RRV-DIAGNOSTICS

How good are they?

A tale about a technological journey that turned into an endeavor...

The RRV-diagnostic team
TEAM Working & TASKS distribution

- Polyclonal Ab
- Monoclonal Ab
- ELISA/Lateral flow device
- TaqMan RT-qPCR
- RPA (isothermal)
- One primer - multiple chemistries
- Artificial positive control
- LAMP (isothermal)
- HDA + self quenched primers
- Emaravirus - Multiplex RT-qPCR + HRM
- EDNA-Rose

Team speaker of the day: Francisco Ochoa-Corona
1. FAST !!!!
2. Friendly to use... meaning SIMPLE !!!
3. Consistent & reliable !!!
   meaning... VERY specific and sensitive !!!
4. Economic... meaning CHEAP !!!
5. Ideally... all pathogens of interest in A SINGLE TEST !!!

RRV-DIAGNOSTICS
How good are they?

vs.

How good they are demanded to be?
The Rose Virome

26 viruses reported
• 24 Complete genomes sequenced
• 2 No sequence available
• 26 2 DNA / 24 RNA genomes

Taxa distribution at a glance
• 4 Ilarvirus
• 4 +1 Nepovirus
• 4 Tospovirus
• 3 virus species (3 Genera in Betaflexiviridae)
• 10 virus species in different genera/family

Source: List of Rose Viruses 6_14/RRV project
Dimitri’s driven
Artificial roses expressing viral symptoms at retail stores...

Taken for Virus Chasers discussion

What this actually means?
• Even plastic roses get infected...
• Virus acceptance by market?
• Not a cosmetic issue?
• Plastic roses market is cheap...
• Do not buy plastic!

Rose yellow vein virus?
Perhaps Nutritional -hahaha! 😊
2016
Rose antivirus arsenal

<table>
<thead>
<tr>
<th>COMMERCIALLY AVAILABLE ASSAYS:</th>
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<tbody>
<tr>
<td><strong>ELISA:</strong> 13</td>
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<td><em>Lateral flow devices:</em> 4</td>
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<tr>
<td><strong>RT-PCR:</strong> 1</td>
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<table>
<thead>
<tr>
<th>RESEARCH/PUBLISHED ASSAYS (MOLECULAR)</th>
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</thead>
<tbody>
<tr>
<td><strong>RT-PCR:</strong> 22</td>
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</tbody>
</table>

... and we should:

- Minimize the number of assays
- Minimize assay testing time
- Minimize complexity of assays
- Transfer friendly assays to Diagnostic networks & Industry*

* Minimize complexity of assays
Combating RRD: Project’s Diagnostic Objectives

Key to this effort to detect the virus and to control the disease is the development of efficient diagnostic tools to enable rapid, easy-to-use, accurate and affordable detection of the virus. Specifically –

Reagents will be designed and developed
- RRV-specific primers/probes (for nucleic acid-based assays)
- Monoclonal and/or polyclonal antibodies (for serological antibody-based assays)

Techniques & Assays will be developed and refined for lab and field use
- ELISA and immuno-dipstick tests
- RT-LAMP (Reverse transcription-Loop mediated isothermal amplification)
- Self-quenched primer (SqP) technologies (for laboratory detection systems)
- Lateral flow devices (LFD; both Ab & NA-based detection assays)
Rose rosette disease (RRD) symptoms.

The RRV Disease Triangle

Rose rosette virus
(Emaraviruses)


Phyllocopetes fructiphilus

F.M. Ochoa-Corona

Image: G. Bauchan, R. Ochoa

Image courtesy ViralZone

Enveloped, spherical.

Jordan & Hammond, USDA-ARS, US National Arboretum
<table>
<thead>
<tr>
<th>Actual project output</th>
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<tbody>
<tr>
<td><strong>Developing reliable user friendly serological assays:</strong></td>
</tr>
<tr>
<td>• <em>ELISAs</em> and <em>Immuno lateral flow device</em> for rapid and user friendly detection</td>
</tr>
<tr>
<td>• To include monoclonal &amp; polyclonal antibodies.</td>
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<tr>
<td><strong>Developing reliable user friendly molecular assays:</strong></td>
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<tr>
<td>• Reference method based on Endpoint RT-PCR</td>
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<tr>
<td>• Exploring user-friendly isothermal methods:</td>
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<tr>
<td>- <em>Loop mediated amplification of DNA (LAMP)</em></td>
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<tr>
<td>- <em>Helicase dependent amplification (HAD) with self quenched primers</em></td>
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<tr>
<td>- <em>Recombinase Polymerase Amplification (RPA)</em></td>
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<tr>
<td>- <em>Exploring a broad detection &amp; discrimination for Emaravirus (RT-qPCR+HRM)</em></td>
</tr>
<tr>
<td>- <em>EDNA-Rose (Electronic Diagnostic Nucleic-acid Analysis)</em></td>
</tr>
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</table>
Progress Toward Development of a Serological Assay for the Detection of Rose Rosette Virus - 6

Current & Newly Developed Nucleic acid-based Diagnostics -Seven scientific articles-

Endpoint RT-PCR; RT-qPCR and SYBR Green RT-qPCR with High Resolution Melting analyses & Artificial Positive Control


**Taq-man qRT-PCR**


**SYBR Green qRT-PCR**


**RT-exoRPA (25-min isothermal assay)**

Development and Characterization of RRV virus-specific PcAb & McAb antibodies


- A cloned and bacterially-expressed RRV 316 aa nucleocapsid (NP) was purified and used as a source of RRV NP for immunization of rabbits and mice and as a positive control protein in immunoassays.
Development and Characterization of RRV-specific PcAb & McAb antibodies

Rabbit - Polyclonal antibodies

- Protein A-purified rabbit polyclonal antibodies react to RRV NP in Western-bLOTS and in both antigen coated plate (ACP-) and triple antibody sandwich (TAS-) ELISAs. [Titer > 1:512,000 in ACP-ELISA].

![Western blot image](image)

- eNP, 36.4 kDa
Development and Characterization of RRV-specific PcAb & McAb antibodies

Mouse - Monoclonal antibodies

- **Monoclonal antibodies from 10 mouse hybridoma cell lines** reacted with NP in ACP-ELISA and western-blots; 5 were selected for further study: Three that exhibited strong reactivity in both ACP- and TAS-ELISA and two that exhibited strong reactivity in ACP- and weak to zero reaction in TAS-ELISA.

Table 1. Immunoreactivities of selected monoclonal antibodies (McAb) to bacterially-expressed purified *Rose rosette virus* nucleoprotein (RRV-NP) in an antigen-coated plate ELISA (ACP-ELISA) and triple-antibody ELISA (TAS-ELISA).

| McAb Cell Line | ACP-ELISA | TAS-ELISA | ug mL⁻¹ | RRV-NP
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<td>NC</td>
<td>0.057³</td>
<td>0.002</td>
<td>ND</td>
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<tr>
<td>PTY-1</td>
<td>0.007</td>
<td>0.000</td>
<td>10.000</td>
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<td>1E6A10</td>
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<tr>
<td>3D5H6</td>
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<td>0.139</td>
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<td>0.948</td>
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<tr>
<td>8D2F4</td>
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<td>2.255 **</td>
<td>5.124</td>
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<tr>
<td>8E9E6</td>
<td>2.609 **</td>
<td>1.933 **</td>
<td>3.743</td>
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<tr>
<td>9A7E4</td>
<td>1.624 **</td>
<td>0.051 **</td>
<td>14.255</td>
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<tr>
<td>10D8E10</td>
<td>1.983</td>
<td>2.049</td>
<td>4.107</td>
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</table>

Jordan & Hammond, USDA-ARS, US National Arboretum
Mouse - Monoclonal antibodies

- Protein A-purified McAb 8D2F4 also reacts to bacterially-expressed RRV NP in Western-blot analysis.
Development and Characterization of RRV-specific PcAb & McAb antibodies

**Rabbit - Polyclonal antibodies**
- Protein A-purified rabbit polyclonal antibodies react to RRV NP in Western-blots and in both antigen coated plate (ACP-) and triple antibody sandwich (TAS-) ELISAs.
- Alkaline phosphatase-conjugated PcAb did **not** work in DAS-ELISA as detecting Ab.

**Mouse - Monoclonal antibodies**
- Protein A-purified McAb 8D2F4 also reacts to bacterially-expressed RRV NP in ACP- and TAS-ELISA, and Western-blots.
- Alkaline phosphatase-conjugated McAb 8D2F4 did work well in DAS-ELISA as the detecting Ab (with PcAb as trapping antibody).
Antigenic and Structural Analysis

- Predicted antigenic regions (dark red arrows) in the 316 amino acid sequence of RRV NP.
- Predicted secondary structure motifs in the protein (symbols above the NP sequence).

- Twelve synthetic 25-aa peptides containing the various antigenic regions were produced.
Progress Toward Development of a Serological Assay for the Detection of Rose Rosette Virus - 13

Development and Characterization of RRV-specific PcAb & McAb antibodies

PcAb and McAb reactivity to RRV-NP peptides
- Peptides 1-12: 25-aa NP peptides; Peptide 13: RRV-NP.
- Peptide 14-15: Flexiviridae Carlavirus coat protein peptides (Flex-CP 1 & 2)
- Peptide 16: Buffer blank.

✓ Note strong reactions with antigenic regions #3 and #11 (#13 = NP)
Development and Characterization of RRV-specific PcAb & McAb antibodies

Reactivity of 10 RRV-specific McAbs to RRV-NP and seven selected peptides
• Peptides 1, 3, 7, 9, 11, 13 (NP), and 16 (Flexi-CP 2)
• NC: negative control (no antibody)
• PTY-1: Potyvirus-specific McAb

▲ Note strong differential reactions with antigenic regions #3 and #11 (#13 = NP) by McAbs 8D2 and 8E9
3D model folding of RRV-NP [using Phyre2]
“NP surface-located” peptide regions 3 and 11 are identified [arrows].

- Peptide region #3 reactivities = PcAb, 1E6, 3A9, 8E9, 9A7
- Peptide region #11 reactivities = PcAb, 8D2, 10D8
Progress Toward Development of a Serological Assay for the Detection of Rose Rosette Virus - 16

Development and Characterization of RRR-specific PcAb & McAb antibodies

- Antigenic region #3 bound by PcAb and/or McAb 8E9
- Antigenic region #11 bound by PcAb and/or McAb 8D2

Proposed TAS-ELISA -->
1. Rabbit PcAb as trapping antibody.
2. Antigen/extract.
3. Admix of McAb 8D2 and 8E9 as detecting antibodies.
4. Then, Goat anti-mouse AP-conjugate.

<table>
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<tr>
<th>Trapping</th>
<th>Antigen</th>
<th>Detecting</th>
<th>Conjugate</th>
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<tbody>
<tr>
<td>PcAb</td>
<td>$^{3}$NP$^{-11}$</td>
<td>McAb 8D2</td>
<td>anti-M*</td>
</tr>
<tr>
<td>PcAb</td>
<td>$^{11}$NP$^{-3}$</td>
<td>McAb 8E9</td>
<td>anti-M*</td>
</tr>
</tbody>
</table>

- Now – Evaluate various extraction buffers (16).
- Now – Test fresh and frozen healthy and infected samples.
- Now – Compare side-by-side with PCR-based protocols.
- Next – Protocol and reagents will be sent to collaborators for their in-house validation.
- Next – Test parameters for immune-capture [IC-RT-PCR].
- Next – Immuno-Electron Microscopy (plants and mites).
- Next – Test/Develop Lateral Flow Assay [LFA; ImmunoStrips].
Direct antigen-capture (Binoy, et al., 2017)

1. Grind 100mg of leaves and petals in 1 mL of PBS-T (pH 7.4).
2. Add 50µL of sap to the PCR tube and incubate in ice for 2min.
3. Denature at 95°C for 1min.
4. Add 30µL of water nuclease free with RNAsin.
5. Remove the sap.
6. Wash the tube twice with 50µL PBS-T (pH 7.4).

4µL of extracted RNA was used to prepare cDNA (Dobhal, et al., 2016)
TaqMan RT-qPCR assay for *Rose Rosette Virus*

- A TaqMan RT-qPCR assay for RRV for multiple gene targets
- Primers & probes designed made on genomic RNA2 & RNA3
- TaqMan probes: FAM (reporter) and IOWA black (quencher) used
- The assays were specific as tested against common rose infecting viruses (exclusive and inclusive to *Emaravirus*)
- Sensitivity to 1 fg

![Graphs showing fluorescence over cycles for different RRV targets](image)

University of Florida

Development of Recombinase polymerase amplification (RPA) assay for *Rose Rosette Virus*

- RT-RPA is isothermal (37–42°C)
- Specific & sensitive (1 fg) primers/probe for RT-RPA
- Rapid direct capture of RRV directly on PCR tube (>5 min) and a single standardized
- Tested with different tissues
- Tested with a number of rose varieties
- Testing time: 15-20 min

Samples
L1/L2 = Leaves
S1/S2 = Stem
P1/P2 = Petals
R1a/R1b = Primary roots
R2a/R2b = Secondary roots
Multi-target Artificial Positive Control (APC) harboring a synthetic RRV target of 125 bp

Maintained in soluble paper (APC Technology patented in our lab)

Artificial Positive Control
Field samples

125 bp
108 bp
Detection of Rose Rosette Virus Infection in Field Samples Using RT-PCR and APC

**RRV qRT-PCR preliminary test**
Green lines are positive controls, red lines are plasmid containing a RRV fragment, blue are healthy tissue and yellow non template control (NTC)

The method includes the way to overcome problems posed by inhibitors which are abundant in roses.

BSA /50 mM
PVP40 /10%
OBJECTIVE 1

To Compare the sensitivity of pH-sensitive dyes, for visual detection of Loop-Mediated Isothermal Amplification (LAMP) for diagnosis of *Rose rosette virus*

- **P3 gene**
- **LAMP**
- **P4 gene**

**Bst 2.0 WarmStart DNA Polymerase**
- (cDNA)

**OptiGene GspSSD Mastermix ISO-004**
- (cDNA or RNA)

- **Hydroxynaphtol Blue (HNB)**
- **Cresol Red (CR)**
- **Malachite Green (MG)**

A. Salazar-Aguirre
Isothermal – NO thermocycler

- **LAMP – Optigene**
  with P4 primers

- **HDA**
- **HDA + Self Quenched primers**

- **Direct trapping**
  RRV in PCR tube

- **Visualization with pre-casted gels**

- **LAMP – Bst 2.0 WarmStart DNA Polymerase**
  with P4 primers

- **Replicase Polymerase Amplification (RPA)**
How much did we progress?

- RT-qPCR + direct RRV trapping in plastic
- Artificial Positive control RRV+ other 5 virus infecting ornamentals
- Helicase Dependent Amplification (HDA)
- Helicase Dependent Amplification (HAD) + to Self Quenched primers
- Multiplex RT-PCR for Emaravirus
- LAMP-P3 Bst 2.0 WarmStart DNA Polymerase + Hydroxynapthol blue
- LAMP-P4 OptiGene (Realtime LAMP & precasted gel)
Progress Toward Development of a Reliable, Efficient, Cost-effective, User-Friendly Diagnostic Assay for the Detection of Rose Rosette Virus

Table. Comparative analysis of the different potential diagnostic methods

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sensitivity</th>
<th>Skill required</th>
<th>Equipment needed</th>
<th>High throughput</th>
<th>Time required</th>
<th>Cost</th>
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<tbody>
<tr>
<td><strong>Nucleic acid-based Assays</strong></td>
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<tr>
<td>RT-PCR</td>
<td>High</td>
<td>High</td>
<td>Yes</td>
<td>Low</td>
<td>6-8 hrs</td>
<td>Med</td>
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<td>RT-LAMP</td>
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<td>Med</td>
<td>Yes</td>
<td>Med</td>
<td>1-3 hrs</td>
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<td>RT-exoRPA</td>
<td>High</td>
<td>Med/Low</td>
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<td>Med</td>
<td>20-30 min</td>
<td>Med</td>
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<td>NGS</td>
<td>Med</td>
<td>High</td>
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<td>Low</td>
<td>Months</td>
<td>High</td>
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<tr>
<td>EDNA</td>
<td>Low?</td>
<td>Med/Low</td>
<td>Yes [Laptop]</td>
<td>High</td>
<td>10 min</td>
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<td><strong>Antibody-based Assays</strong></td>
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<tr>
<td>ELISA</td>
<td>Med</td>
<td>Med</td>
<td>Yes/No</td>
<td>High</td>
<td>4-18 hrs</td>
<td>Low</td>
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<tr>
<td>Immunostrip</td>
<td>Low/Med</td>
<td>Low</td>
<td>No</td>
<td>Med</td>
<td>10-30 min</td>
<td>Med</td>
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</tbody>
</table>
New from our kitchen

Rose Microbudding
a faster alternative for assessment RRV
Rose varietal resistance

Microbudding was Developed by Dr. M. Skaria (Texas A&M) during the late 90’s for high throughput propagation of citrus

USE OF MICROBUDDING TO EXPEDITE PRODUCTION OF EXPERIMENTAL CITRUS HOST FOR USE FOR BIOLOGICAL INDEXING OF CITRUS PATHOGENS.

F. M. Ochoa¹, M. G. H. Dakkers¹, M. Skaria² and R. F. Lee¹. ¹University of Florida, CREC, Lake Alfred, FL USA. ²Texas A&M University, Kingsville Citrus Center, Weslaco, TX USA.

Fig. 1. Micro-grafting technique. 1A) Micro-bud protected by 200 µl pipette tip. 1B) Micro-bud flushing after approximately 6 weeks.
Rose Microbudding steps for RRV screening of germplasm resistance

Tested Germplasm

RRV + rootstock

Micro-pipette tip

2 months
12 hours light/dark
20°C/18°C
79-80 RH
TWO MONTHS AFTER MICROBUDDING-INOCULATION

RRV- Knock out®/ RRV+ Sweet® drift

RRV+ control Knock out®

Root callus initiation

RRV- healthy control Knock out®/Knock out®
Is this of interest for rose breeders?

- Potentially a 3-4 months assay to assess varietal resistance of rose progenies
- Will require an infected block in a infested area for sourcing of infected canes
- Easy to combine with a serological or molecular method of choice

RRV+ ‘Sweet drift®’ in Oklahoma
Moving toward the Future

Consolidating EDNA–ROSE
*a windows based bioinformatics portal for rapid processing of NGS generated files*

G.R.A. & Doctoral student

Dr. Andres Espindola

an ‘All IN ONE’ Rose-virus Assay
Combining NGS and BIOINFORMATICS
The EDNA concept

1. **Cucurbit Genome**
2. **Cucurbit Virus Genomes**
3. **Near Neighbor Genomes**
4. **Next Generation Sequencing Simulator (MetaSim)**
5. **Electronic Probe Design**
6. **Mock Sample Database (MSD)**
7. **E-probes**
8. **BLASTn**
9. **Plant Sample**
10. **Next generation sequence (NGS)**
11. **Sample Sequence Database**
12. **In vitro stage**
13. **In silico stage**

- **Pathogen absent**
- **Pathogen present**
- **Match/hit?**

---

**EDNA**

Electronic Diagnostic nucleic acid Analysis
EDNA Advantage:
Highly reduced size of screening database
Highly curated database

Traditional approach

Traditional approach

EDNA approach

E-probe database 0.0001 Gb

LARGE REFERENCE DATABASE
Public database

SMALL REFERENCE DATABASE
Highly curated database
### SIMULATED RESULTS USING SOFTWARE - DATA FROM EDNA2 SERVER

**ILLUMINA SIMULATOR**

**TEN MILLION READS GENERATED**

**SENSITIVITY ASSAY in silico**

- 10% VIRUS – 90% PLANT
- 1% VIRUS – 99% PLANT
- 0.1% VIRUS – 99.9% PLANT
- 0.01% VIRUS – 99.99% PLANT
- 0.001% VIRUS – 99.999% PLANT
- 0.0001% VIRUS – 99.9999% PLANT

### eprobeDB

<table>
<thead>
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CONCLUSIONS

• e-probes for 24 Rose virus showed specificity over ten million MSDS simulating single and multiple viral infections.

• EDNA2 can accurately detect viral infections *in silico* in a *Rosa multiflora* metagenomic database infected with RRV and PNRSV.

• EDNA2 limit of detection *in silico* is 0.001% viral reads in single and multiple infections output databases.

• Metagenomic data processing in EDNA2 server take minutes.
VII International Symposium on Rose Research and Cultivation

Section Ornamental Plants

Broad detection strategies for multiple targets of rose viromes using next generation sequencing and bioinformatics (Lizbeth Peña-Zúñiga)

Lizbeth Peña-Zúñiga, winner of the ISHS student award for the best oral presentation.

Mi-Fi a FRIENDLY USER INTERFACE

E-PROBE DATABASE AVAILABLE

EDNA Rose

TIP E-PROBES: You can use the search box below to filter your e-probes by virus species name. Then select your intended e-probes and click on create new list. You can create lists that contain e-probes of your interest. Then you can use them in the Metagenomes section to run EDNA detection on your uploaded metagenomes.

We have 26 Rose virome e-probe database(s) available for you

<table>
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<tr>
<th>E-probe ID</th>
<th>E-probe name</th>
<th>IF</th>
<th>Target Genome</th>
<th>Host</th>
<th>Status</th>
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<td>Raspberry ringspot virus</td>
<td>rose</td>
<td>READY</td>
</tr>
</tbody>
</table>
REAL DATA ANALYSIS with true NGS files from FPS (blind test)
FUTURE APPLICATION: VIRUS FREE ROSE

Breeding Blocks

F1, F2, F3....

Foundation blocks (Mother stocks)

Propagation blocks

Commercial Propagation Growers

hundreds

NGS

EDNA2

thousands

TIME
Combating Rose Rosette Disease

USDA National Institute of Food and Agriculture (NIFA) Specialty Crop Research Initiative project, “Combating Rose Rosette Disease: Short Term and Long Term Approaches” (2014-51181-22644/SCRI)

USDA-OKLAHOMA DEPARTMENT OF AGRICULTURE FOOD & FORESTRY (ODAFF) SCBGP
Diagnostic Nucleic Acid Analysis EDNA for accurate detection & discrimination of Rose Viruses.
Questions?