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Establishing a validated nanopore sequencing workflow for untargeted plant virus detection in diagnostic settings

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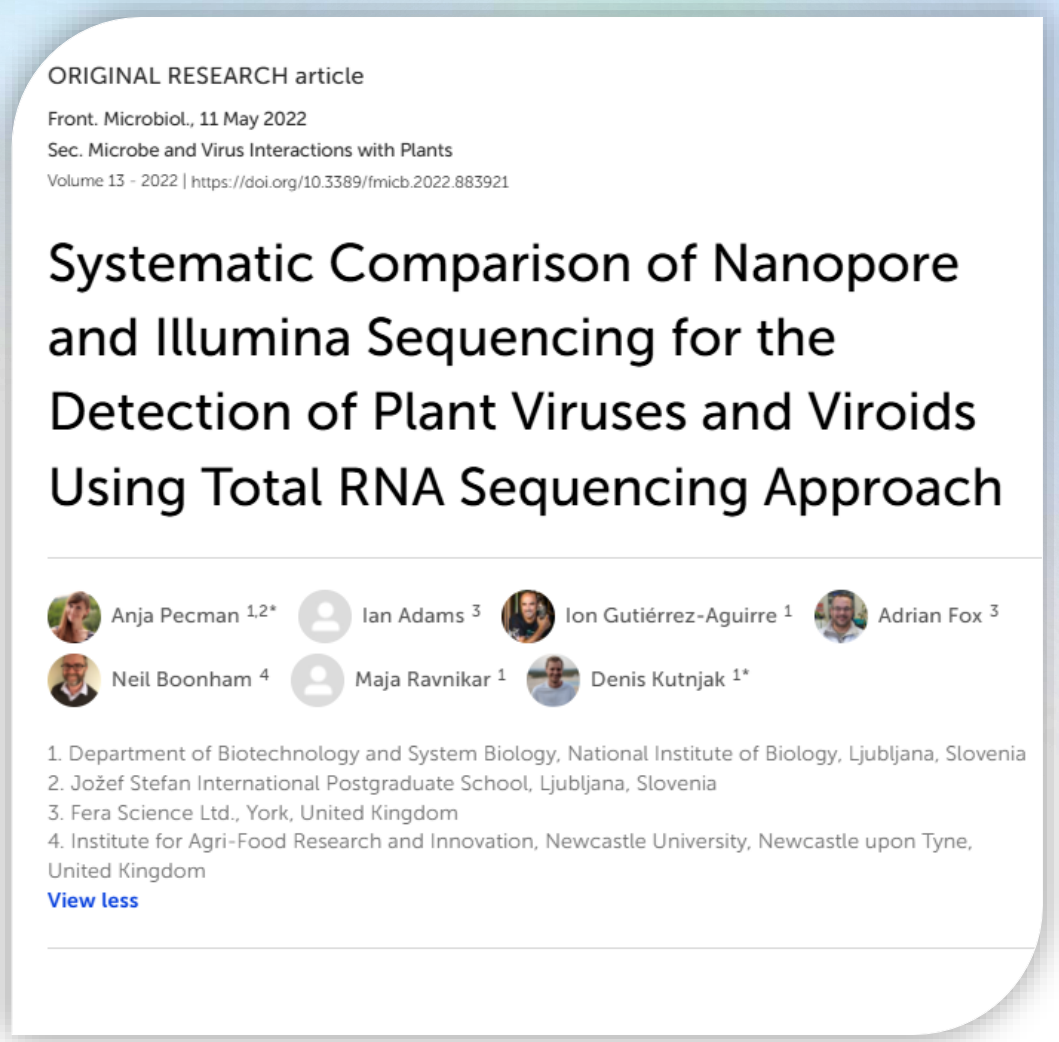
EPPO Conference on Diagnostics of Plant Pests
Recent developments and future trends

2026-04-22/24,
Vienna (AT)

Introducing Nanopore sequencing for plant virus detection

- Sequencing in house (control of all the procedures, e.g., crosstalk across tested samples)
- Faster analysis – from sample to results in days instead of months (External provider -> 1-2 months)
- Suitablity for low throughput (MinION)
- Lowering the cost by using barcodes and pooling samples



ORIGINAL RESEARCH article

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

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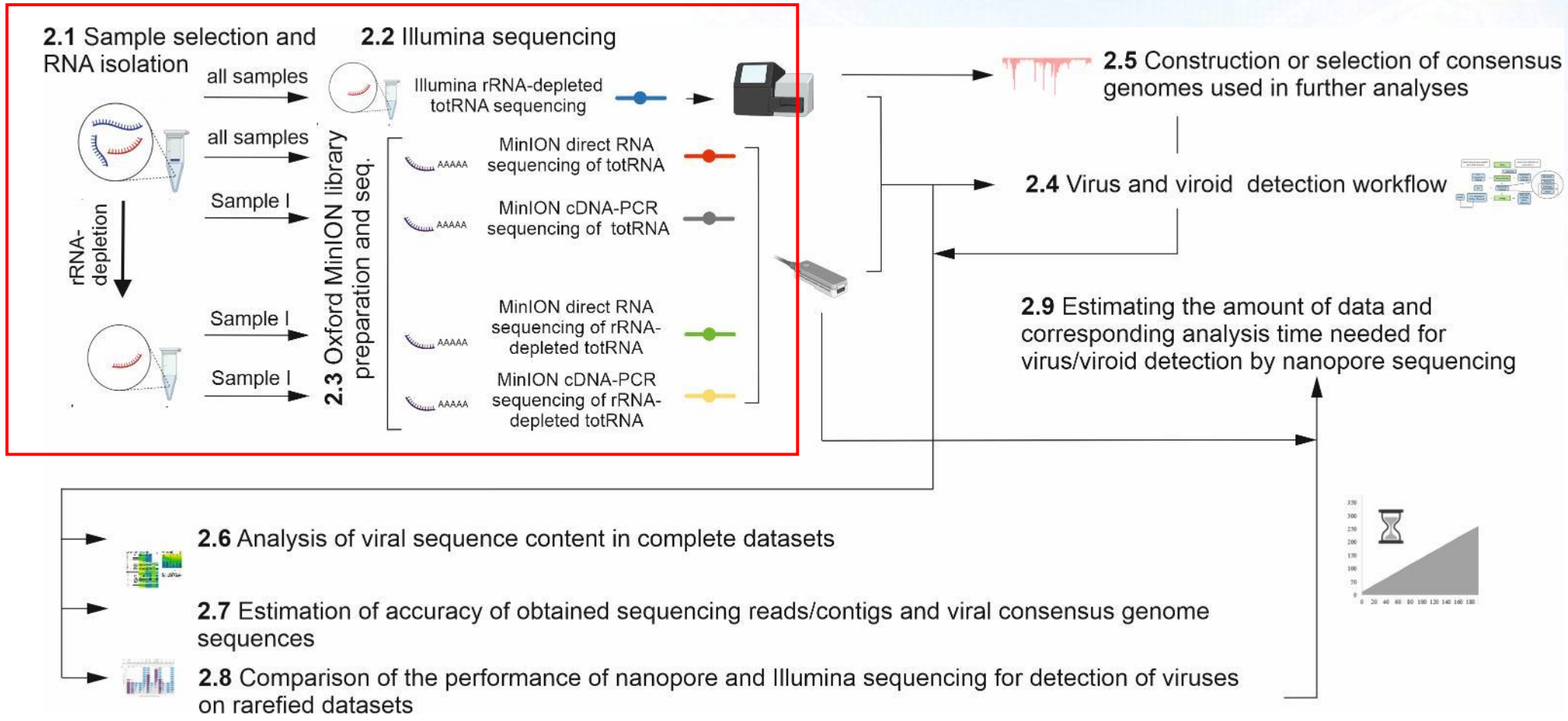
Nanopore sequencing for plant virus detection

Nanopore vs Illumina – Comparison



Nanopore sequencing for plant virus detection

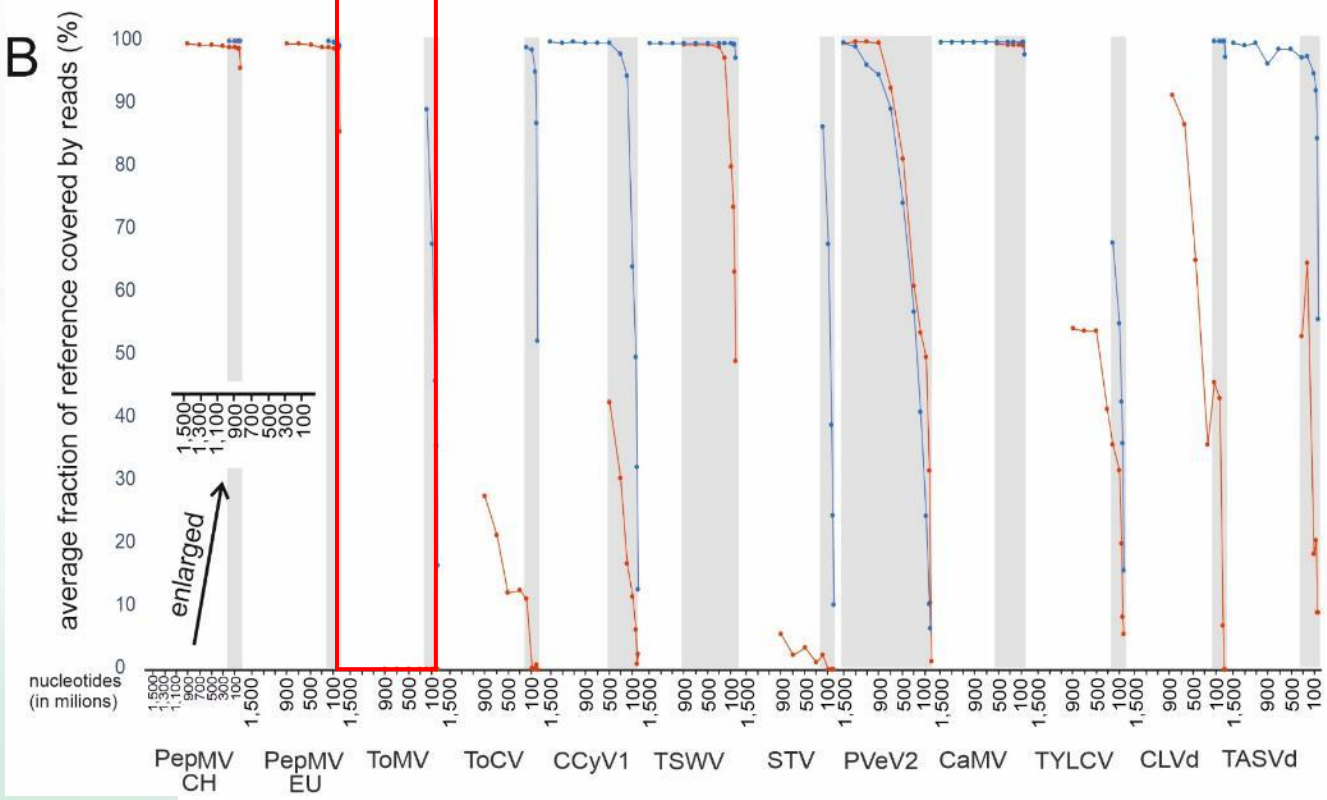
Nanopore vs Illumina – Comparison

- nanopore sequencing using total RNA as RNA input for direct RNA library preparation resulted as the fastest but also as least sensitive: using this approach, we failed to detect one virus that was present in a sample at an extremely low titer

A

	ssRNA+				ssRNA-		dsRNA		dsDNA -RT	ssDNA	circularRNA	
% of mapped reads	Pep-MV CH (Sample I)	Pep-MV EU (Sample I)	ToMV (Sample I)	ToCV (Sample I)	CCyV1 (Sample II)	TSWV (Sample III)	STV (Sample I)	PVeV2 (Sample V)	CaMV (Sample II)	TYLCV (Sample I)	CLVd (Sample I)	TASVd (Sample IV)
MinION seq. directRNA of totRNA	1.1528	0.5638	0.0000	0.0007	0.0022	0.2101	0.0001	0.0043	0.7548	0.0020	0.0005	0.0002
Illu. rRNA-dep. totRNA seq.	12.3547	10.1102	0.0139	0.1584	0.0381	4.5122	0.0069	0.0116	3.4109	0.0809	0.5523	0.0083

B



Nanopore sequencing for plant virus detection

Nanopore vs Illumina – Comparison

- nanopore sequencing using total RNA depleted ribosomal RNA as RNA input for PCR-cDNA library preparation **resulted in very comparable outputs (yellow)** in comparison to Illumina results (blue)

A

% of mapped reads	ssRNA+				dsRNA	ssDNA	viroid
	PepMV CH	PepMV EU	ToMV	ToCV	STV	TYLCV	CLVd
MinION directRNA seq. of totRNA	1.1528	0.5638	0.0000	0.0007	0.0001	0.0020	0.0005
MinION directRNA seq. of rRNA-dep. totRNA	0.6243	0.4552	0.0097	0.0442	0.0030	0.0006	0.0063
MinION cDNA- PCR seq. of totRNA	27.1932	15.7794	0.0847	0.0773	0.0006	0.0486	0.0040
MinION cDNA-PCR seq.of rRNA-dep. totRNA	11.2630	9.7091	0.0452	0.1303	0.0029	0.0132	0.0054
Illumina rRNA-dep. totRNA seq.	12.3547	10.1102	0.0139	0.1584	0.0069	0.0809	0.5523

Nanopore sequencing for plant virus detection

Further use:

1. Sequencing official samples (as bulk samples) using ribosomal RNA-depleted total RNA and a PCR-cDNA barcoding kit (also sequenced on an Illumina platform in parallel).

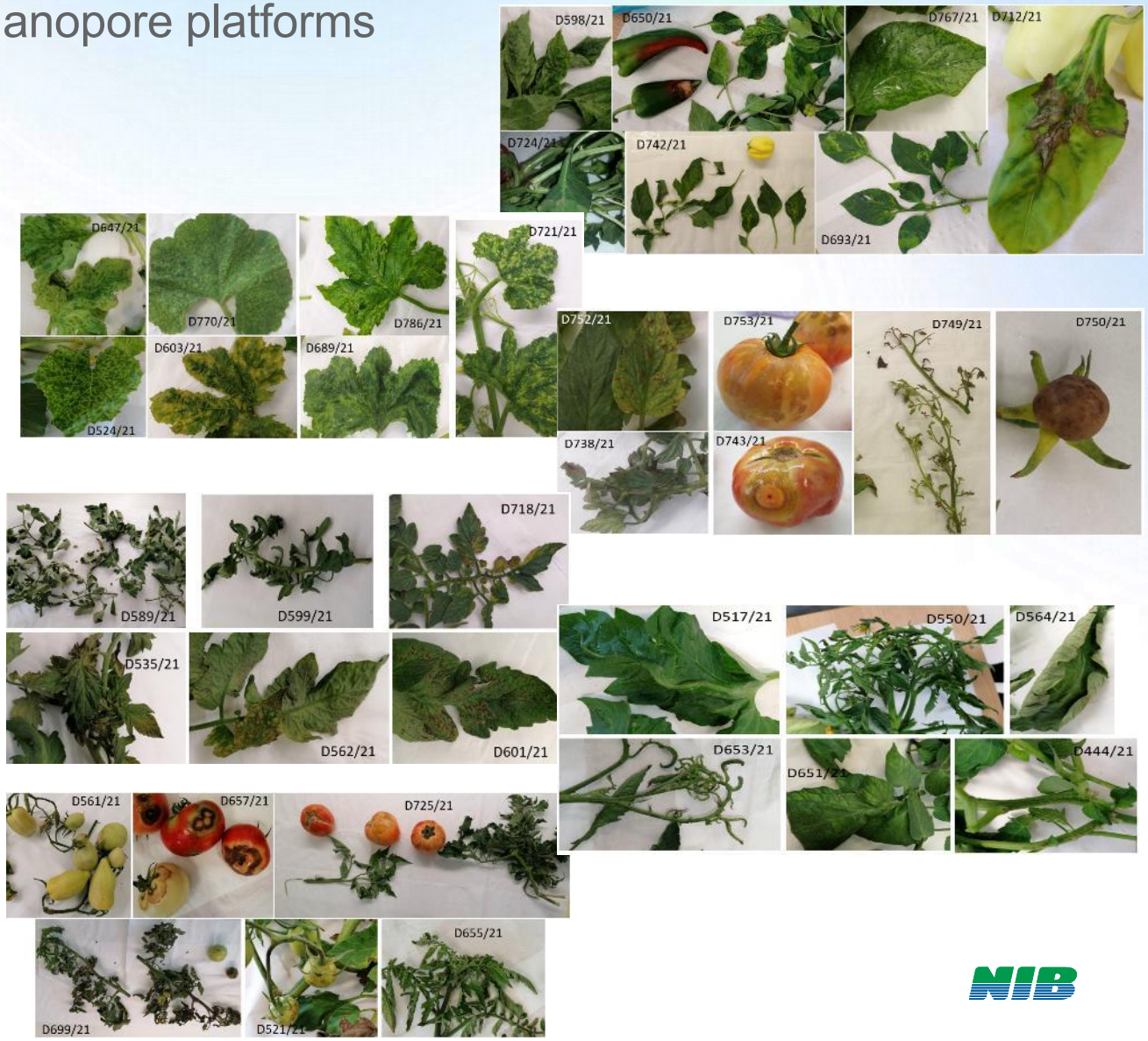
2. Validation: sequencing of various quarantine viruses (as listed in EU Regulation 2021/2285) using the same RNA depletion and barcoding method.

3. Sequencing of bulk samples collected during official surveys in next years.

Nanopore sequencing for plant virus detection

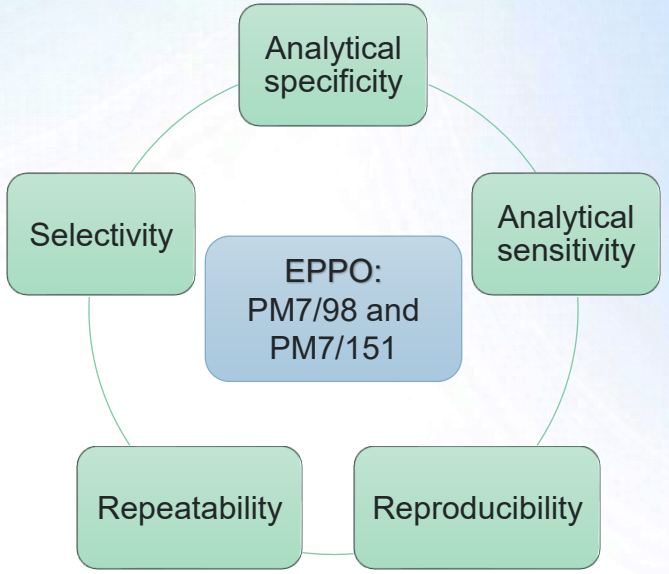
1. Sequencing official samples – Illumina and Nanopore platforms

- **Six bulk samples**: one from pepper, one from zucchini, and four from tomatoes.
- **Comparison of results** was performed by mapping reads to the viral RefSeq database using a normalized dataset (i.e., equal total nucleotide counts). Viruses were considered detected if more than 30% of their reference sequence was covered by reads.
- **Results demonstrated comparable performance** between the Illumina and MinION platforms in terms of the percentage of the reference covered. However, MinION showed improved performance in terms of average coverage (sequencing depth).



Nanopore sequencing for plant virus detection

2. Validation of Nanopore sequencing for one selected assay-virus-matrix combination



- For a **range of quarantine** viruses **no** appropriate targeted assay is designed yet:
  - beet curly top virus (BCTV), *Geminiviridae, Curtovirus*
  - 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 (TMMoV), *Potyviridae, Ipomovirus*** → full validation: for ONE selected **assay-virus-matrix** combination
  - …etc.

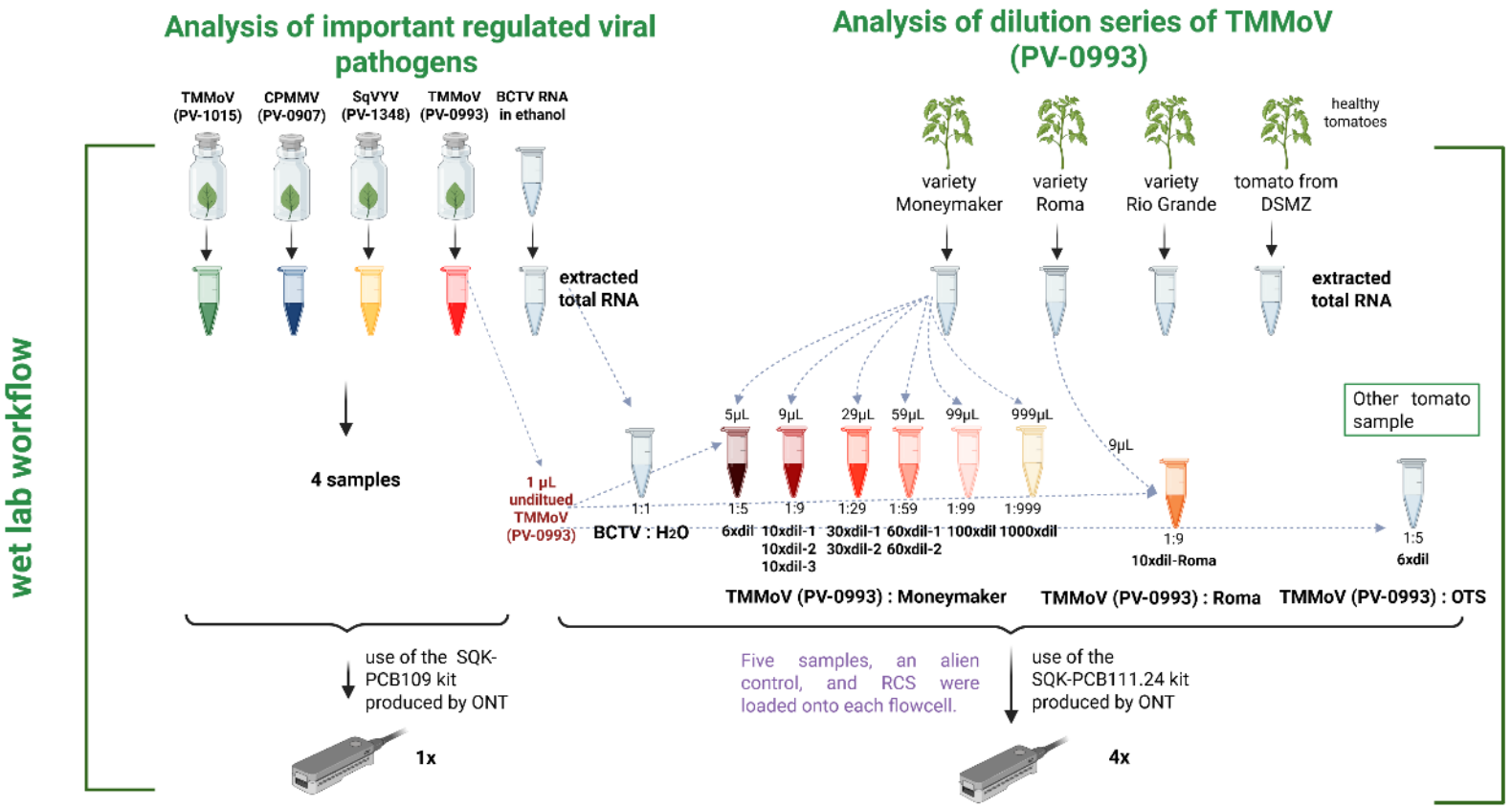
- as assay ….We sequenced **Ribosomal RNA-depleted total RNA** using a **PCR-cDNA barcoding approach** (SQK-PCB111.24) using the established standard operating procedure.

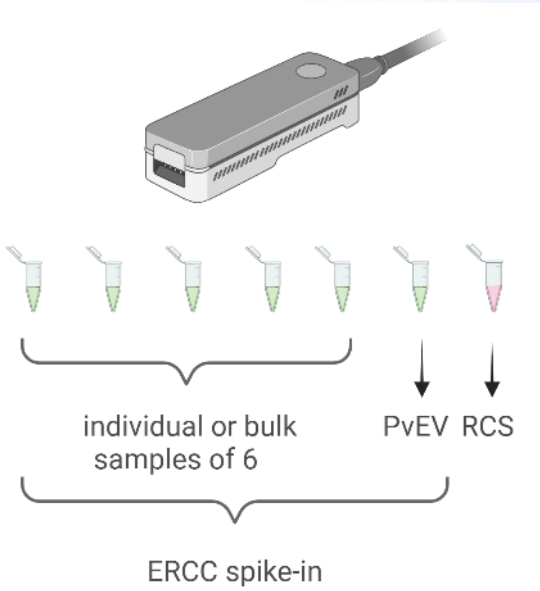
- We **validated** an optimized nanopore sequencing protocol for detecting plant viruses.

Nanopore sequencing for plant virus detection

2. Validation of Nanopore sequencing for one selected assay-virus-matrix combination

matrix → tomato leaves
target → tomato mild mottle virus (TMMoV)
assay → Ribosomal RNA-depleted total RNA using a PCR-cDNA barcoding approach

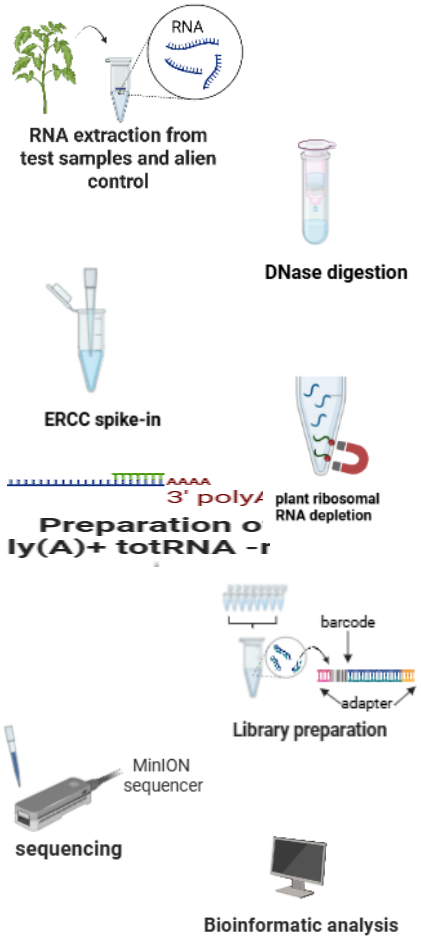


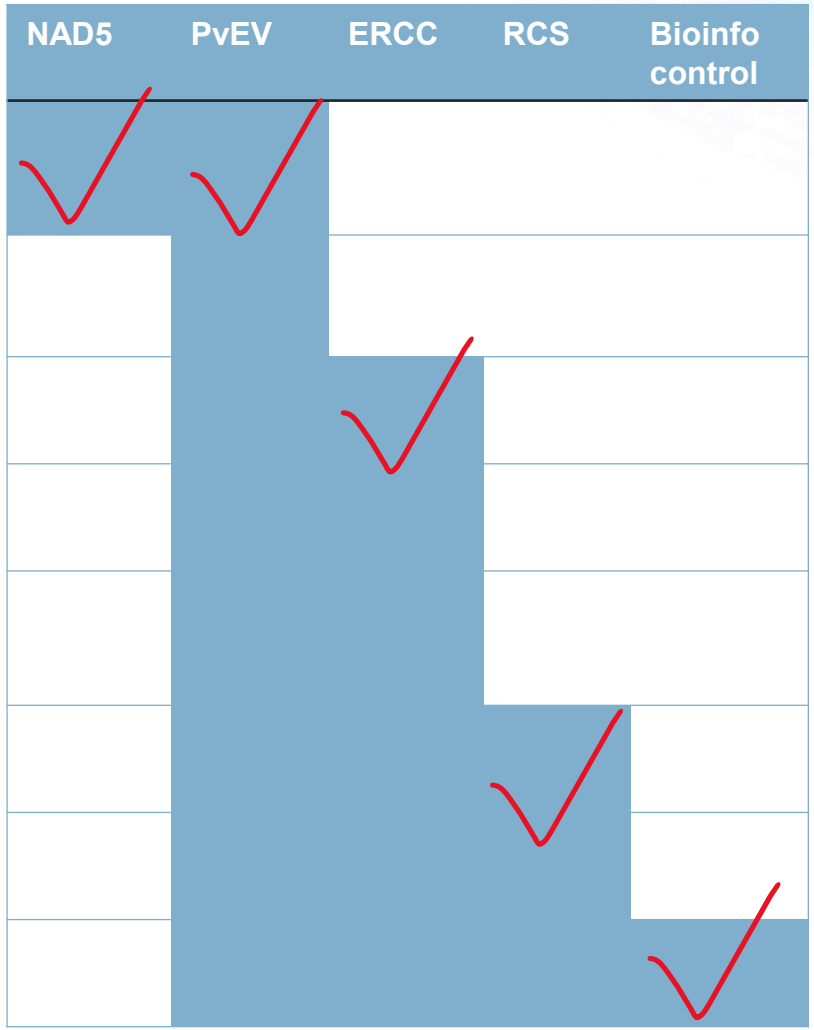


Nanopore sequencing for plant virus detection

2. Validation of Nanopore sequencing - CONTROLS

✓ enable monitoring across both wet-lab and bioinformatics analyses (EPPO PM7/151).





nad5 – an internal positive control for RNA extraction, tested by RT-qPCR (Botermans et al., 2013).

PvEV – alien control; RNA extracted from *Phaseolus vulgaris* infected with endornaviruses (Kesanakurti et al., 2016), processed in parallel with samples. Used to monitor cross-talk and detect low-titer viruses.

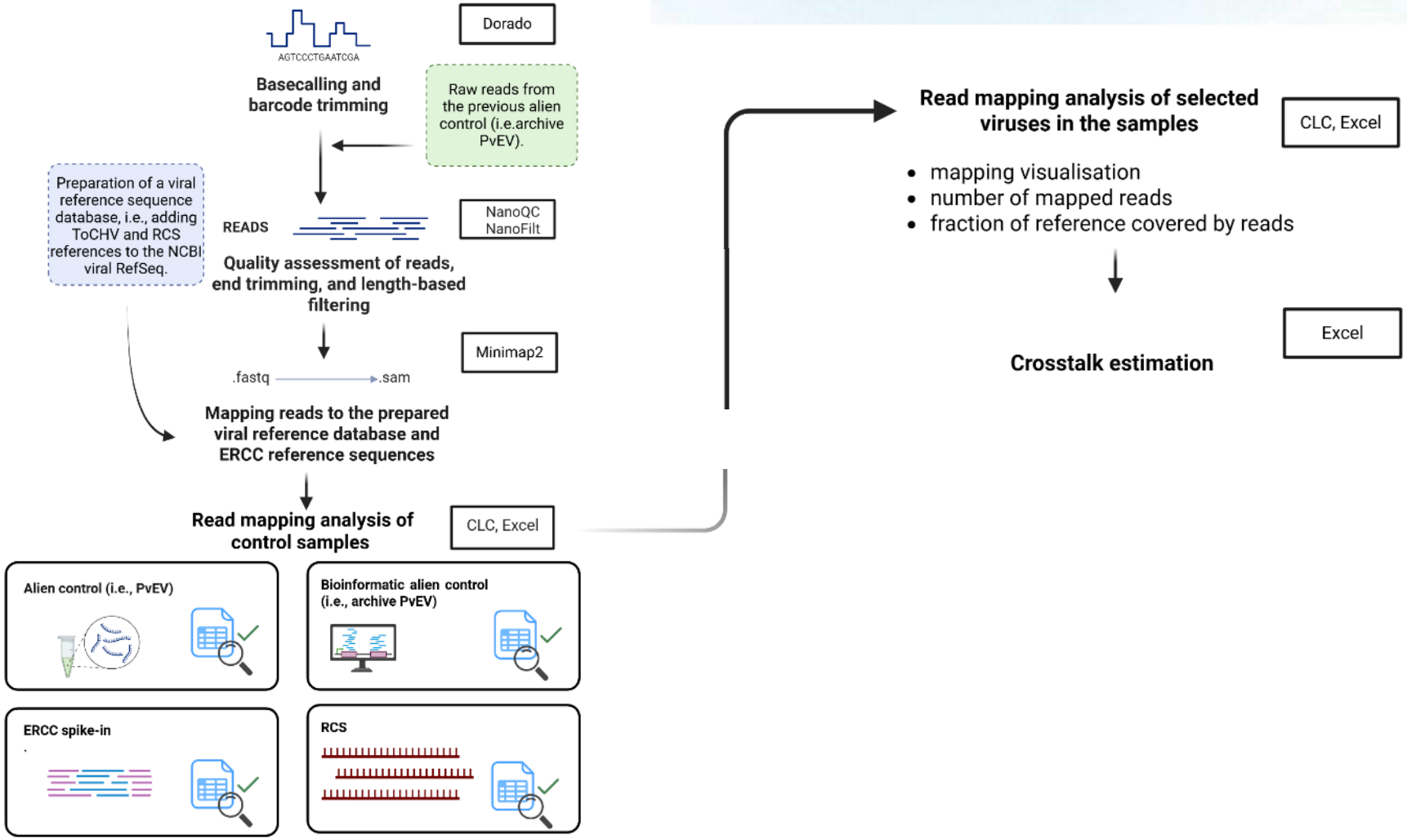
ERCC RNA Spike-In Mix (Invitrogen) – an internal control added to every sample to evaluate all steps following DNase treatment.

RCS – RNA Calibration Strand (by ONT); a high-titer artificial spike-in used to monitor the success of library preparation and sequencing, and for cross-talk estimation.

Bioinformatic pipeline control (PvEV) – an alien control from a previous run, used to evaluate the bioinformatics pipeline.

Nanopore sequencing for plant virus detection

2. Validation of Nanopore sequencing – Bioinformatic analysis



Nanopore sequencing for plant virus detection

2. Validation of Nanopore sequencing – Bioinformatic analysis –Crosstalk calculation

$$RCS\ control\ cross\text{-}talk = \frac{Sum\ of\ reads\ mapped\ to\ the\ RCS\ in\ non-RCS\ samples\ and\ controls}{Sum\ of\ reads\ mapped\ to\ the\ RCS\ in\ all\ samples\ and\ controls}$$

	number of mapped reads						
Virus name	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**PVeV**	**RCS**
RCS	6	5	6	3	5	9	57907
PVY	110	0	0	0	0	0	0
TMMoV	175	23	3	0	0	0	0
TMV	42351	8	5	0	0	6	0
PVeV	0	0	0	0	0	101	0

$$\frac{SUM(6+5+6+3+5+9)}{SUM(6+5+6+3+5+9+57907)} = 0.000587$$

$$Expected\ virus\ cross\text{-}talks = Sum\ of\ mapped\ reads\ for\ a\ specific\ virus\ in\ all\ samples\ and\ controls\ in\ the\ same\ run * RCS\ control\ cross-talk$$

	number of mapped reads							Expected virus
Virus name	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**PVeV**	**RCS**	**cross-talk**
RCS	6	5	6	3	5	9	57907	*0.0006*
PVY	110	0	0	0	0	0	0	0.0645
TMMoV	175	23	3	0	0	0	0	0.1179
TMV	42351	8	5	0	0	6	0	24.8629
PVeV	0	0	0	0	0	101	0	0.0593

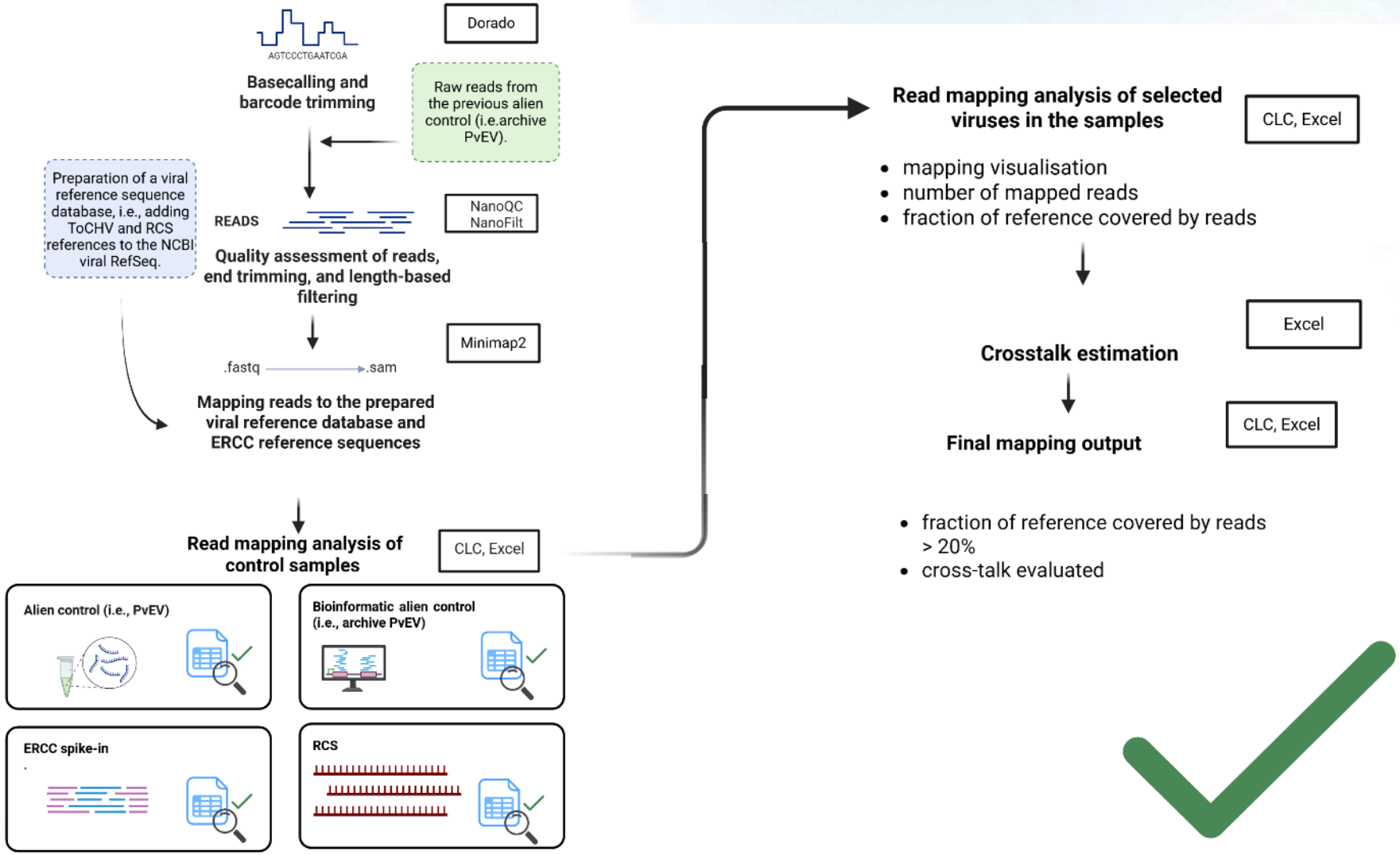
Expected virus cross-talk

SUM(110+0+0+0+0+0+0) **x** 0.000587

5+6+8 = 19

Nanopore sequencing for plant virus detection

2. Validation of Nanopore sequencing – Bioinformatic analysis



Nanopore sequencing for plant virus detection

3. Sequencing of bulk samples collected during official surveys in last years

In last years, we analyze 60 individual samples, pooled into 10 bulk samples (6 individuals per pool).

- We detect viruses that were previously reported in Slovenia.

  - **2022:** We identified the nucleotide sequence of broad bean wilt virus 2 (the first record in Slovenia) and detected a virus from the family *Bromoviridae.*

  - **2023:** We detected nucleotide sequences of a virus from the genus *Polerovirus* and of solanum nigrum illarvirus 1. The latter had previously been identified in Slovenia only in *Physalis* and other wild plants as part of the INEXTVIR research project.

  - **2024:** We identified nucleotide sequences of eggplant mottled crinkle virus in a tomato bulk sample—previously reported in Slovenia only from water samples collected during the INEXTVIR project. We also observed potential detections of a virus from the genus *Cavemovirus* and a double-stranded RNA (dsRNA) virus.

  - **2025:** We identified in cucurbits two viruses that had not previously been known to occur in Slovenia.

Conclusion

- Nanopore-based high-throughput sequencing (HTS) is ideal for small laboratories handling a limited number of samples, where comprehensive control of the entire process is required.

- Using ribosomal **RNA-depleted total RNA** as input and a **PCR-cDNA barcoding kit,** the nanopore sequencing workflow proved to be an effective method for plant virus detection, demonstrating comparable results to the sequencing with Illumina platform.

This approach was successfully applied for the accurate diagnosis of tomato mild mottle virus (TMMoV) in tomato leaves. The protocol was validated according to the European and Mediterranean Plant Protection Organisation (EPPO) guidelines (PM7/98 and PM7/151), supporting its **ISO 17025 accreditation — the first of its kind globally**.

Acknowledgements

…and the whole NIB team

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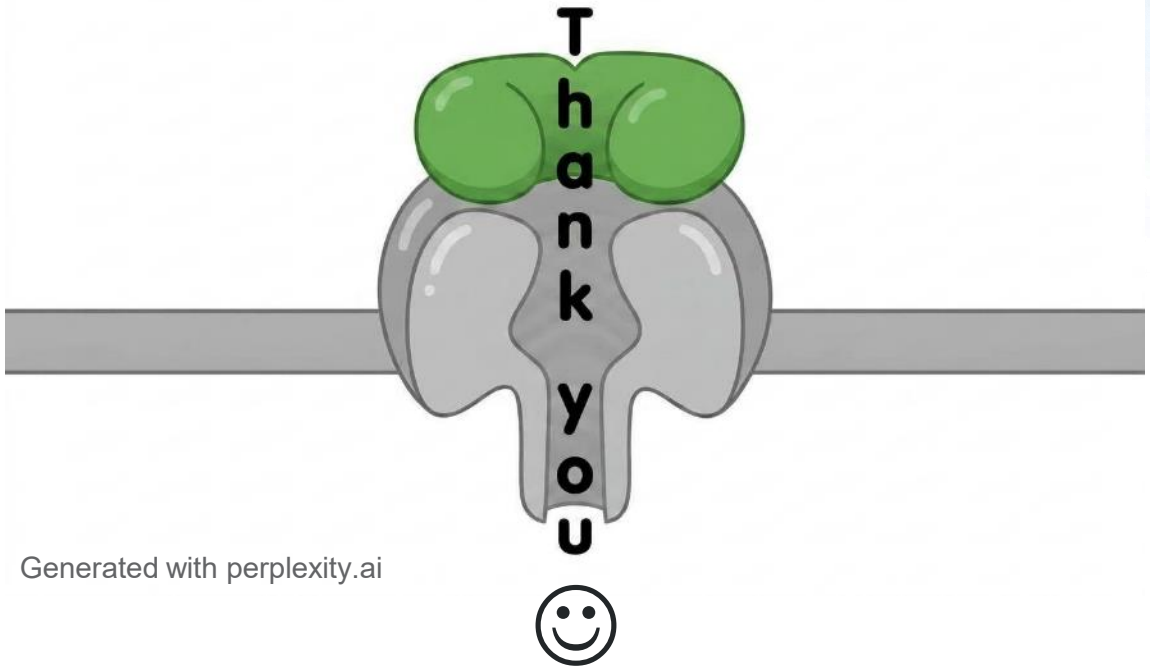

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