



# High Throughput Sequencing (HTS) as a Tool for Viral Pathogen Diagnosis and Expedited Release of Quarantined Propagative Plant Material

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**UCDAVIS FPS**



# FPS CLEAN STOCK PROGRAM

## Foundation Plant Services (FPS) Mission



- Produce, test, maintain and distribute elite disease-tested plant propagation material.
- Provide plant importation and quarantine services, virus testing and virus elimination.
- Coordinate release of UC-patented varieties.
- Link researchers, nurseries, and producers.

# Registration and Certification of Grapevines

## 3 CCR § 3024-3024.8

Last updated 2010

The Grapevine Registration & Certification Program is a **voluntary program that provides for the testing of source vines for significant grape pathogens**. Registered sources and certified nursery stock are then inspected by CDFA staff and maintained by the participant in a manner to protect them from exposure to regulated diseases.

# California Registration and Certification Programs

## Grapes



## Fruit and Nut Trees

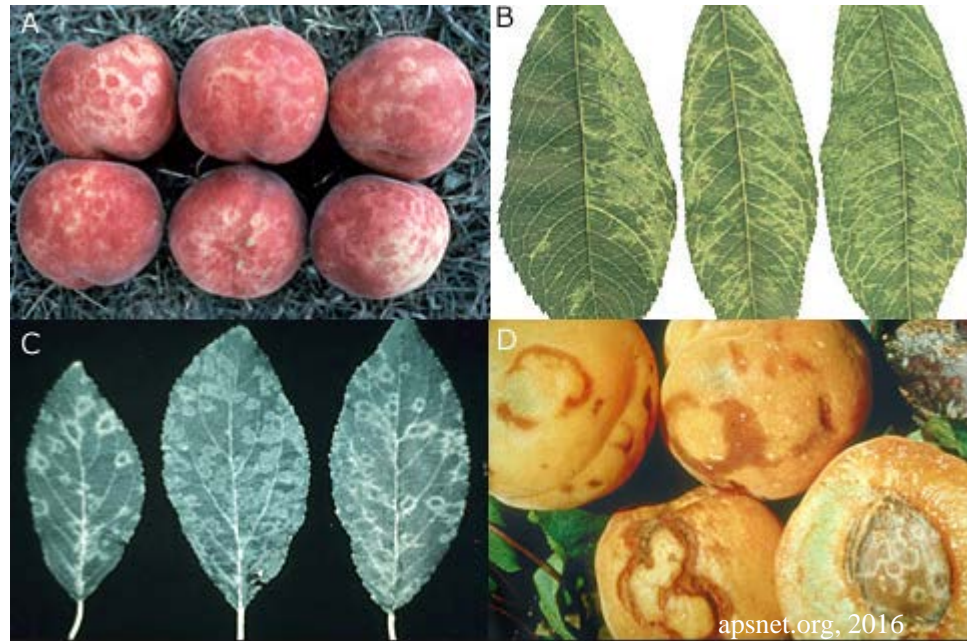
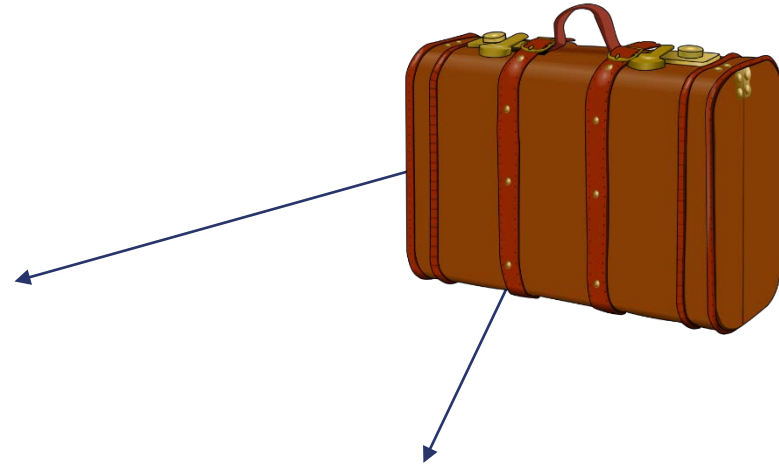


## Strawberries



# The Dangers of Samsonite Importation

## Pierce's Disease



**Plum pox virus on stone fruit**

# National Grapevine Importation Program: Foreign Imports

- Housed at FPS
- Largest nationally-recognized program for importing grape selections into the US
- Serves as both an importation and quarantine facility



# APHIS – Animal Plant Health Inspection Service



## Plant Health (PPQ)

- **APHIS' Plant Protection and Quarantine (PPQ) program safeguards U.S. agriculture and natural resources against the entry, establishment, and spread of economically and environmentally significant pests, and facilitates the safe trade of agricultural products.**



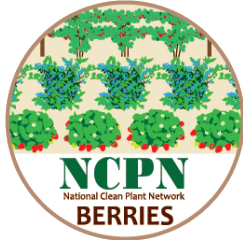
# National Clean Plant Network (NCPN)

- A national network of clean plant centers, scientists, educators, state and federal regulators, and growers and nurseries.
- Focused on providing healthy planting stock of vegetatively propagated specialty crops to nurseries and growers.

**UC DAVIS**  
UNIVERSITY OF CALIFORNIA

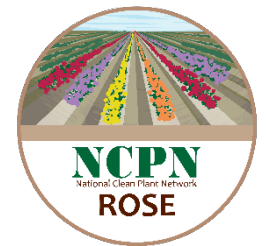




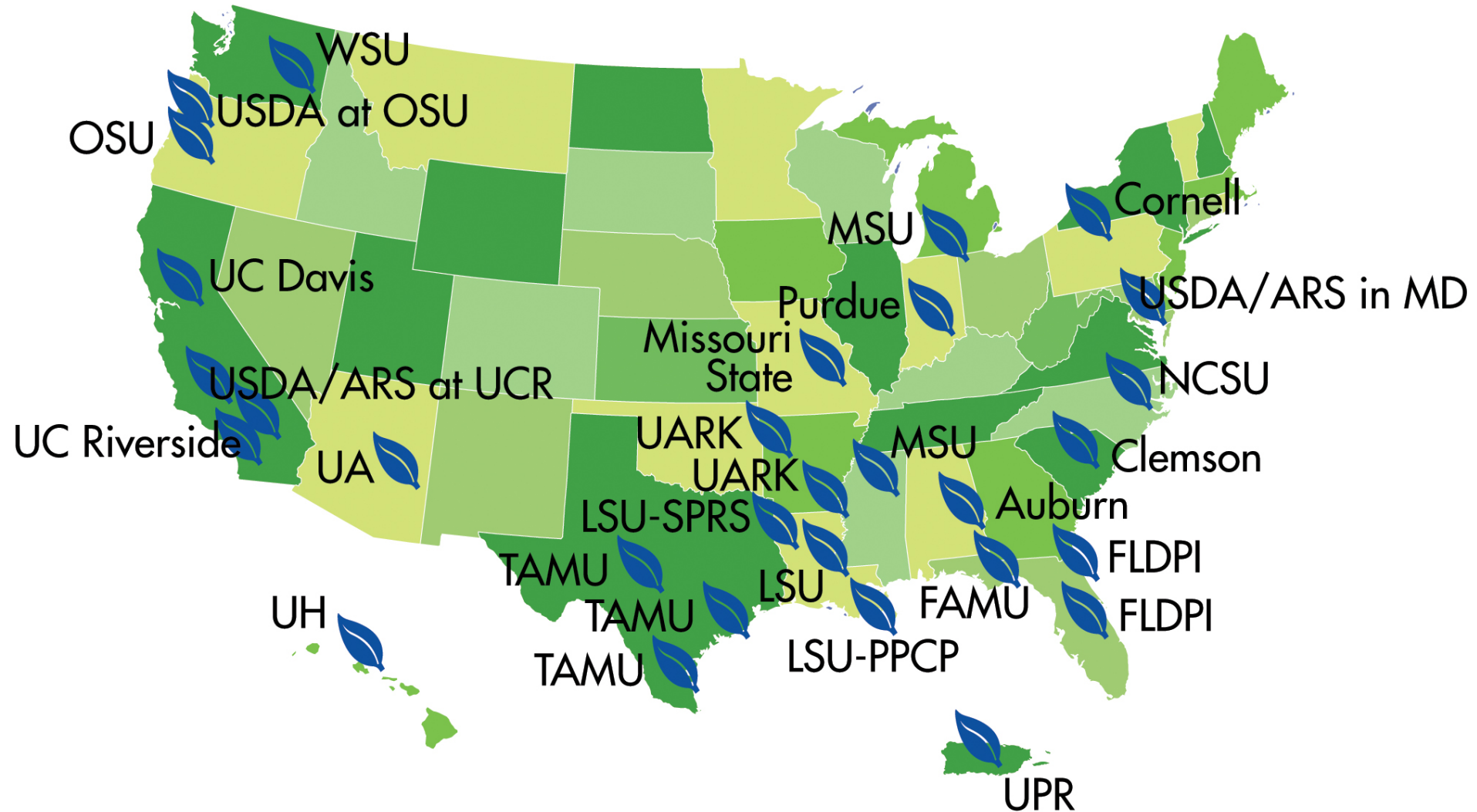


# Participating Crops

1. Fruit Trees
2. Grapes
3. Berries
4. Hops
5. Citrus
6. Sweet potatoes
7. Roses

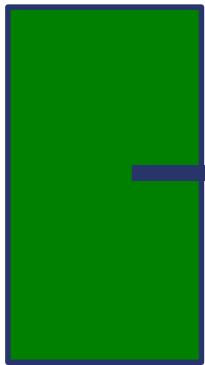


# The NCPN Clean Plant Centers



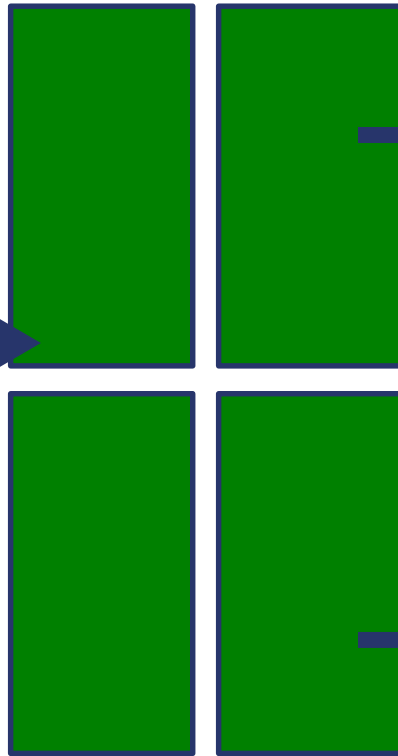
**START CLEAN**

**Foundation**



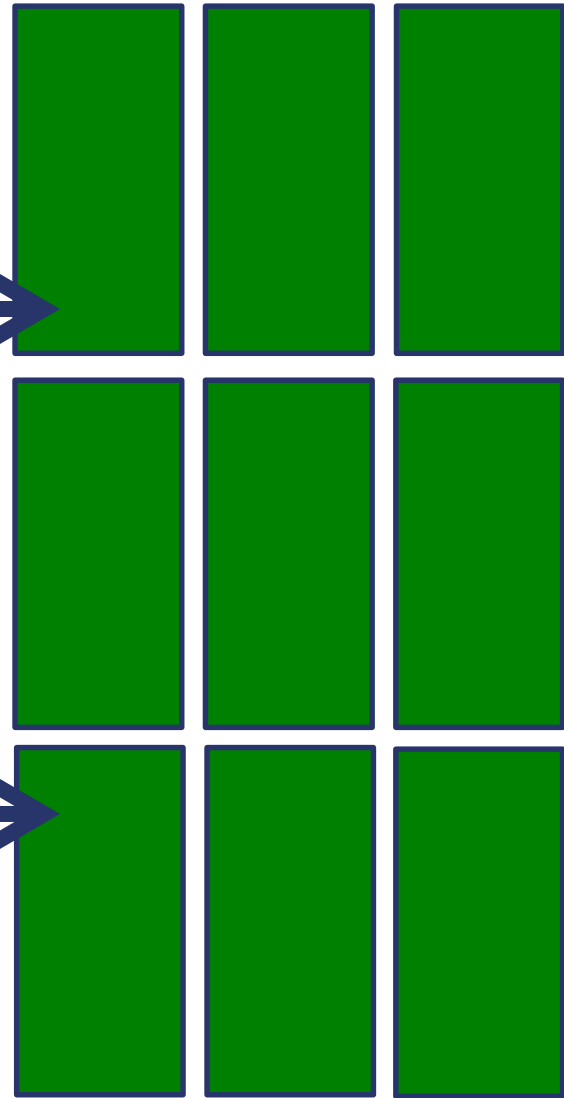
**FPS**

**Registered  
Increase Blocks**



**Nurseries**

**Production Vineyards**



**Growers**

**STAY CLEAN**

# FPS Impact

20 – 30 million grapevine plants sold per year trace back to FPS



# FPS testing protocol

New Grape Selection

- ▶ Foreign imports
- ▶ Domestic selections
- ▶ New cultivars

Release time

2-6 years

Disease Testing

Retesting

Tests positive

Disease Elimination Therapy

- ▶ Tissue culture
- ▶ Heat treatment

all tests negative

FOUNDATION **G1**

Provisional Foundation Vines

Professional Identification

ID not correct  
REMOVE

ID verified correct

Registered Foundation Vines **G1**

To Nurseries

Registered Stock **G2**

To Growers

Certified Stock **G3**

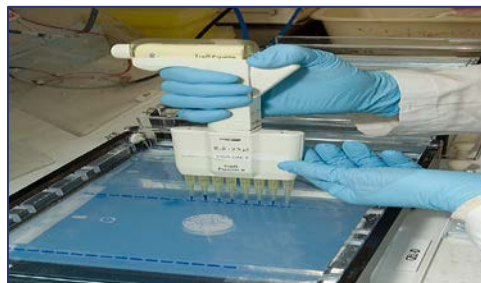
# Tests required by APHIS and CDFA for certification

Standard detection methods for a range of suspected 'known' viruses using a panel of specific tests: ELISA, RT-PCR and RT-qPCR

**ELISA**



**RT-PCR**



**RT-qPCR**



## Biological Indexing: A broader techniques

**Herbaceous Index**



**Woody Index**



# Can HTS replace the field indexing requirement ??

We need to scientifically demonstrate the advantages of HTS over Biological Indexing

Run a side-by-side comparison

## Comparison of Next-Generation Sequencing Versus Biological Indexing for the Optimal Detection of Viral Pathogens in Grapevine

Maher Al Rwahnih, Steve Daubert, Deborah Golino, Christina Islas, and Adib Rowhani

Department of Plant Pathology, University of California, Davis 95616.  
Accepted for publication 2 February 2015.

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### ABSTRACT

Al Rwahnih, M., Daubert, S., Golino, D., Islas, C., and Rowhani, A. 2015. Comparison of next-generation sequencing versus biological indexing for the optimal detection of viral pathogens in grapevine. *Phytopathology* 105:758-763.

vines being tested. We compared the bioassay against next-generation sequencing (NGS) analysis of grapevine material. NGS is a laboratory procedure that catalogs the genomic sequences of the viruses and other pathogens extracted as DNA and RNA from infected vines. NGS analysis was found to be superior to the standard bioassay in detection



# Estimated cost for conventional virus testing

Assay	cost/selection
PCR assays (37 pathogens)	\$1,200
ELISA (4 pathogens)	\$250
Herbaceous host indexing	\$100
Woody host indexing	\$350
<b>Total</b>	<b>\$1,900</b>

**Minimum release time: 2 – 3 years**

# With HTS testing option

- Cost \$ 300 per selection
- Total testing time is 1 - 2 months

## Main advantage:

- Detects ALL known and unknown viruses (all strains)



# Summary

HTS analysis has advantages over the standard bioassay in:

- **Detection of viruses of agronomic significance (including low titer viruses)**
- **Comprehensiveness**
- **Speed of analysis**
- **Discovery of novel, uncharacterized viruses**

- FPS has a **new import permit** that allows the use of HTS analysis
- When HTS use is accepted for grapevine certification and registration in place of the current industry standard, growers will be able to start propagative increase and virus elimination programs with most new accessions **years earlier** than they can now



United States Department of Agriculture  
Animal and Plant Health Inspection Service  
4700 River Road  
Riverdale, MD 20737



**Controlled Import Permit to Import Restricted or Not Authorized Plant  
Material**

**Regulated by 7 CFR 319.6**

# The improved testing protocol

## New Grape Selection

- ▶ Foreign imports
- ▶ Domestic selections
- ▶ New cultivars

## Disease Elimination Therapy

- ▶ Tissue culture
- ▶ Heat treatment

Disease Testing

Retesting

Tests positive

Provisional release time

Final release time

all tests negative

### FOUNDATION

Provisional Foundation Vines

Professional Identification

ID not correct  
**REMOVE**

ID verified correct

Registered Foundation Vines

To Nurseries

Registered Stock

To Growers

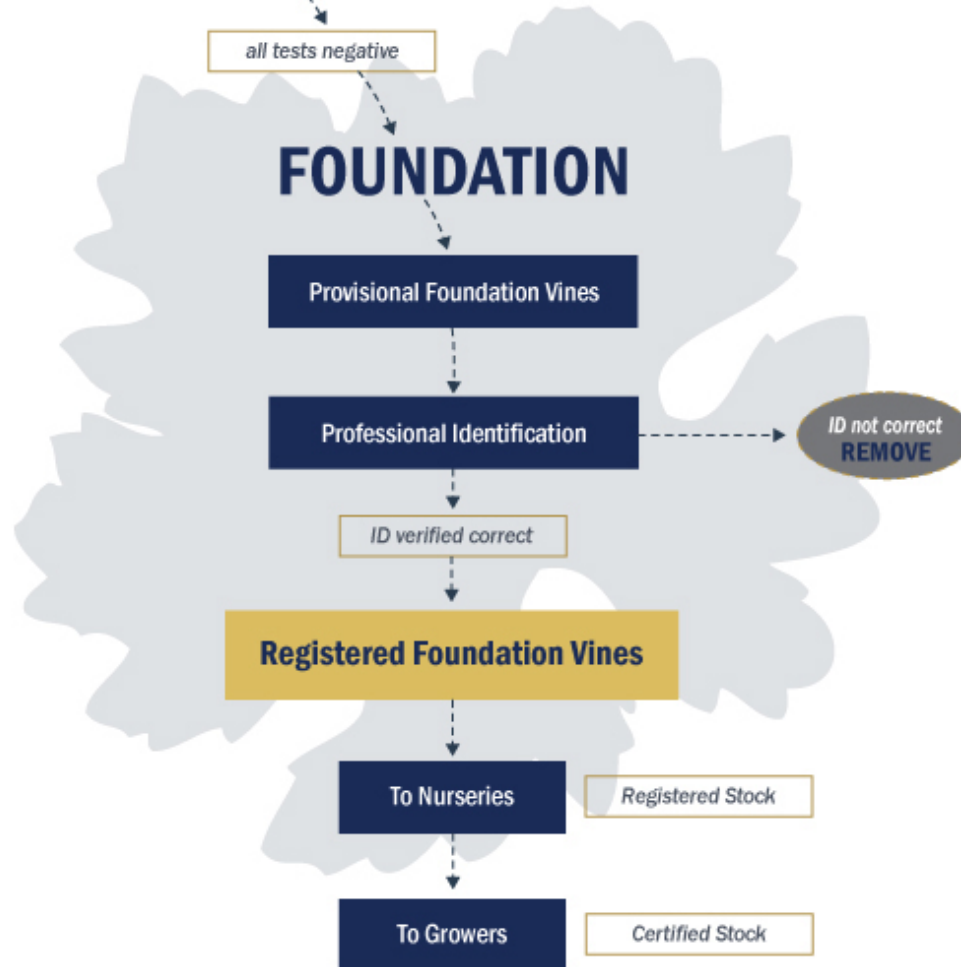
Certified Stock

2-4 months

\*No virus-like agents detected with HTS

2 years

\*No virus-like agents detected with biological indexing



# What is next??

- **Run side-by-side studies to accumulate further comparative data, until the replacement of the bioassay is accomplished**
- **Coordinate with other countries, which are pursuing a similar protocol revision.**

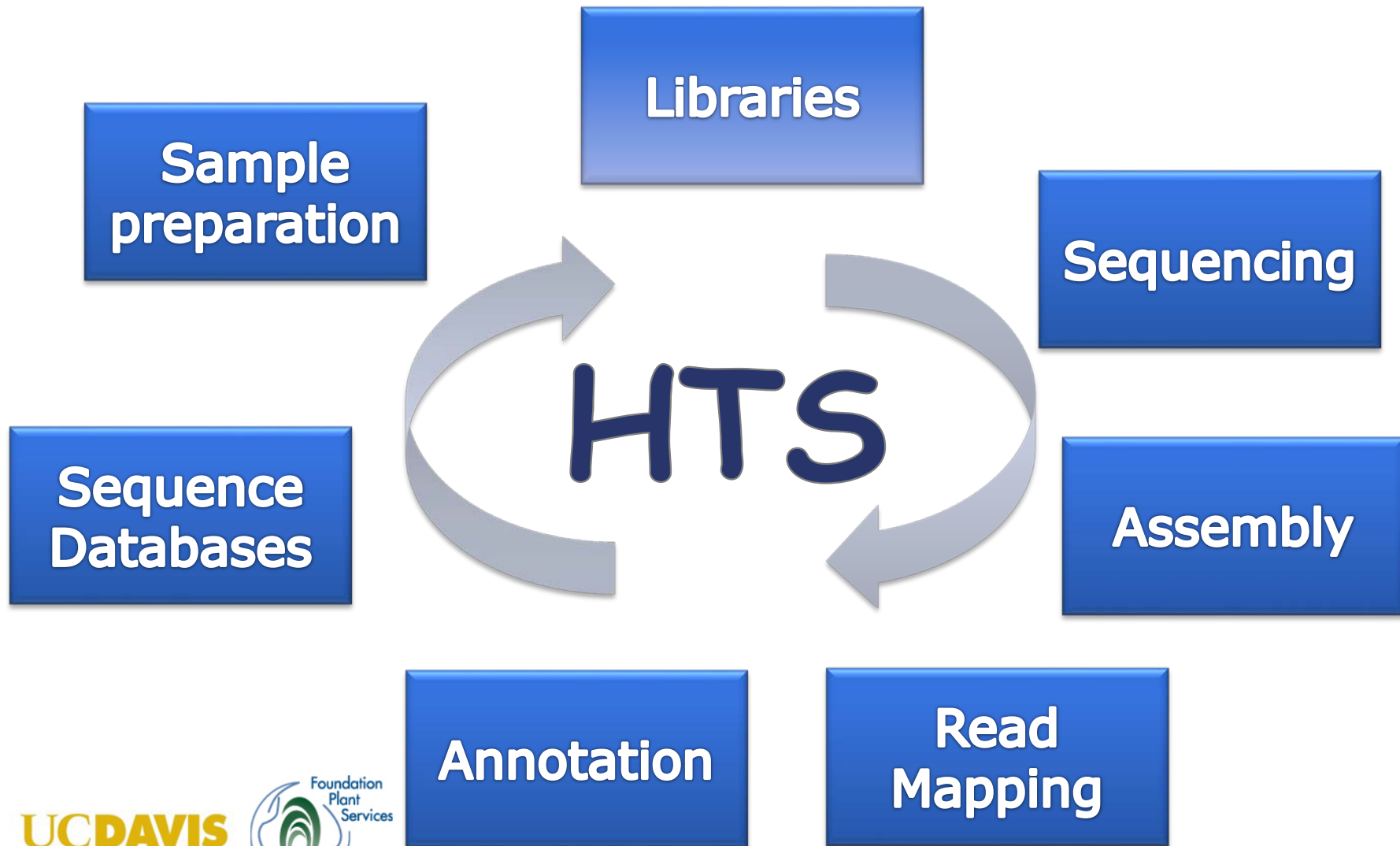


# However, there are limitations...

1. Technical challenges

2. Establishment of biological significance

# HTS technical challenges





# HTS: Technological challenges

## The use of HTS as a routine diagnostic tool

- **Developing efficient sample preparation methods for large scale application.**
- **Developing bioinformatics algorithms to efficiently separate pathogen and host sequences**

**Validation....Validation.... Validation!!!!**



# Establishment of Biological Significance

# Novel grapevine viruses discovered by HTS

- 2009 Al Rwahnih et al.: Description of Grapevine Syrah virus 1 (California, USA)
- 2011 Giampetruzzi et al.: Description of **Grapevine Pinot gris-associated virus** (Italy)
- 2011 Zhang et al.: Description of Grapevine vein clearing virus (Midwest USA), the first DNA virus found in *Vitis*.)
- 2012 Al Rwahnih et al.: Description of Grapevine virus F (California, USA)
- 2012 Al Rwahnih et al.: Identification of **Grapevine red blotch-associated virus** (California, USA). (Same as Grapevine Cabernet franc-associated virus: New York. Krenz et al., 2012)
- 2013 Al Rwahnih et al.: Identification of plant virus satellite. (California, USA)
- 2014 Maliogka and Katis: A putative badnavirus identified in vines affected by Roditis leaf discoloration (Greece)
- 2015 Al Rwahnih et al.: A putative reovirus identified in Cabernet Sauvignon vines (California, USA, Brazil) (Same as Summer Grape latent virus - Mississippi USA) Sabanadzovic et al., 2012
- 2016 Al Rwahnih et al.: A putative Fabavirus identified in Nagano Purple vines (South Korea, India)
- 2016 Al Rwahnih et al.: A novel monopartite geminivirus and its defective DNA molecule characterized from grapevine (*Vitis vinifera* L.). **Grapevine geminivirus A (GGVA)**
- 2016 Silva, et al.: Molecular characterization of grapevine enamolike virus, a novel putative member of the genus enamovirus (Brazil)

**And many more novel grapevine viruses are currently in the pipeline....**

# Bioinformatic analysis cannot prove pathological causality

- **Detection of a given pathogen sequence does not mean that pathogen is responsible for the disease.**
- **Koch's postulates cannot be satisfied using only HTS-based data.**

# Establish the significance of HTS findings

- HTS discovery of novel viruses is based solely on genomics information!!!
- But **NO decision** can be made on the importance of a novel virus without information about its biological effects...

## **Biological effects are assessed by:**

- **Performing graft transmission**
- **Fulfillment of Koch's postulates**
- **Spread and distribution studies**
- **Assessment of (symptoms) agronomic significance**

# A novel virus in a domestic selection: Grapevine red blotch virus (GRBV)



**Fig. 1.** Symptoms of grapevine red blotch disease on leaves of **A**, ‘Cabernet Franc’ clone 214 and **B**, ‘Cabernet Sauvignon’ clone 7 in fall. **C**, Red secondary and tertiary veins of a leaf from affected Cabernet Franc grapevine. **D**, Basal leaves on the shoots of a mature Cabernet Franc clone 214 grapevine showing red blotch symptoms in fall.

## Association of a DNA Virus with Grapevines Affected by Red Blotch Disease in California

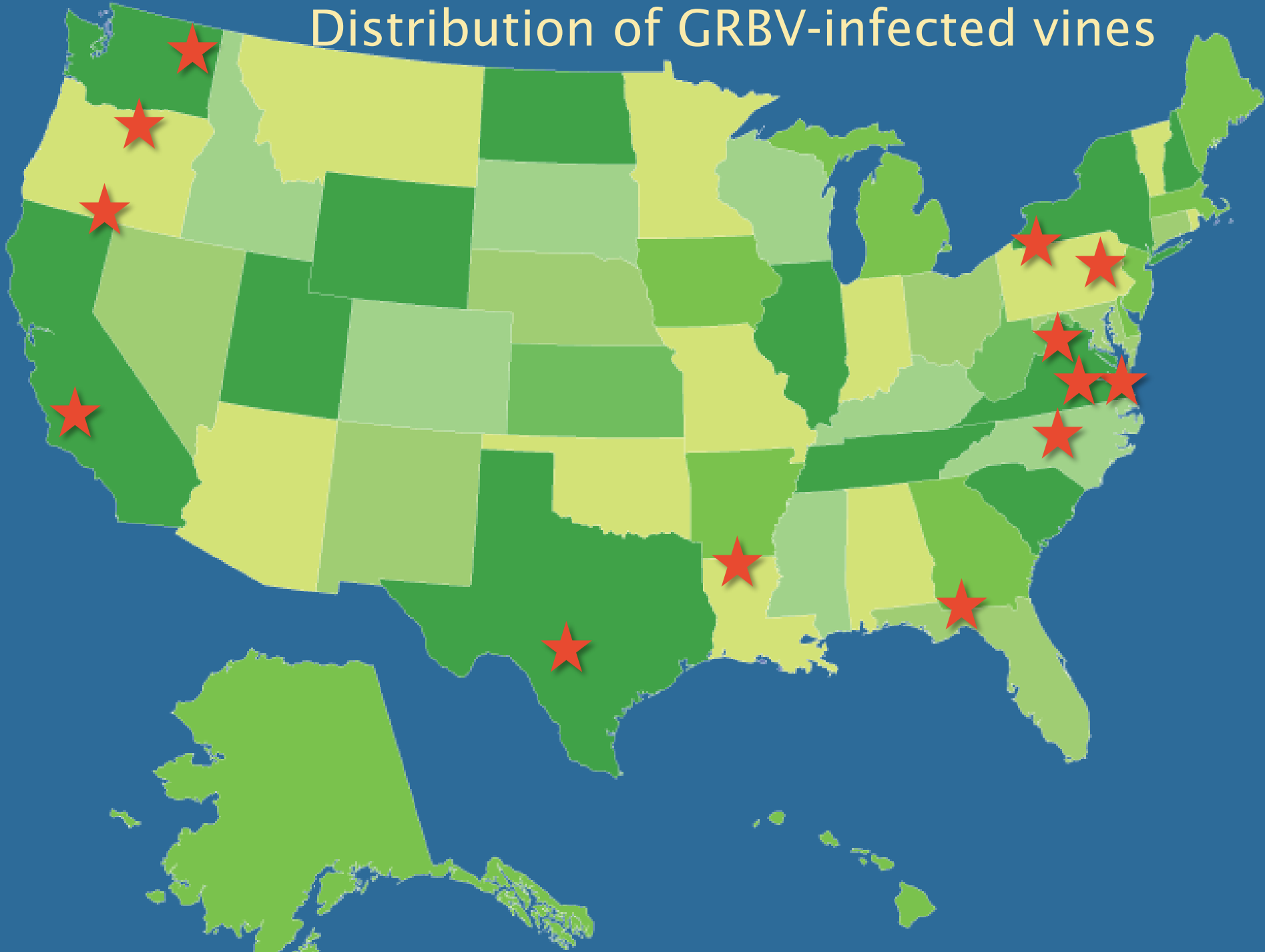
Maher Al Rwahnih, Ashita Dave, Michael M. Anderson, Adib Rowhani, Jerry K. Uyemoto, and Mysore R. Sudarshana



# GRBV: Chip bud inoculations

Source vines		Tested	PCR +ve	
			Petioles	Roots
a) Grapevines	Cabernet franc	5	4	2
	Cab Sauvignon	5	2	4
	Zinfandel	5	0	4
b) Controls	Healthy	3	0	0
	Ungrafted	3	0	0

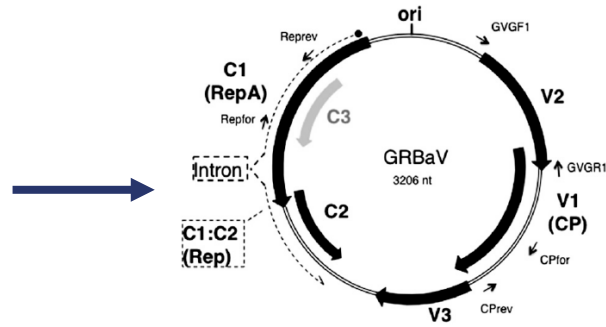
# Distribution of GRBV-infected vines



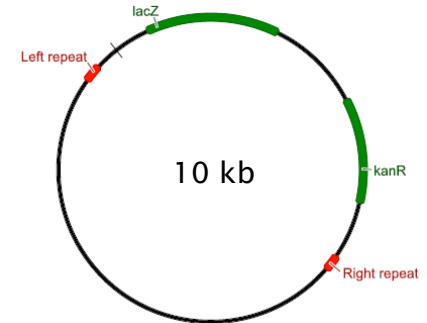
# Fulfillment of Koch's postulates



Red blotch



Grapevine red blotch virus



Plasmid vector



Healthy grape



*Agrobacterium tumefaciens*

# ***Grapevine red blotch-associated virus* Is Widespread in the United States**

B. Krenz, J. R. Thompson, H. L. McLane, M. Fuchs, and K. L. Perry

## ***Grapevine red blotch-associated virus* is widespread in California and U.S. vineyards.**

M. R. SUDARSHANA (1), A. Gonzalez (1), A. Dave (1), A. Wei (2), R. Smith (3), M. M. Anderson (3), A. M. Walker (3)

## **Detection and genetic diversity of Grapevine red blotch-associated virus isolates in table grape accessions in the National Clonal Germplasm Repository in California**

Maher Al Rwahnih<sup>a</sup>, Adib Rowhani<sup>a</sup>, Deborah A. Golino<sup>a</sup>, Christina M. Islas<sup>a</sup>, John E. Preece<sup>b</sup>  
& Mysore R. Sudarshana<sup>c</sup>

## **Grapevine Red Blotch-Associated Virus, an Emerging Threat to the Grapevine Industry**

Mysore R. Sudarshana, Keith L. Perry, and Marc F. Fuchs

# Regulation Updates

**California, Oregon, Washington, and New York have added GRBV to the list of regulated viruses**

## **Novel viruses in a foreign import:**

**Grapevine fabavirus (GFabV), a novel putative member of the genus Fabavirus**

**Grapevine geminivirus A (GGVA), a novel gemini-like virus**

# New introduction (quarantine material)

Two accessions of Japanese table grapes, introduced to the FPS from South Korea in 2013

A: chlorotic ringspots on NP



B: asymptomatic BB



C: chlorotic ringspots on Cab franc indicator graft inoculated with NP



D: leaf roll symptoms on Cab franc indicator graft inoculated with BB



# HTS Results



## Nagano Purple

- **Novel Gemini-like virus: Grapevine geminivirus A (GGVA) and its defective DNA molecule (GGVA D-DNA)**
- **Novel Fabavirus (GFabV)**
- **GLRaV-3, GFkV, GSyV-1, and viroids**
- **GLRaV-2**



## Black Beet

- **GGVA only with no defective DNA**
- **GFabV**
- **GLRaV-3, GFkV, GSyV-1, and viroids**
- **GVE, GRSPaV, GRVfV**



# Genome of Grapevine fabavirus (GFabV)

- **GFabV shared 23-34% sequence identities with polyproteins RNA-1 and RNA-2 of Broad bean wilt virus 1**
- **GFabV only shared 40% with PrVF from Cherry**



## New Viruses, Next Steps...

- Communicate with Plant Protection and Quarantine (PPQ)
- Conduct a survey using new real time PCR assays
- Total of 1,262 vines tested

Source	Number of plants tested	GFabV positive	Variety/ Country of origin	GGVA positive	Variety/Country of origin
UCD Grapevine Virus Collection	585	0	N/A	3	Tamar/Israel
USDA NCGR	230	1	Bhokri/India	15	Koshu Sanjaku/Japan Muscat Angel/Japan Super Hamburg/Japan Pione/Japan J-167-045/China Kyoho/Japan Neo-muscat/Japan Longyan/China Scol. Kiralynoje/Hungary Pearl of Zola/US
FPS New Introductions	250	0	N/A	4	BB/S.Korea, NP/S.Korea, Nehelescol/Japan, Shine Muscat, S. Korea
Commercial Vineyards	197	0	N/A	0	N/A

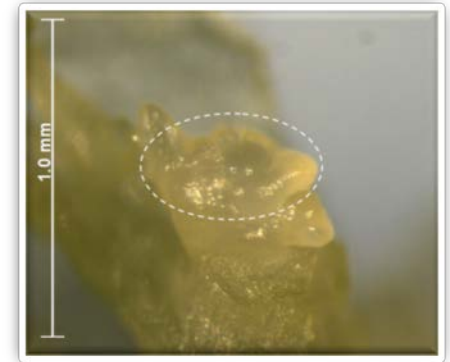
# GGVA Relevance Study: NCGR Grapevine Germplasm Collection



# Virus Elimination Therapy



Collect apical shoot tips



Excise meristem dome and 1-2 pairs of leaf primordia



Plants develop a shoot first, then root in 5 to 12 months

## 4 years after tissue culture treatment:



**Cv. 'Nagano Purple 0.1'**

**Tests results:**

**PCR: negative for all viruses**

**Bioassay: negative in all indicators**



**Cv. 'Black Beet 0.1'**

**Test results:**

**PCR: positive only for GFabV**

**Bioassay: positive in Cv. 'St George'**

Spring 2017



**Indicator plants Cv. 'St George' grafted with Black Beet 0.1**

## PCR results

Virus	Result
Grapevine fleck virus (GFkV)	NEG
Grapevine geminivirus A (GGVA)	NEG
Grapevine leafroll-associated virus 3 (GLRaV-3)	NEG
Grapevine rupestris stem pitting-associated virus (GRSPaV)	NEG
Grapevine virus E (GVE)	NEG
<b>Grapevine fabavirus (GFbV)</b>	<b>POS</b>



- Repeat the indexing in 2017
- HTS on TCE plant and St George indicator
- Infectious clone-Koch's postulates



# Open questions:

- **Fulfill of Koch's postulates**
- **Spread or vector**
- **Distribution studies**
- **Assessment of (symptoms) agronomic significance**

# Mixed infection

The most pathogenic viruses in a mixed infection cannot be distinguished from the rest by NGS data.

Grapevine syrah virus-1  
(GSyV-1)



Grapevine vein clearing  
virus (GVCV)



Grapevine Pinot gris virus  
(GPGV)



mixed infection with:

GRVFV, GRSPaV, GLRaV-9  
AGVd, GYSVd HSVd

GFLV, GRSPaV, ToRSV

GRVFV, GRSPaV, GSyV-1  
GYSVd1, HSVd

# Discovery of insignificant background viruses

- Cryptic viruses
- Latent viruses
- Asymptomatic viruses



Our main goal is to single out the viruses with **high economic impact** (regulated viruses)

Science & Society

CellPress

*Omics: Fulfilling the Promise*

**Virus discovery: are we scientists or genome collectors?**

**Marta Canuti and Lia van der Hoek**

Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity (CINIMA), Academic Medical Center (AMC), University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

## What do we need?

**We are in need of standard protocols/minimal requirement for HTS use.**

**Establish a framework for the evaluation of risks posed by new virus discovered by HTS.**

## Other Challenges!!!

- **Naming the novel viruses!!**
- **Releasing the sequences!!**
- **Publishing!!**



THANK YOU

**UCDAVIS FPS**

