

The use of HTS in virology (NIB experience)

NATAŠA MEHLE, ANJA PECMAN, ANA VUČUROVIĆ,
VERONIKA BUKVIČ, JAKOB BRODARIČ, IRENA
BAJDE, MAJA RAVNIKAR, DENIS KUTNJAK

5th EPPO Workshop for Heads of Plant Pest Diagnostic Laboratories,
19th April 2023, Oeiras (PT)



NACIONALNI INŠTITUT ZA **BIOLOGIJO**
NATIONAL INSTITUTE OF **BIOLOGY**

NATIONAL INSTITUTE OF BIOLOGY



- Independent public research institut
- Established by the Government of the Republic of Slovenia in 1960
- Basic, developmental and applicative research in the fields of biotechnology, biophysics, biomedicine and system biology
- ~190 employees

Department of
Biotechnology
and Systems
Biology

Department of
Genetic
Toxicology and
Cancer Biology

Department of
Organisms and
Ecosystems
Research

Marine Biology
Station Piran

Corporate
Services

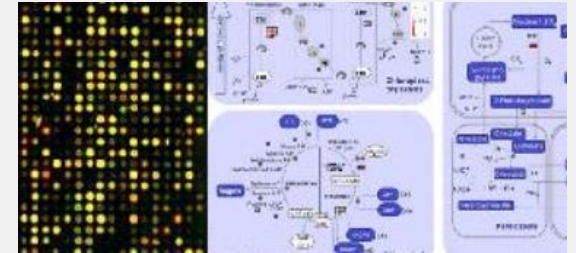
DEPARTMENT OF BIOTECHNOLOGY AND SYSTEMS BIOLOGY

Research

Genetically modified organisms



Systems Biology



Plant pathogens and other organisms pathogenic to human and animals

Bacteriology & Metrology

Laboratory for detection of bacteria

- Official laboratory
- NRL
- Partner in EURL-Bacteriology

Virology

Laboratory for detection of viruses, viroids and phytoplasmas

- Official laboratory (viruses and viroids of ornamentals and vegetables, phytoplasmas)
- NRL (consortium: NIB+KIS+IHPS)
- Partner in EURL-Virology



- Detection of GMOs: 2004 ->
- Detection of plant pathogens: 2012 ->

Type of scope: FLEXIBLE

(possibility of introducing minor modifications to the method or additional parameters)

Method

Molecular methods:

- qPCR
- RT-qPCR
- PCR

Pathogen

- *Xylella fastidiosa*
- *Clavibacter sepedonicus*
- *Ralstonia solanacearum*
- Stolbur phytoplasma (BN, MR)
- Elm yellows phytoplasma (GFDP)
- 'Ca. P. mali' (AP)
- 'Ca. P. pyri' (PD)
- 'Ca. P. prunorum' (ESFY)
- ToBRFV
- Begomoviruses

Matrix

- Plants
- Seeds
- Insects

HTS from research to use for analysis of official samples

2012 ->



American Society for Microbiology
Journal of Clinical Microbiology
Volume 51, Issue 11, November 2013, Pages 3818-3825
<https://doi.org/10.1128/JCM.01531-13>

Virology

High Similarity of Novel Orthoreovirus Detected in a Child Hospitalized with Acute Gastroenteritis to Mammalian Orthoreoviruses Found in Bats in Europe

Andrej Steyer^a, Ion Gutiérrez-Aguirre^{b,e}, Marko Kolenc^a, Simon Koren^c, Denis Kutnjak^b, Marko Pokorn^d, Mateja Poljšak-Prijatelj^a, Nejc Rački^b, Maja Ravnikar^{b,e}, Martin Sagadin^a, Adela Fratnik Steyer^a, and Nataša Toplak^c

Virus Research 191 (2014) 45–50



ELSEVIER

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

Complete genome sequences of new divergent potato virus X isolates and discrimination between strains in a mixed infection using small RNAs sequencing approach

Denis Kutnjak^a, Rocio Silvestre^b, Wilmer Cuellar^{b,1}, Wilmer Perez^b, Giovanna Müller^b, Maja Ravnikar^a, Jan Kreuze^{b,*}



Journal of
Virology®

GENETIC DIVERSITY AND EVOLUTION
1 May 2015 Volume 89 Issue 9
<https://doi.org/10.1128/JVI.03685-14>

Deep Sequencing of Virus-Derived Small Interfering RNAs and RNA from Viral Particles Shows Highly Similar Mutational Landscapes of a Plant Virus Population

Denis Kutnjak^{a,b}, Matevž Rupar^a, Ion Gutierrez-Aguirre^a, Tomaž Curk^c, Jan F. Kreuze^d, and Maja Ravnikar^a



NACIONALNI INŠTITUT ZA BIOLOGIJO
NATIONAL INSTITUTE OF BIOLOGY

HTS from research to use for analysis of official samples



SOP

Sample



RNA extraction



small RNA/ rRNA-depleted totRNA & Illumina sequencing



user-friendly automatic bioinformatics pipeline in CLC Genomics Workbench

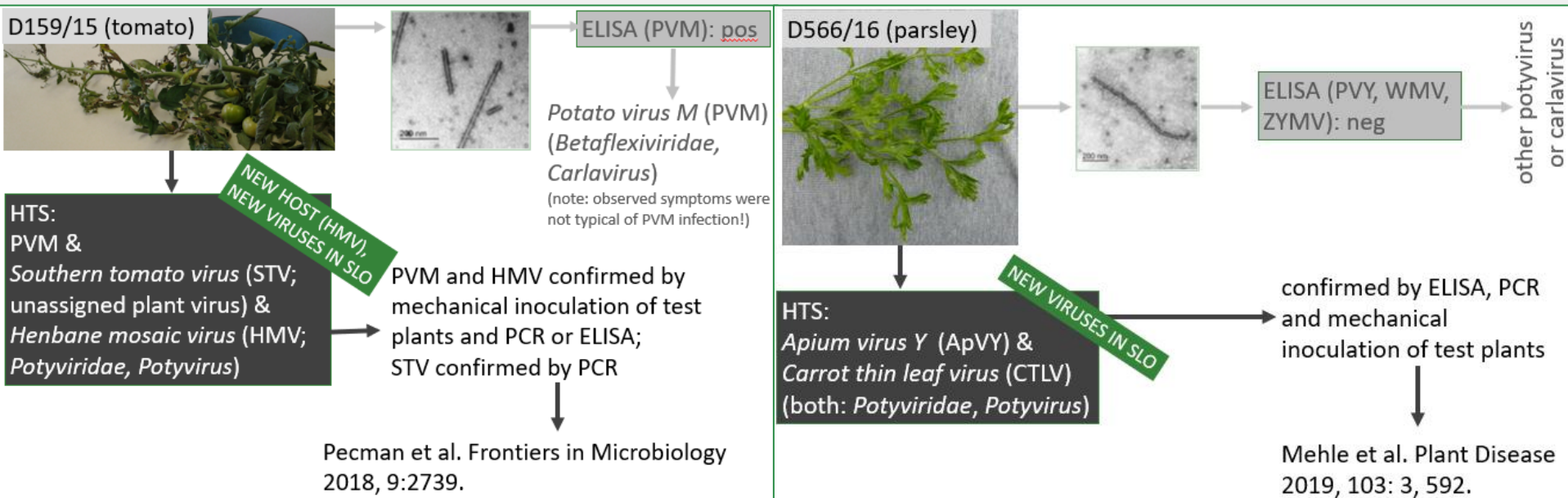


confirmation (EM, ELISA, PCR,...) & study of biological impact

HTS for analysis of official samples

2015 -> 2016

analysis of **individual samples** with unknown symptoms



The costs per sample are too high!

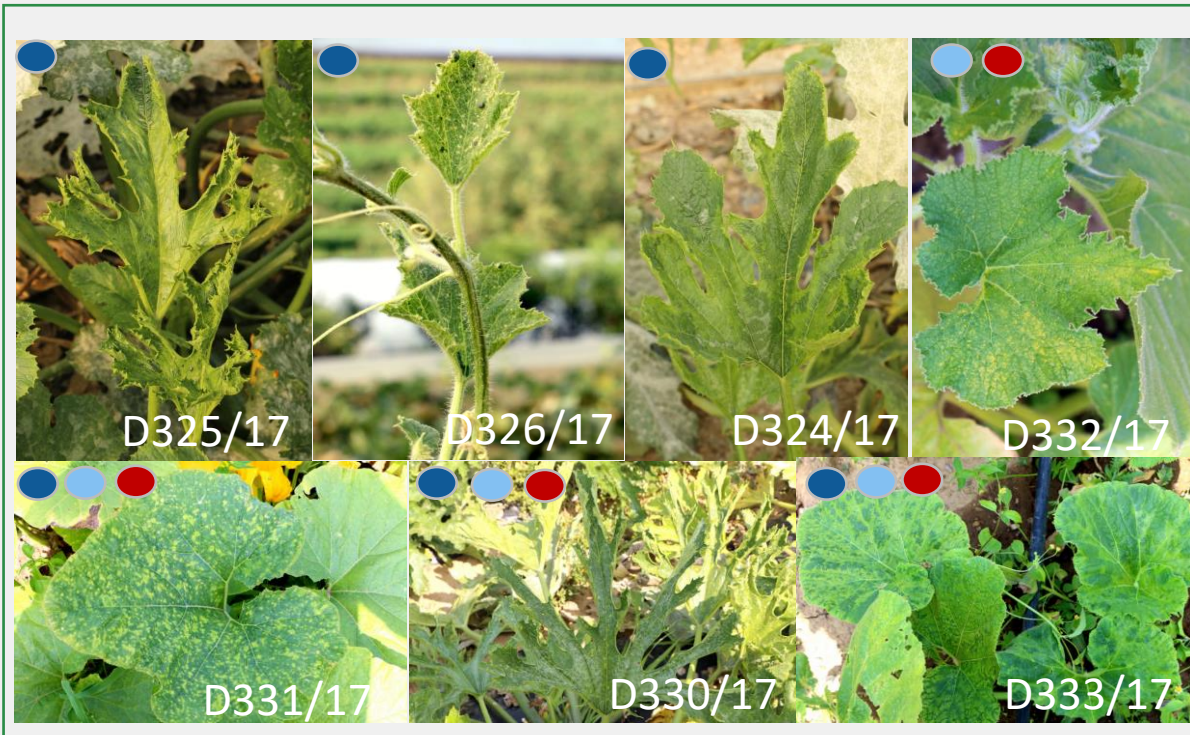
HTS for analysis of official samples

2017 -> 2021

analysis of **bulk samples** with the aim of finding out which viruses, in addition to the known ones, are present in the crops grown in Slovenia

CABYV new in Slo

HTS:
WMV-2, ZYMV,
Cucurbit aphid-borne
yellows virus (CABYV)



- WMV-2
- ZYMV

Analysis of each
individual sample
by CABYV specific
RT-PCR

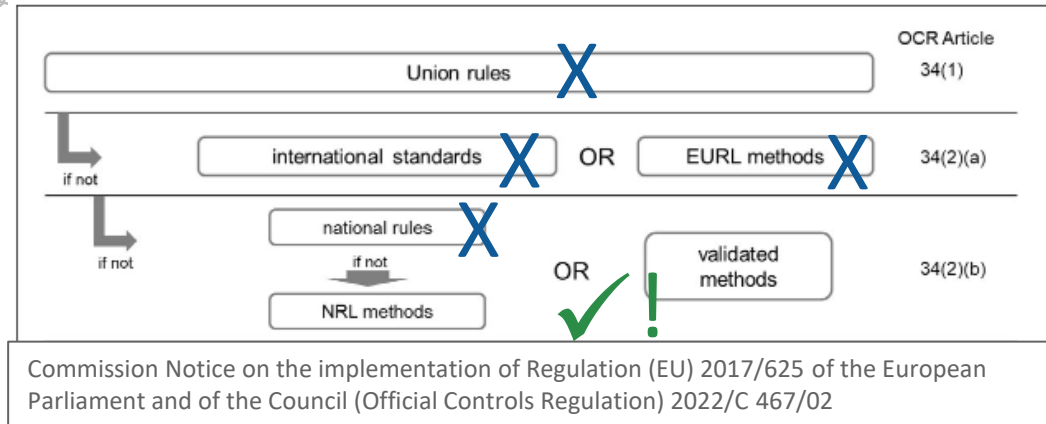
CABYV: ●

Mehle et al. Plant Disease
2020, 104:2, 599

2022 ->

Extension of national surveillance (monitoring) to several viruses listed in Annex II, Part A of (EU) 2019/2072

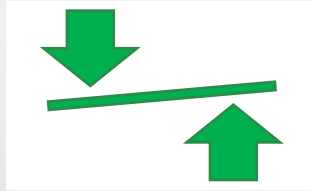
- Cowpea mild mottle virus – CPMMV (Betaflexiviridae, Carlavirus)
- Lettuce infectious yellows virus - LIYV (Closteroviridae, Crinivirus)
- Melon yellowing-associated virus- MYaV (Betaflexiviridae, Carlavirus)
- Squash vein yellowing virus - SqVYV (Potyviridae, Ipomovirus)
- Tomato chocolate virus - ToChV (Secoviridae, Torradovirus)
- Tomato marchitez virus - ToMarV (Secoviridae, Torradovirus)
- Tomato mild mottle virus - ToMMoV (Potyviridae, Ipomovirus)
- ...



HTS yes, but:

- Only 10 bulk samples per year (2023: 7x bulk tomato samples, 3x bulk samples of Cucurbitacea)
- Results should be available in time!

HTS platform?



Illumina rRNA-depleted totRNA sequencing

frontiers | Frontiers in Microbiology

ORIGINAL RESEARCH
published: 11 May 2022
doi: 10.3389/fmicb.2022.853921



	totRNA	rRNA-depleted totRNA
MinION direct RNA sequencing		
MinION cDNA-PCR sequencing		

Systematic Comparison of Nanopore and Illumina Sequencing for the Detection of Plant Viruses and Viroids Using Total RNA Sequencing Approach

Anja Pecman^{1,2*}, Jan Adams³, Ion Gutiérrez-Aguirre¹, Adrian Fox³, Neil Boonham⁴, Maja Ravnikar¹ and Denis Kutnjak^{1*}

Nanopore sequencing has been found to provide comparable results to Illumina sequencing, while being faster and better suited for small laboratories

New SOP for HTS

	NAD5	Alien control	ERCC	RCS
RNA extraction	IPC	<i>Phaseolus vulgaris</i> infected with endornavirus PvEV (RNA extraction and all further steps in parallel with samples) -: Monitoring contamination +: Ensuring detection of target (at low concentration)		
Bulk sample preparation				
DNase digestion				
Ribosomal RNA depletion, RNA concentration			+ : Spike-in ERCC	
Polyadenylation (polyA tailing)			control (Invitrogen)	
Library preparation: <ul style="list-style-type: none"> ○ Reverse transcription and strand-switching ○ PCR and barcoding ○ Adapter ligation and library loading onto flowcell 			(control added to every sample and PvEV)	+ : RNA Control Expansion (Oxford Nanopore Technologies, UK)
MinION sequencing				
Bioinformatic analysis for data visualisation: CLC Genomic Workbench (Qiagen, USA) (Pecman et al., 2017)				

New SOP for HTS

One MinION flowcell:

- 5 bulk samples
 - Each bulk sample: up to 6 samples
- > Results for up to 30 individual samples

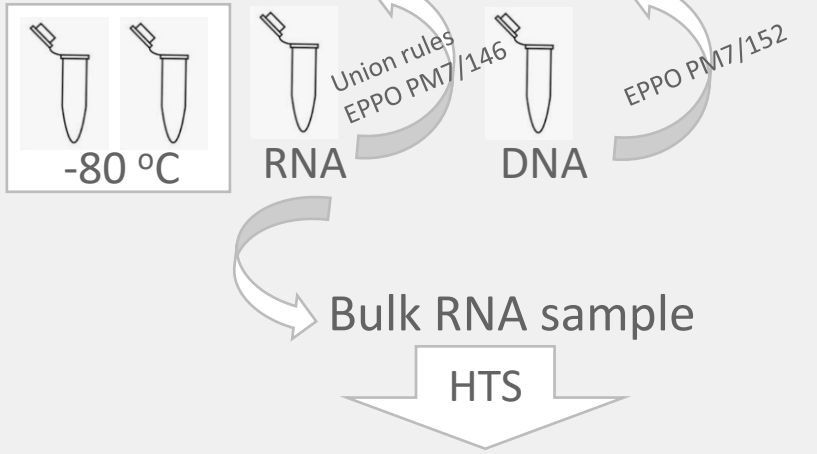
Wet lab (4 days)	MinION sequencing (weekend)	Dry lab (bioinformatic analysis; 3 days): <ol style="list-style-type: none">1. Review of controls (analysis of reads mapped to RCS, ERCC, and PvEV)2. Check for the presence of quarantine viruses (mapping to reference database with nucleotide sequences of all target quarantine viruses)3. Check for the presence of other known viruses4. Check for the presence of unknown/new viruses
----------------------------	---------------------------------------	---

If ≥ 1 read of virus from Annex II, Part A of (EU) 2019/2072 is detected, individual samples will be tested with HTS or with target tests.

HTS for the analysis of samples from national monitoring



	ToBRFV	Begomoviruses
D1105/22	neg	neg
D1181/22	neg	neg
D1268/22	neg	/
D1269/22	neg	neg
D1271/22	neg	/
D1274/22	neg	neg



- Annex II, Part A of (EU) 2019/2072: No
- Other viruses: PVY, TSWV, ToMV, PhCMoV, ToFBV

Full validation only for the detection of ToMMoV in tomato leaves

Analytical specificity: 100%	<p>A reference database of sequences of ToMMoV, BCTV, CPMMV, LIYV, MYaV, SqVYV, ToChV, and ToMarV has been created.</p> <ul style="list-style-type: none">• Samples containing 2x ToMMoV, 1x BCTV, 1x CPMMV, 1x SqYV• No. of samples that did not contain quarantine virus: 10
Analytical sensitivity	<p>ToMMoV dilutions tested: 6x, 10x, 30x, 60x, 100x and 1000x</p> <ul style="list-style-type: none">➤ If more than 1,000,000 filtered reads are generated: 100x➤ If 100,000 filtered reads are generated (the lowest acceptable output): 10x
Selectivity	<p>ToMMV (dilutions 6x and 10x) spiked into leaves of different tomato cultivars</p> <ul style="list-style-type: none">➤ no impact of tomato cultivars
Repeatability: 100%	<ul style="list-style-type: none">• ToMMoV 10x diluted: 2 parallel testing• ERCC: 7 x 6 parallel testing
Reproducibility: 100%	<p>No. of MinION flowcells (run on different days):</p> <ul style="list-style-type: none">• ToMMoV 10x diluted: 3• ERCC: 7; RCS: 4; PvEV: 8• No. of wet lab operators: 4; No. of dry lab operators: 3



➤ External audit
(Autumn 2023)

➤ 2nd Internal audit
(May 2023)

✓ 1st Internal audit
(14.4.2023)

✓ Validation

✓ SOP