Netherlands Institute for Vectors, Invasive plants and Plant health

Netherlands Food and Consumer Product Safety Authority
Ministry of Agriculture, Nature and Food Quality

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Validation of a high throughput sequencing test within an ISO17025 accredited plant health laboratory

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How high throughput sequencing can be used as a generic tool to identify viruses in a regulatory framework

Marcel Westenberg

ISTA 100 YEARS
A CENTURY OF PROGRESS IN SEED QUALITY ASSURANCE

Why using HTS in a regulatory framework

Official Controls Regulation (EU) 2017/625

Laboratories designated by the competent authorities to carry out analyses, tests and diagnoses on samples taken in the context of official controls and other official activities should possess the expertise, equipment, infrastructure and staff to carry out such tasks to the highest standards. To ensure sound and reliable results, those laboratories should be accredited for the use of these methods according to standard EN ISO/IEC 17025 on 'General requirements for the competence of testing and calibration laboratories'. The accreditation should be delivered by a national accreditation body operating in accordance with Regulation (EC) No 765/2008 of the European Parliament and of the Council ([1]).

Commission implementing regulation (EU) 2021/2285

'ANNEX II

List of Union quarantine pests and their respective codes assigned by EPPO

TABLE OF CONTENTS

Part A : Pests not known to occur in the Union territory

Part B: Pests known to occur in the Union territory

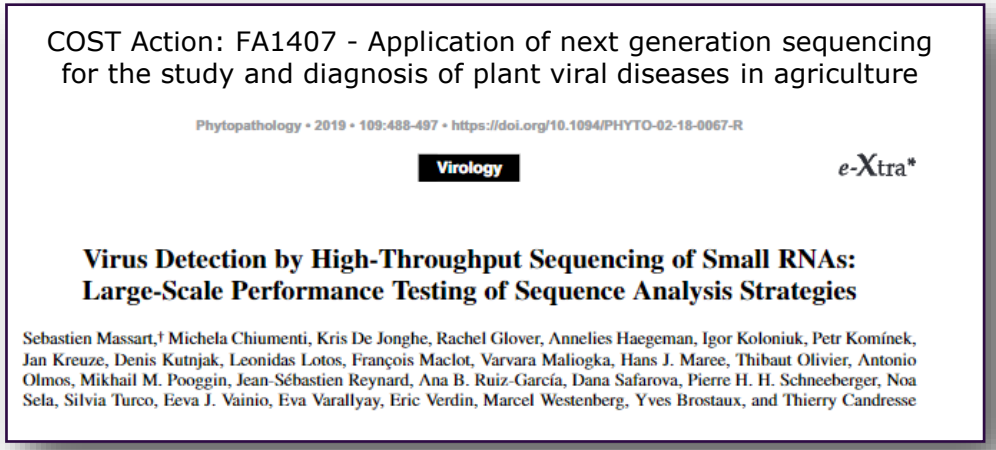
57	virus species
1	begomovirus genus (>400 species)
2	viroids
40	phytoplasmas strains

One single HTS test, instead of implementing and validating specific tests (ELISAs, PCRs) for each regulated pest

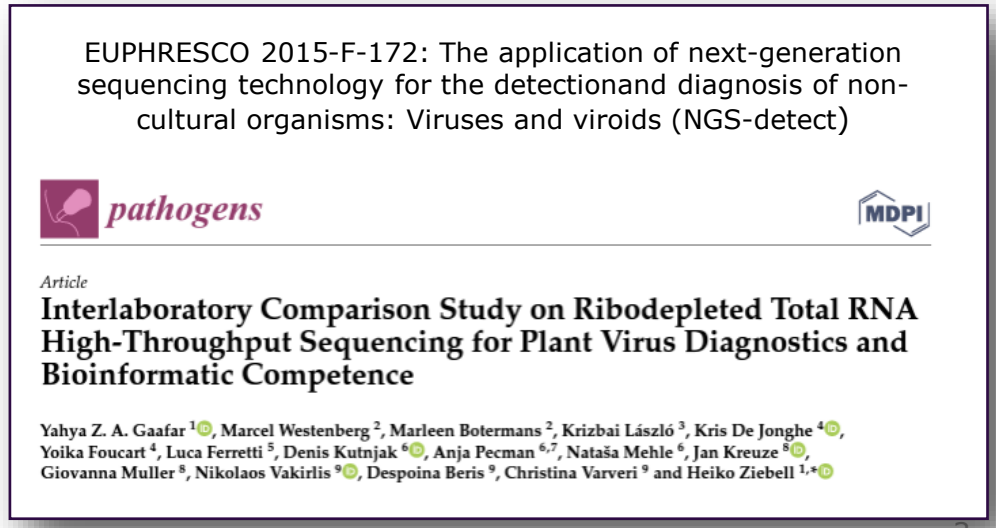
HTS at NIVIP

- Since 2014, HTS (Illumina sequencing) is used in applied research
- Since 2015, virus detection and identification in diagnostic samples
- Pipeline optimization due to PT/TPS organized in frame of international research projects
- Since 2019 from research applications to standardized diagnostics

COST Action: FA1407 - Application of next generation sequencing for the study and diagnosis of plant viral diseases in agriculture

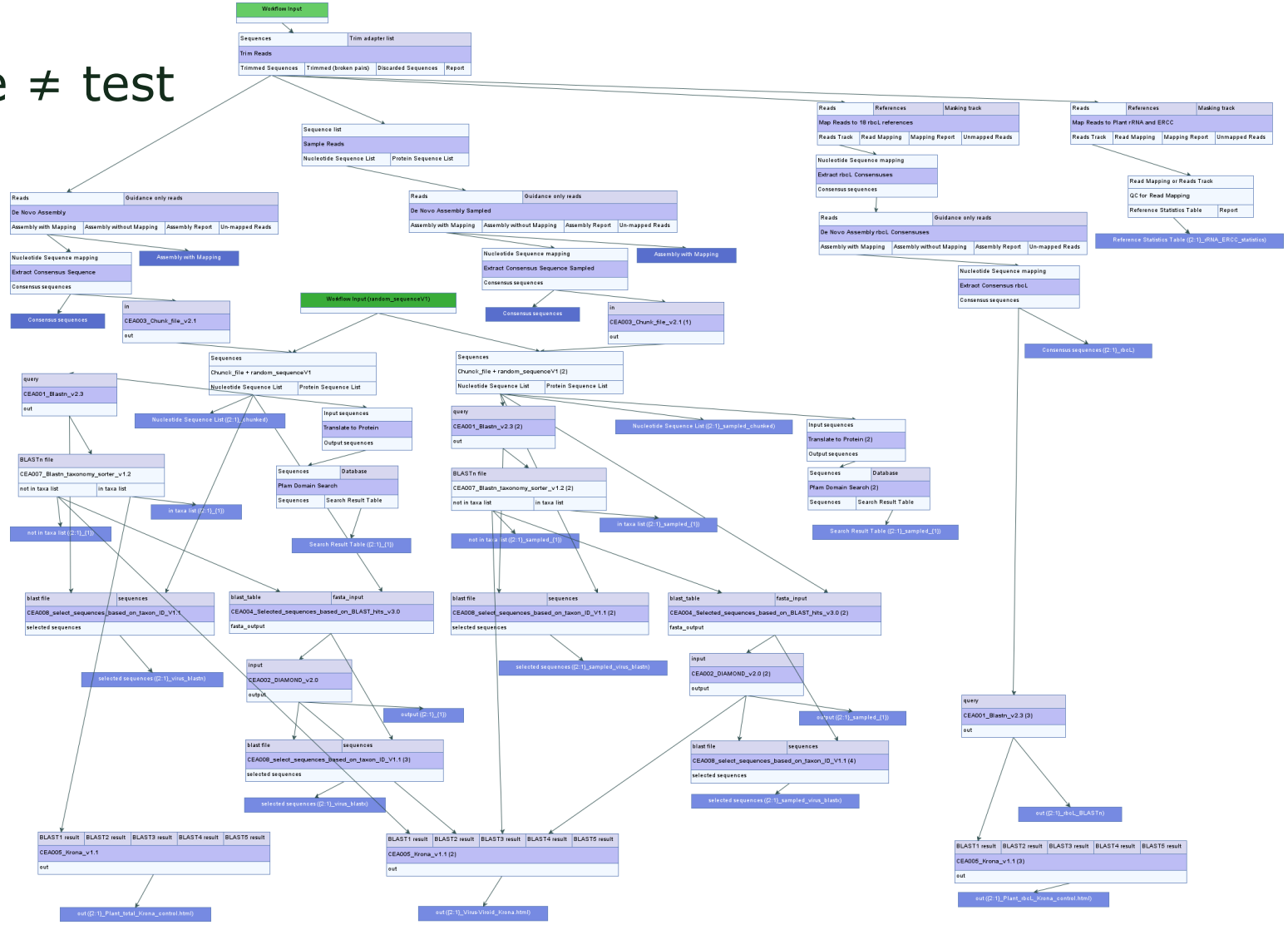


EUPHRESCO 2015-F-172: The application of next-generation sequencing technology for the detectionand diagnosis of non-cultural organisms: Viruses and viroids (NGS-detect)



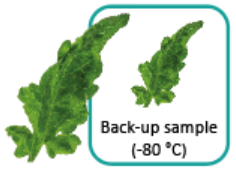
Defining a test

- bio-informatics pipeline ≠ test



Defining the tests (2)

- A test is a specific application of a method (EPPO PM7/76)
- Includes everything from nucleic acid extraction to data interpretation



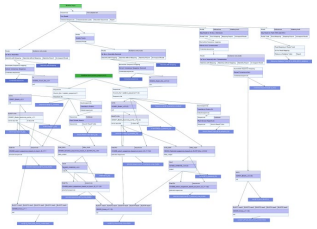
Sampling and packaging

RNA extraction

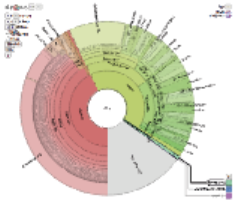
Outsourced at ISO17025 accredited sequence provider

Library preparation

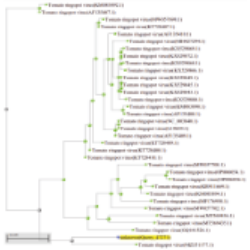
Illumina Sequencing



Bioinformatic pipeline

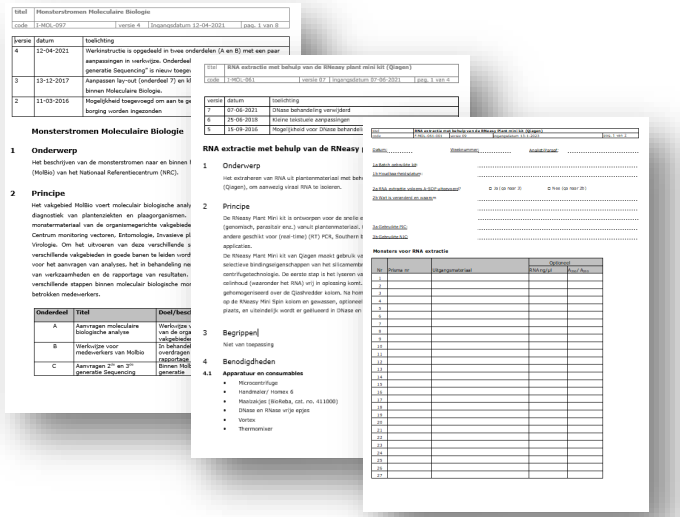


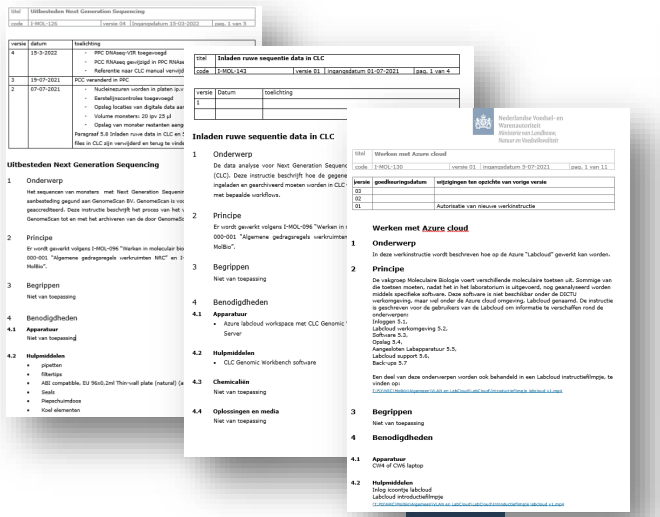
Selection and analysis of putative viral contigs

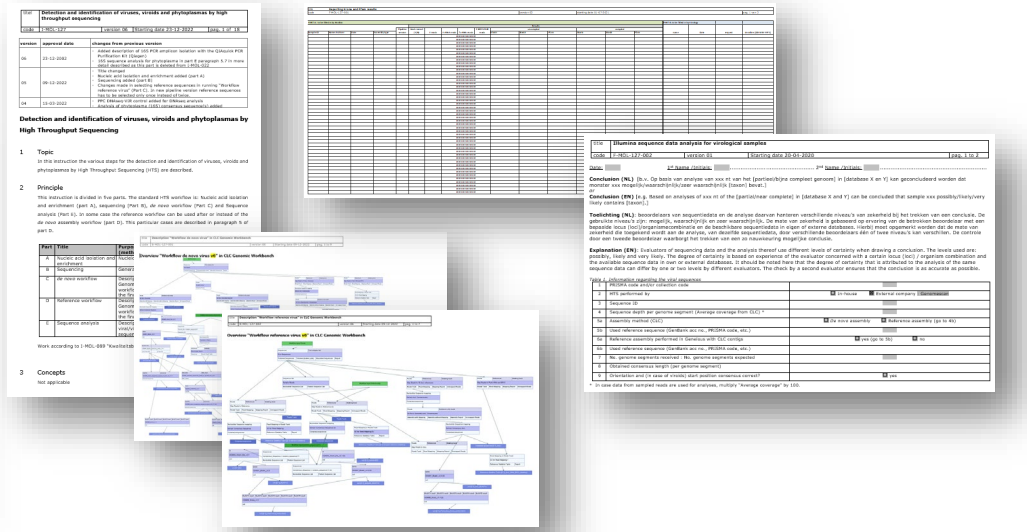


Sequence analysis and reporting

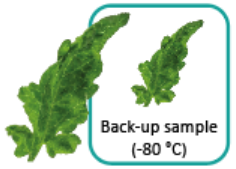
1 2 3 4 5 6 7







One control to serve many purposes



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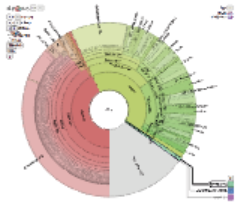
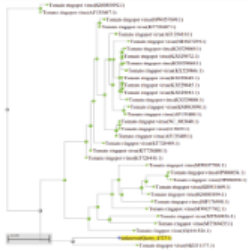


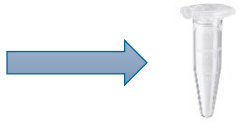
Selection and analysis of putative viral contigs



Sequence analysis and reporting

1 2 3 4 5 6 7

Healthy
N. benthamiana



20 ng/µl RNA Spiked (99:1) with ERCC RNA Spike-In Mix (ThermoFischer)

Positive Process Control batch

- Should pass entry QC and library QC

- Should produce 2Gb of data at >80% PHRED30

- Should complete the bioinformatic pipeline with all output (check on bioinformatic pipeline)
- Identification of plant species based on rbcL sequence (only *Nicotiana* should be identified, serves as positive control and contamination control)
- Recovery of 4 selected ERCC constructs (MRD>10) from positive process control sample (positive control)
- Limited reads (<10) of most abundant ERCC should be identified in diagnostic samples in the same batch (contamination control)
- Assessment of rRNA depletion success (<10% rRNA reads or >1.8 Gb non-rRNA reads)

Validating tests

What to validate or verify?

- Well described analytical standard operating procedures (A-SOP)

Why should we validate or verify?

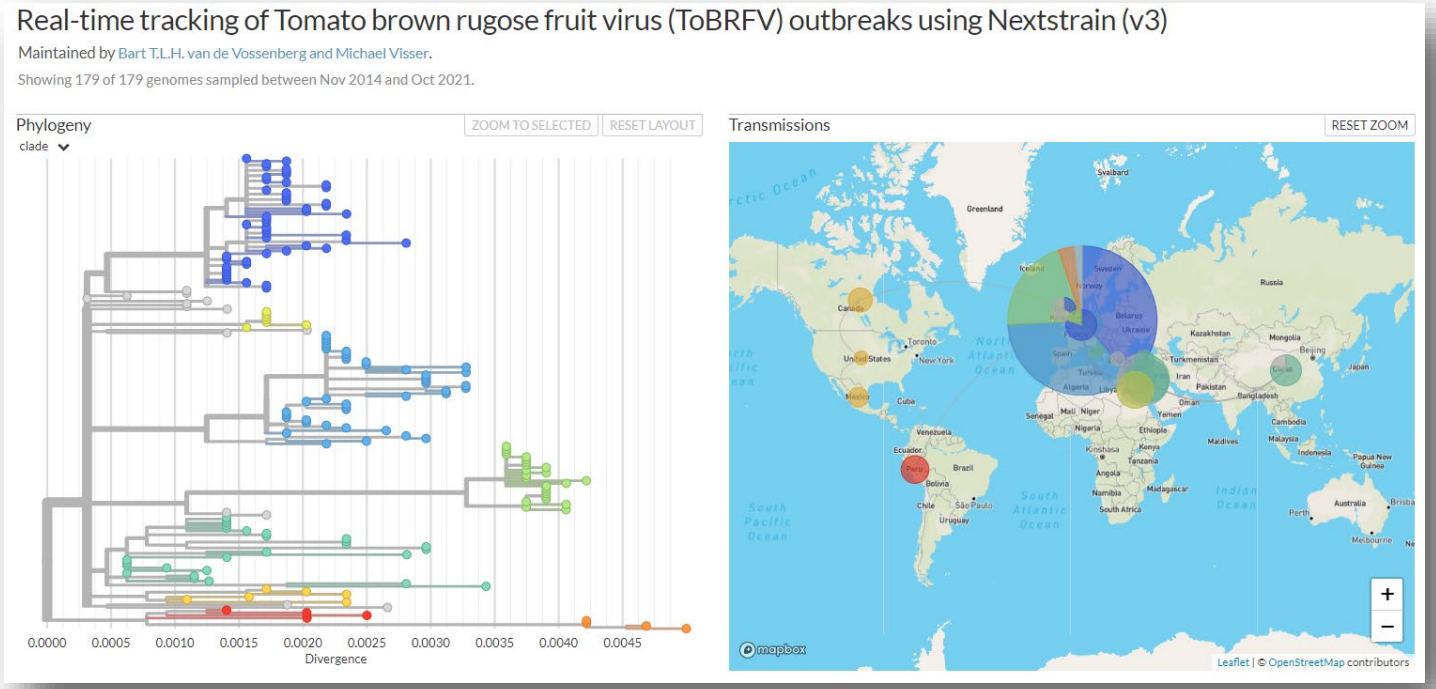
- To assess if a test (A-SOP) is fit for purpose (scope)

How to validate or verify?

- Determination or verification of performance criteria (EPPO PM 7/98)
- Analytical sensitivity, Analytical specificity, Selectivity, Repeatability, Reproducibility
- Well characterized (biological) material

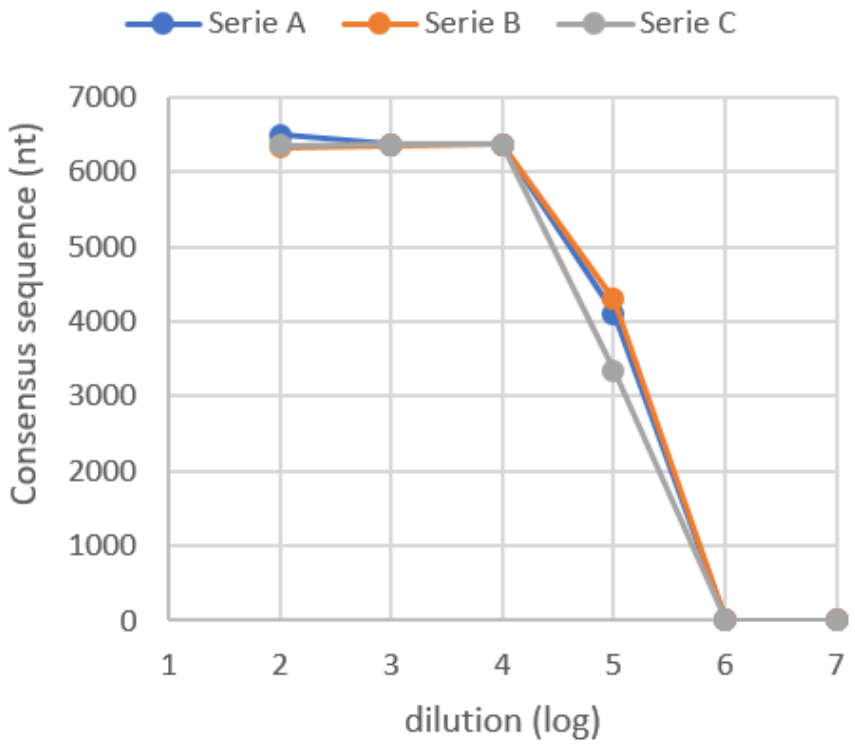
Validation scope

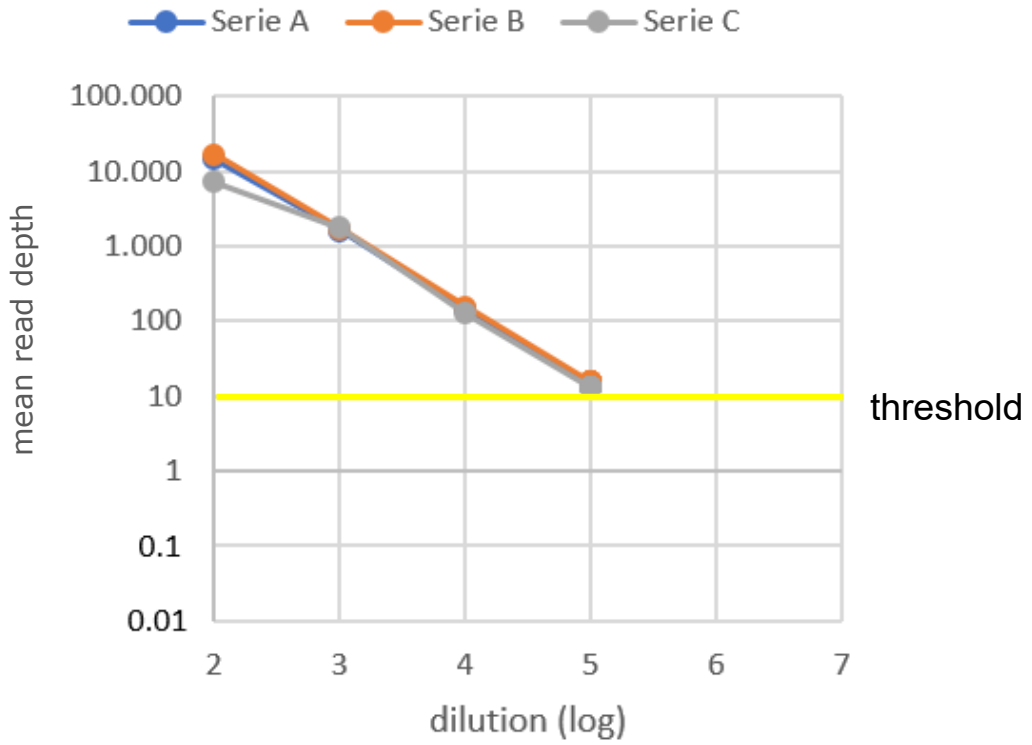
- Detection and identification of ToBRFV in tomato leaf material by HTS (Illumina sequencing)



Analytical sensitivity

- Full length sequences were obtained till a dilution of 10^4
- Species identification still possible with partial sequences at dilution 10^5
- Analytical sensitivity: relative dilution rate of 10^5
- Correlation between average read coverage and dilution.



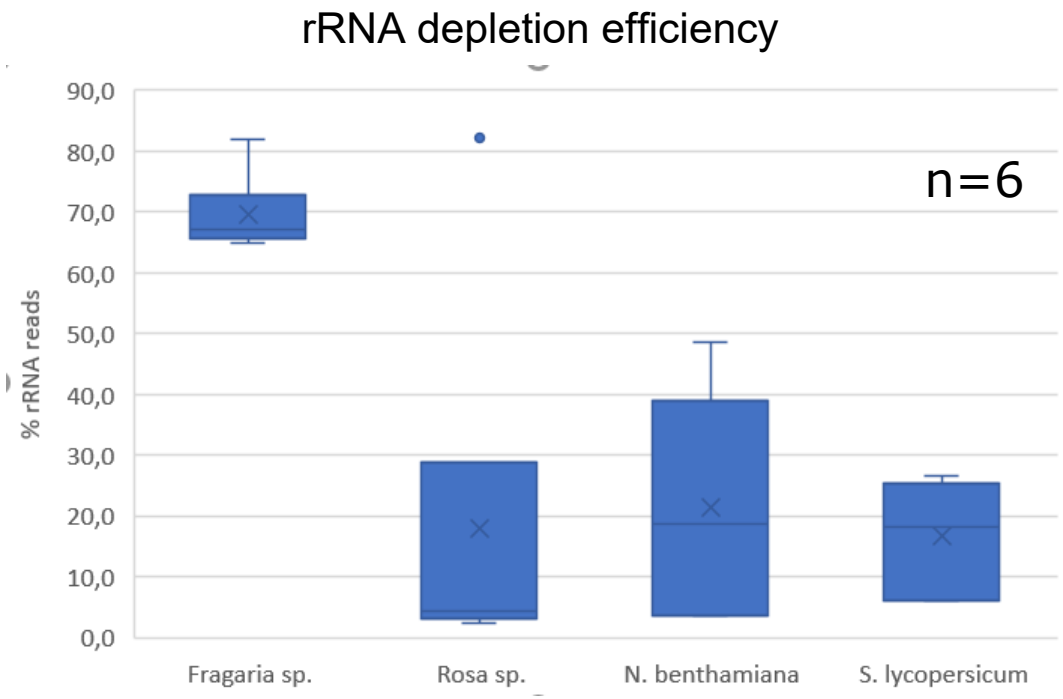


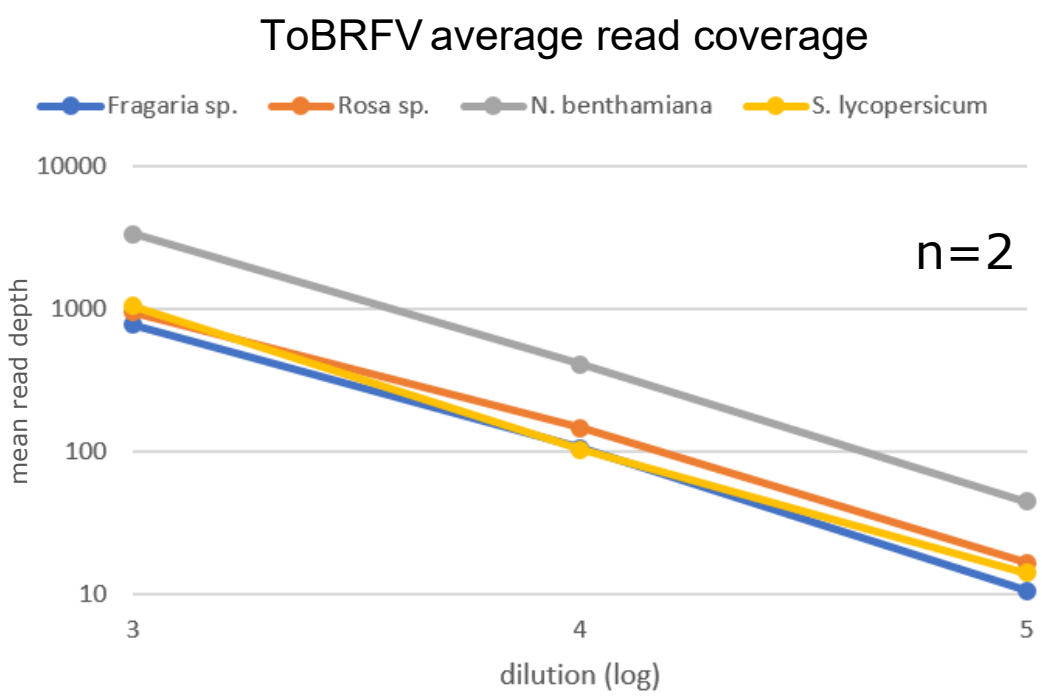
Analytical specificity

- Between 2016-2022 detected and identified over 180 viruses/viroids
- Belonging to 47 genera, 25 families
- All known viral nucleic acid types:
  ssRNA(+), ssRNA(-), dsRNA, CssRNA, dsDNA(-RT), ssDNA
- In a variety of hosts, and plant parts (e.g. leaf, fruit, seed)

Selectivity

- Affect of difficult matrices (*Fragaria* sp., *Rosa* sp.)
- Dilution of ToBRFV-infected plantsap in plantsap of different matrices





matrix has no effect on the analytical sensitivity

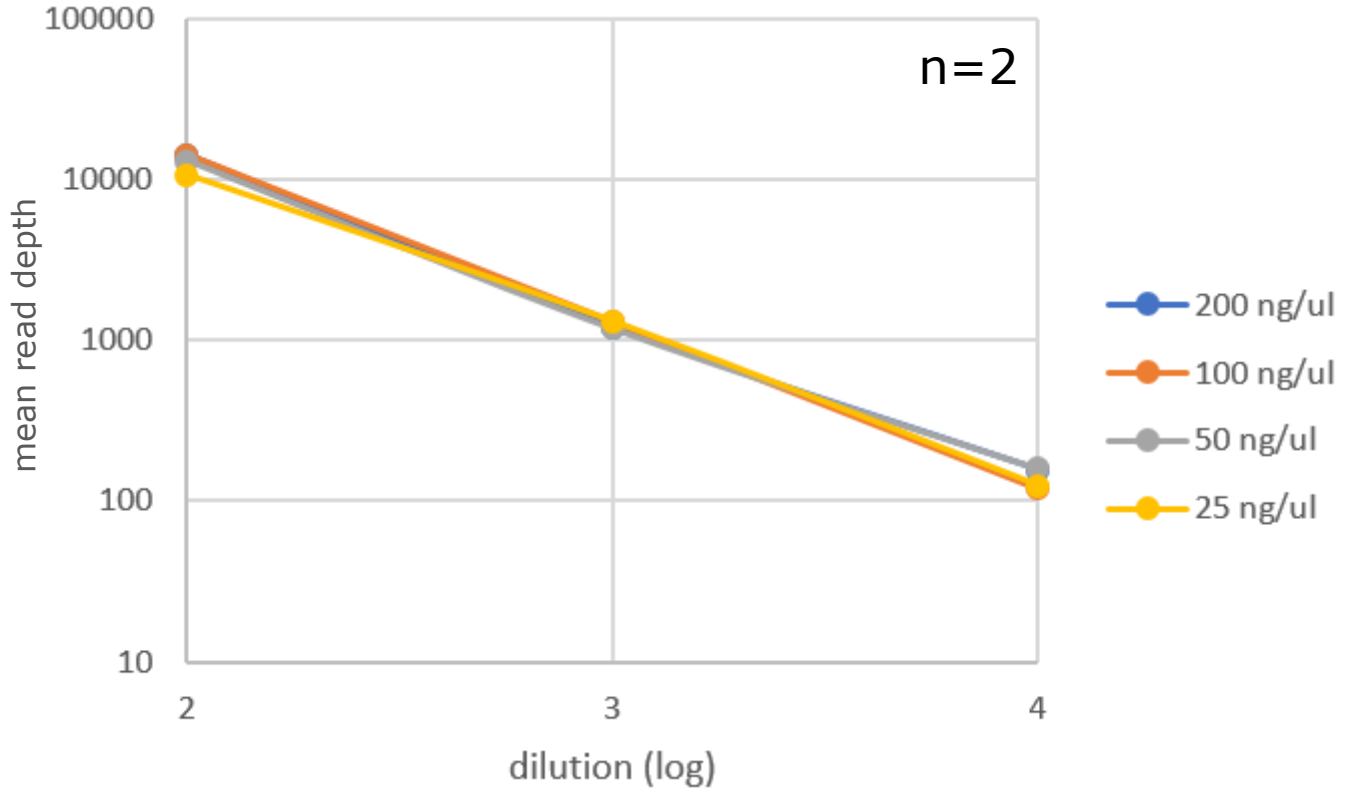
Repeatability and reproducibility

- Reproducibility of the bio-informatics pipeline
  - Data from one serial dilution (10^2-10^5):
    100% reproduceable (max. 3 nt difference in ToBRFV sequence length)

- Repeatability and reproducibility with biological samples
  - At low and medium dilutions (10^2-10^4):
    A single ToBRFV contig (6379-6353 nt) was obtained, all 100% identical
  - At a high dilution (10^5):
    11-13 ToBRFV contigs (Σ=3349-5107 nt) were obtained, all leading to a positive ToBRFV identification

Robustness

- Effect of RNA concentration on virus detection



RNA concentration had no effect on average read coverage

Validation of the HTS test for other viruses/viroids

- Only the analytical sensitivity should be determined
- Make use of - correlation between mean read depth (MRD) and dilution
  - minimal required data (2 Gb)
  - minimal read depth of 10

- Example: received 2.5 Gb data (yield) and obtained viral contig has an MRD of 1500

  analytical sensitivity = (2 Gb/2.5 Gb) x (1500/10) = 0.8 x 150 = relative dilution rate of 120

- So far validated the test for 75 viruses/viroids

Conclusions

- A HTS test can be validated in a similar way as done for other molecular methods
- In 2022 we became ISO17025 accredited for detection and identification of ToBRFV by HTS.
- Due to transferable performance characteristics of the HTS test we were able to validate the HTS test for 74 other viruses/viroids, which were added to our flexible accreditation scope in 2023
- The ISO17025 accredited HTS test allows us, as an official laboratory, to detect and identify potentially all EU-regulated viruses and viroids
- The single HTS test is providing an efficient and appropriate tool to screen for multiple viruses and viroids in surveys

Acknowledgement

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- Pier de Koning
- Marleen Botermans
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- Micheal Visser
- Lucas van der Gouw



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Thank you