPR-Cas Systems

- Bacterial defense system using RNA-guided nucleases to cleave foreign nucleic acids
- CRISPR (**c**lustered **r**egularly **i**nterspaced **s**hort **p**alindromic **r**epeats)
  - Flank "spacer" sequences
    - Segments of acquired foreign nucleic acid
    - Transcribed into RNA "guides" (gRNA or crRNA)
- Cas (CRISPR-associated proteins)
  - System machinery
  - Including nucleases (e.g.: Cas9, Cas12, etc.)
    - Associate with guide RNA
    - Find matching targets and cleave

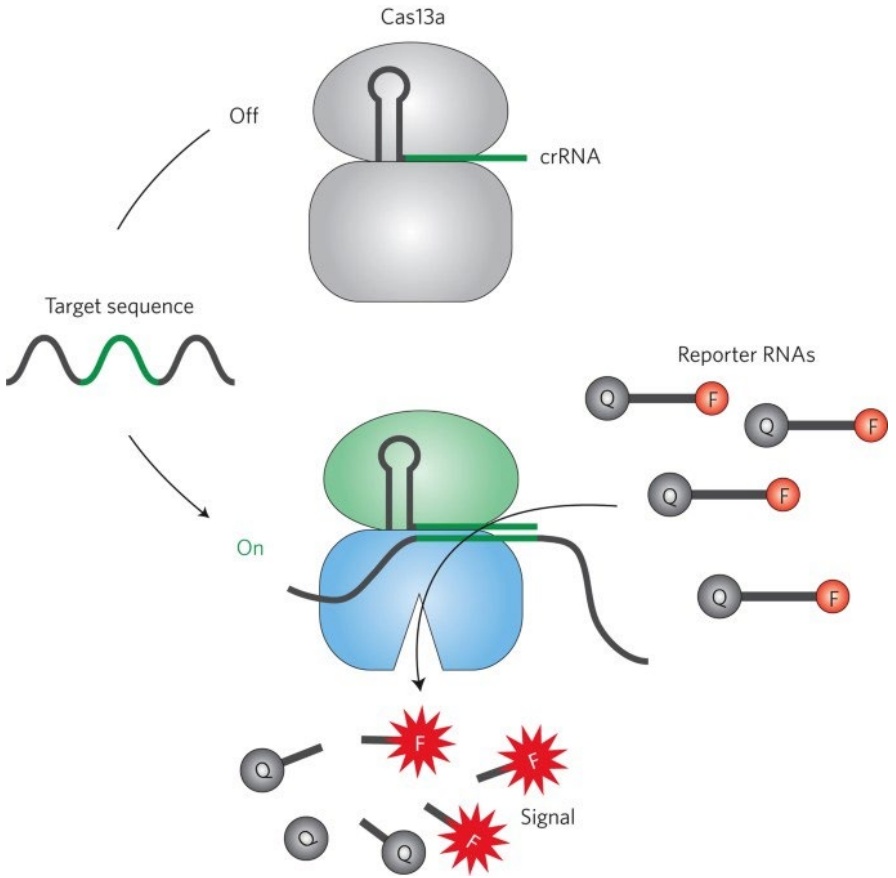


https://innovativegenomics.org/crisprpedia/crispr-in-nature/

**Similar to RNAi, except with "memory"

CRISPR-Cas Diagnostics

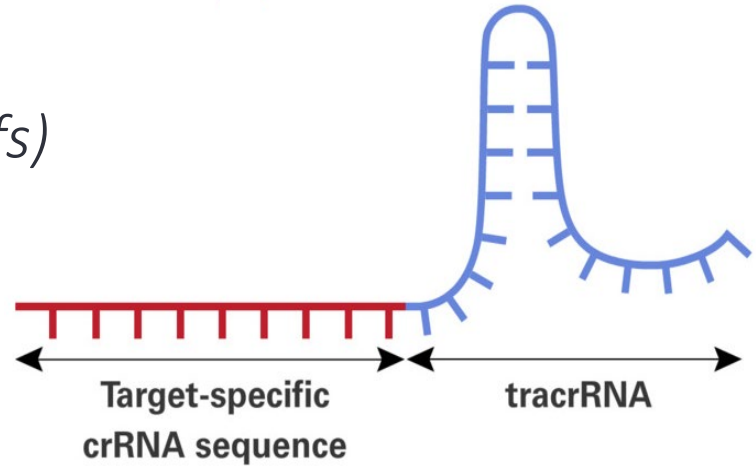
- Synthesize crRNA with target sequence
- Cas-crRNA searches for target sequence
- If found, Cas nuclease is activated
  - Cas enzymes produce off-target cleavage
  - “Collateral or trans- cleavage activity”
    - Can be leveraged for diagnostics
    - Inclusion of short dual-labelled reporters
      - Single stranded nucleic acids (ssDNA or ssRNA)
      - Cleavage by activated Cas produces signal
        - Indirect detection
        - Fluorescence (real-time) or lateral flow (end-point)



Zou et al. 2017

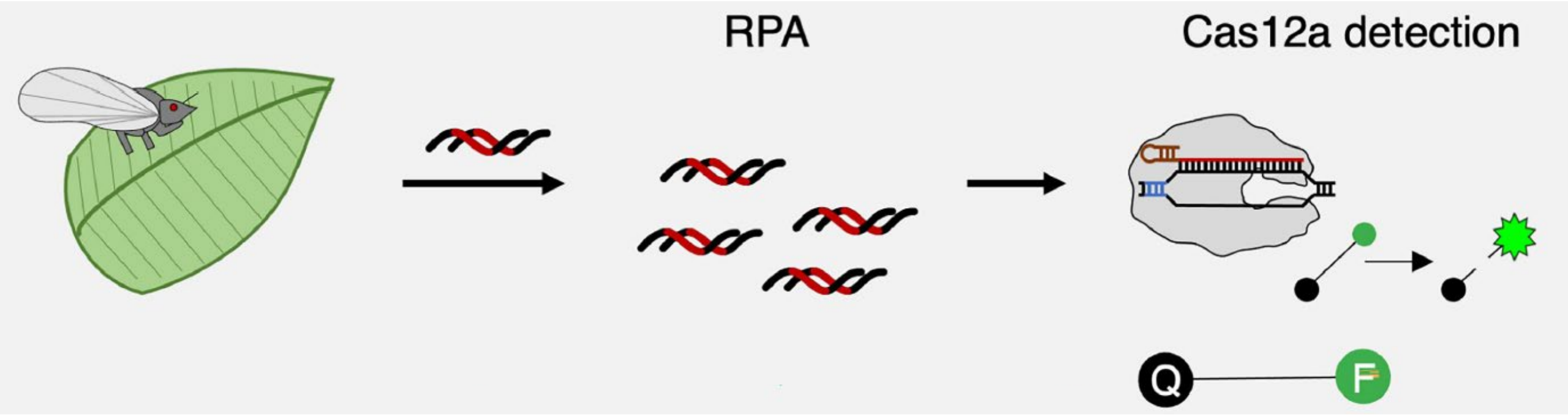
Design of CRISPR-Cas Diagnostics

- crRNA design
  - 20 nt target sequence
    - Must account for PAM (*protospacer adjacent motifs)*
      - Recognition site used by Cas
        - Present in target
        - Not present in own CRISPR array
  - tracrRNA sequence
    - Unique to each Cas enzyme
    - companies have this already designed into the ordering



https://www.takarabio.com/learning-centers/gene-function/gene-editing/gene-editing-tools-and-information/how-to-design-sgrna-sequences

RPA-CRISPR-Cas diagnostics

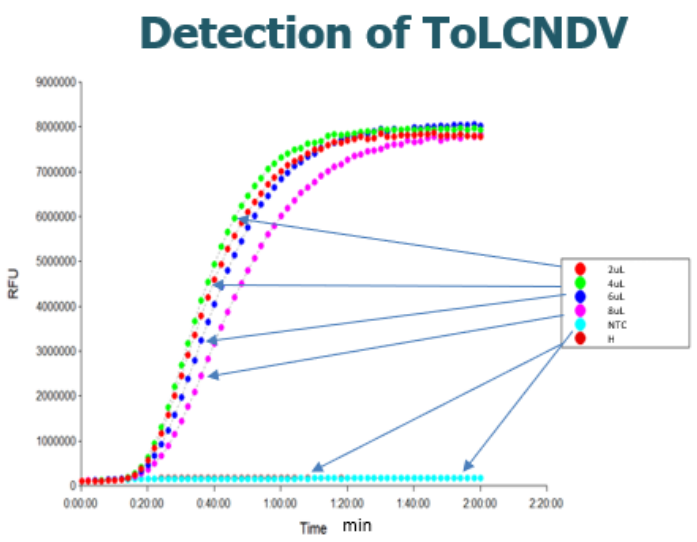


Wheatley et al. 2021

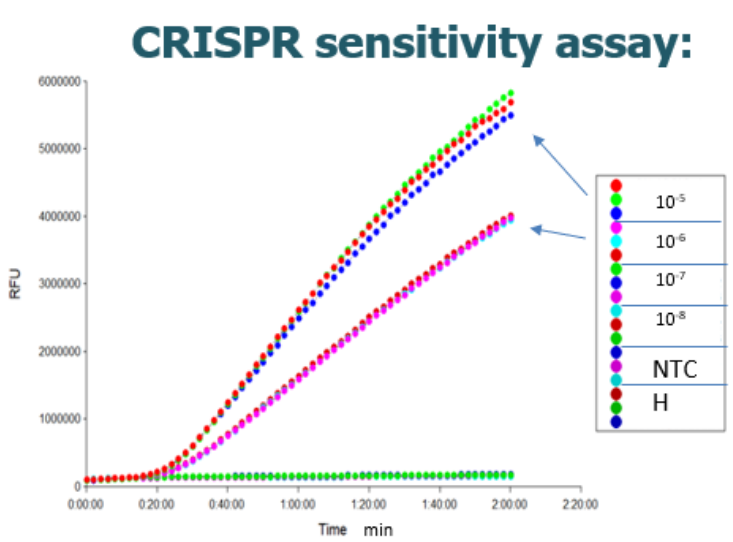
RPA=Recombinase Polymerase Amplification

1. CRISPR-Cas-based detection of ToBRFV and ToLCNDV

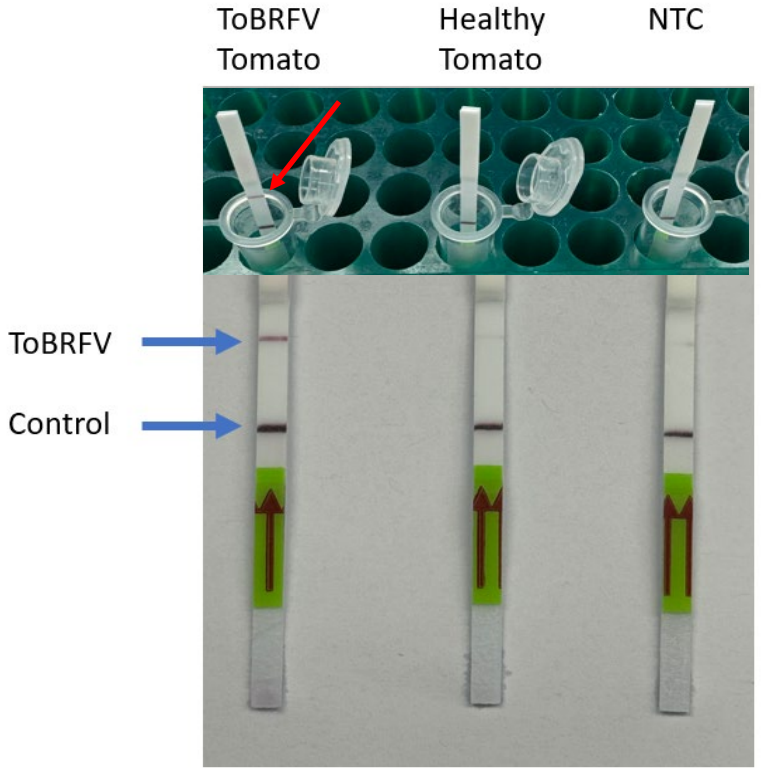
- Developed in collaboration with academic institutions
- Evaluation of lateral flow detection



Using optimized protocol for detecting ToLCNDV



Sensitivity test using infected samples



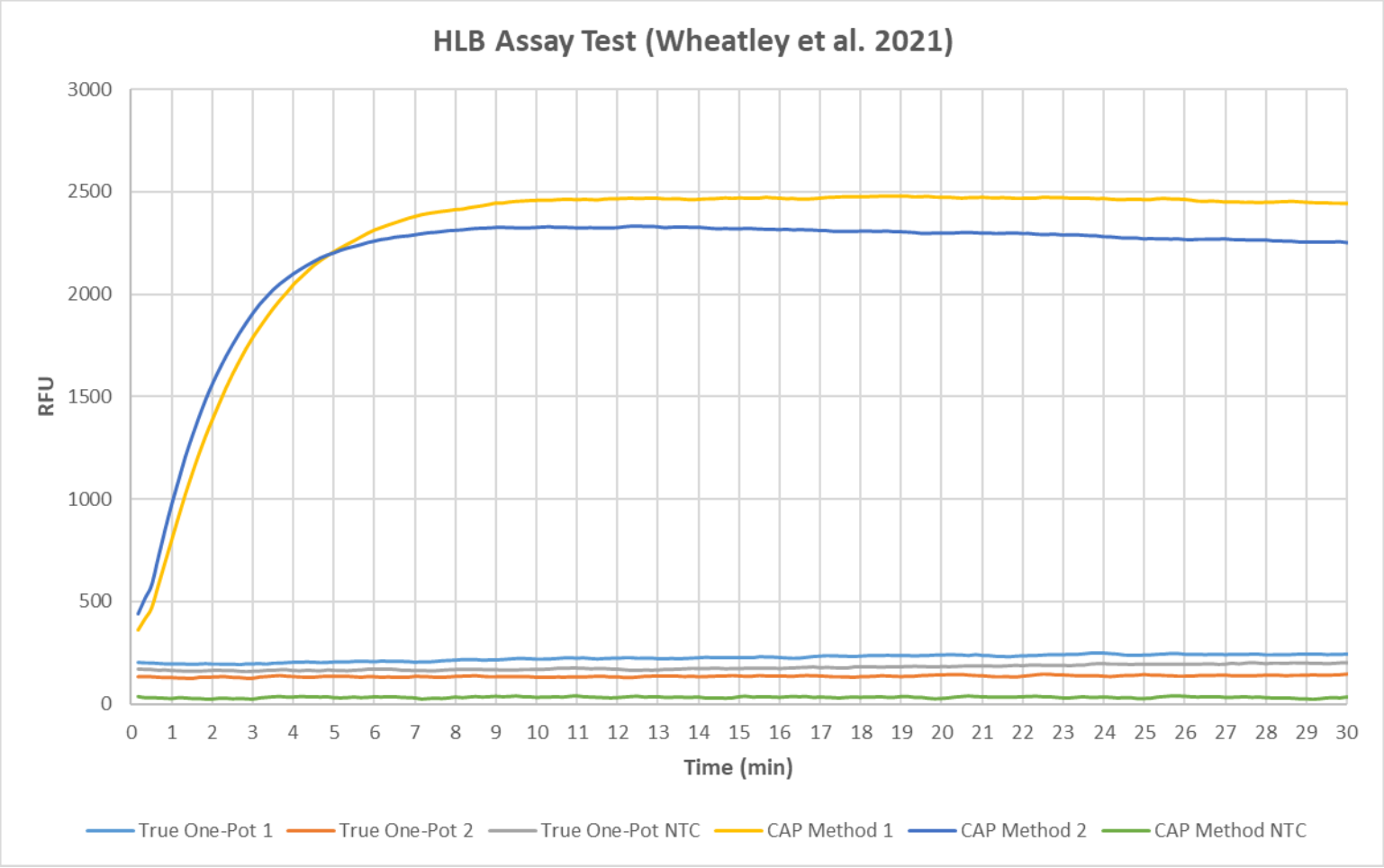
RT-RPA CRISPR Cas12 based Lateral Flow Assay

RPA-CRISPR-Cas diagnostics - Point of Care detection

- "One-pot" assay

- *In literature*
  - Single-tube
  - RPA reagents at bottom and CRISPR-Cas reagents in the cap
    - Run RPA (10-30 min)
    - Spin down tube and read CRISPR-Cas results (30 min-1 hr)
    - Increases complexity - careful pipetting and handling
    - Increases time – two separate incubation step

RPA-CRISPR-Cas diagnostics - Point of Care detection



Need for True "One-pot" CRISPR-Cas Diagnostics

Projects to evaluate this new technology

- Utility as a field-deployable diagnostic
- Several host/pathogen systems
  - *Ralstonia solanacearum* (Rs) R3b2 (phylotype IIB sequevars 1 and 2)
    - brown rot of potato
    - bacterial wilt of tomato and eggplant
    - southern wilt of geranium
  - '*Candidatus* Liberibacter asiaticus'
    - citrus greening
    - huanglongbing

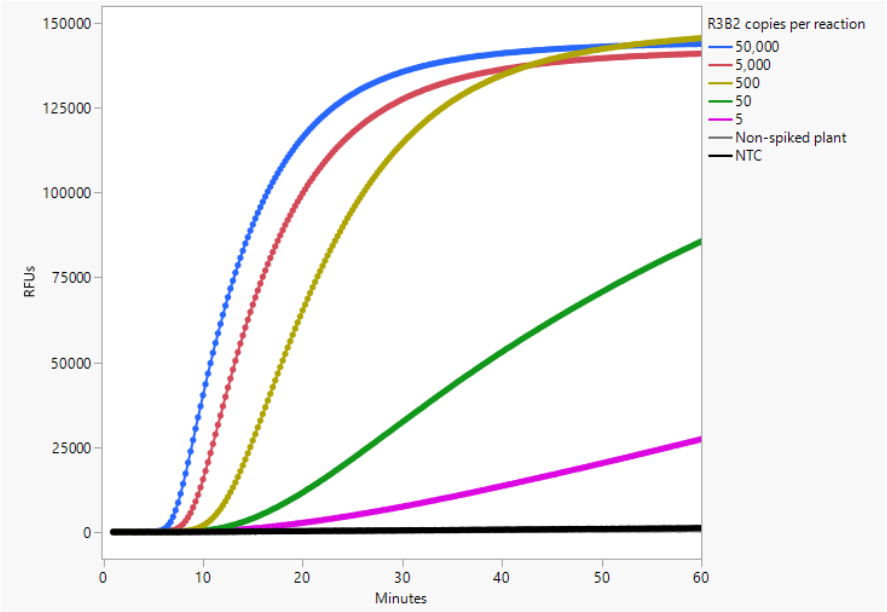
2. RPA-CRISPR-Cas – *Ralstonia solanacearum*

- Optimized design for a ‘one-pot’ reaction
- Evaluated using Genie-III (Optigene.co.uk)
- Limit of detection at 5 copies/reaction

Geranium plants with bacterial wilt symptoms

A fast and sensitive ‘one-pot’ RPA-CRISPR assay is under development

http://www.optigene.co.uk/instruments/instrument-genie-iii/



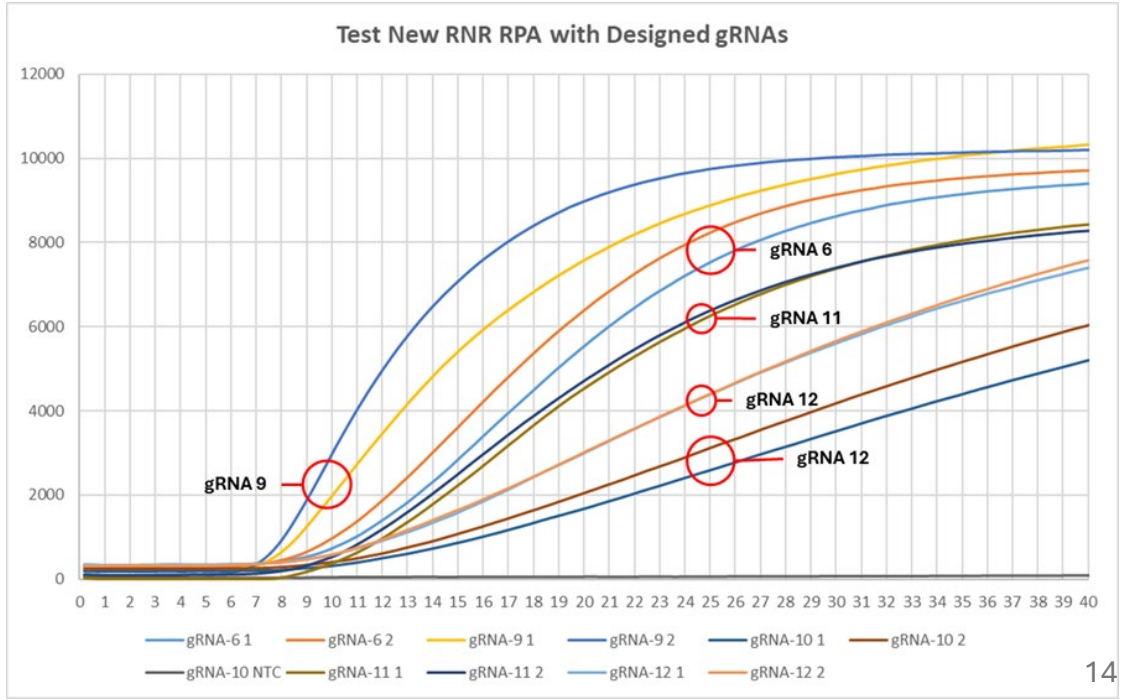
Fluorescence curves generated by a one-pot RPA-CRISPR assay for R3B2

3. RPA-CRISPR-Cas - HLB

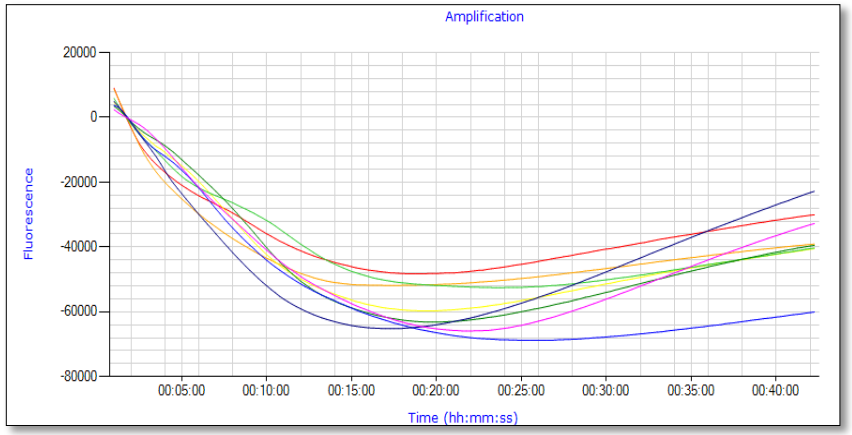
- Optimized design for a ‘one-pot’ reaction
- Evaluated using an Amplifire (Agdia)

https://orders.agdia.com/amplifire

https://iocv.ucr.edu/sites/default/files/huanglongbing



Challenges



Fluorescence curves for a failed RPA-CRISPR experiment

- Reproducibility of published assays
- Lateral flow format is not ideal
- Design of gRNA and availability of PAMs in smaller genomes (viruses/viroids)
- Relevant sensitivity requires upstream amplification (RPA)
- Ultralow cold-chain requirement for reagents
- Requires mixing numerous components to set-up assay

Future and opportunities

- Why CRISPR-Cas?
  - Potential to enhance the sensitivity and specificity of existing isothermal technologies (e.g.: RPA, LAMP)
  - Little added cost
- Potential collaboration with industry
  - Need commercially produced lyophilized “kit” for end users
  - Need for appropriate field extraction
  - Instrumentation (e.g.: AmpliFire)
    - Simple data output for end users
    - No interpretation of curves, just +/-

Thank You!