VALITEST webinar series and training activities

How to organize Test Performance Studies?

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CREA

Council for Agricultural Research and Economics

Research Centre for Plant Protection and Certification

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Introduction of the webinar and training activities

The concept of test validation in Plant Health

Webinar 1	What is test validation and why it matters for reliable diagnostics?	Monday 11th January, 2 pm
Webinar 2	How to adopt a new test in your laboratory?	Friday 15th January, 2pm
Webinar 3	The use and validation of on-site tests	Wednesday 20th January, 2pm
Practical training session 1	Analysis of performance characteristics	Tuesday 26th of January, 2pm to 4:30 pm
Webinar 4	How do companies handle quality control and validation of products and how will the EPDIA charter help in improving this task?	Monday 1st of February, 2pm
Webinar 5	Why is communication on test selection between risk managers and diagnostic laboratories important ?	Monday 15th of February, 2pm
Practical training session 2	The use of kits: training and demonstration	Thursday 22nd of April, 2pm

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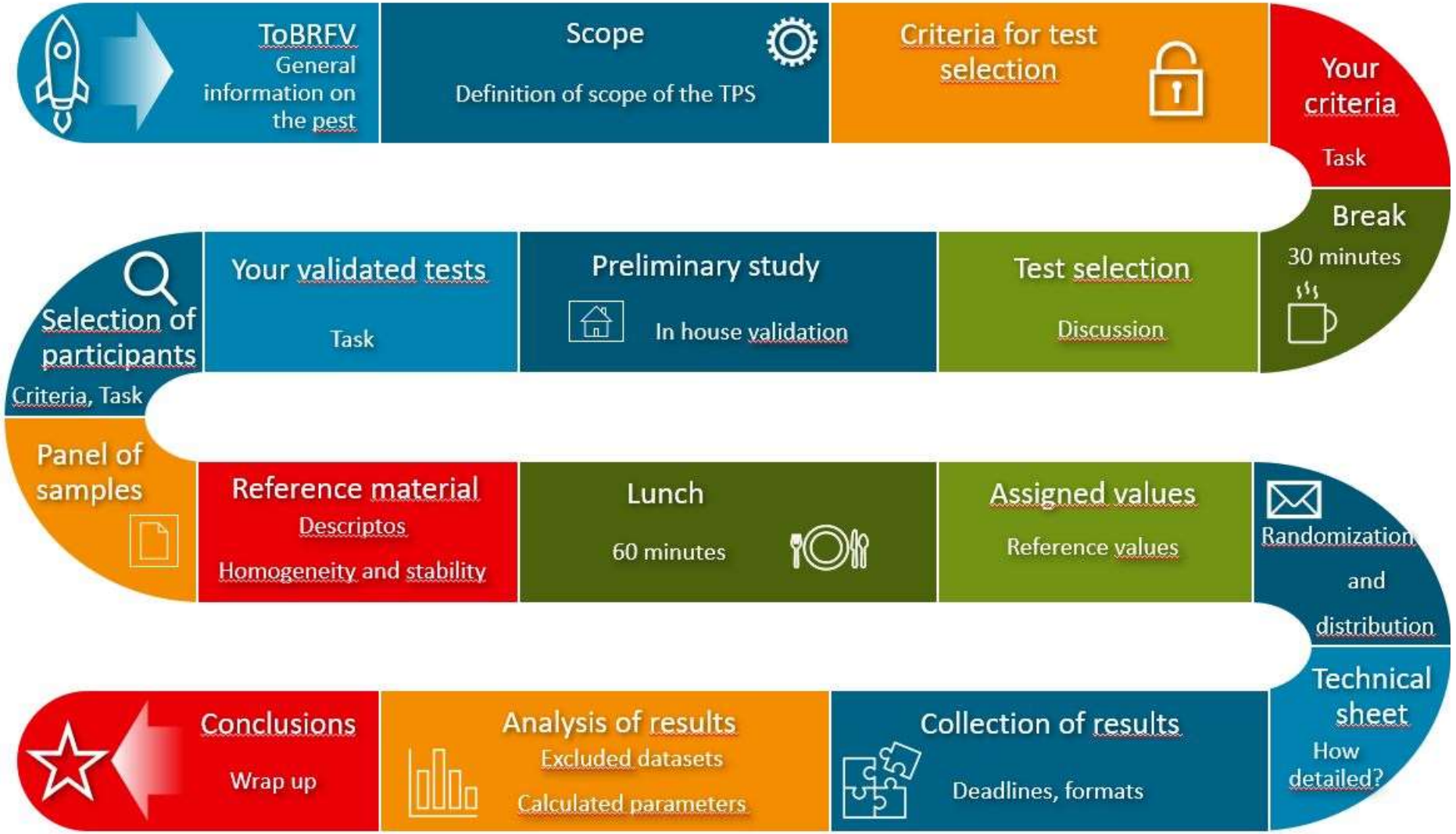
Introduction of the webinar and training activities

Test Performance Studies organisation

Videos	What is a TPS?	On the week 02/15
Videos	VALITEST TPS: selection of the pests and of the TPS organizers	On the week 02/15
Webinar 1	Preparing the TPS plan	Friday 19/02, 11am
Webinar 2	Selection of the tests and associated documents	Wednesday 24/02, 2pm
Webinar 3	Selection of participants and contract	Monday 1/03, 2pm
Webinar 4	Preparation and dispatch of samples	Friday 5/03, 11am
Webinar 5	Production of reference material for TPS	Wednesday 10/03, 2pm
Practical training sessions	How to organise Test Performance Studies?	15-18/03 (4 sessions)
Webinar 6	How to tackle the analysis of TPS results?	Monday 22/03, 2pm
Videos	Calculate performance characteristics of a test and get useful information from your validation data by statistical analysis.	On the week 22/03
Webinar 7	Q&A session: the statistical analysis of TPS results	Monday 29/03, 2pm
Practical training sessions	How to analyse the results of Test Performance Studies?	30/03-1/04 (3 sessions)
Webinar 8	From TPS organisation to analysis of the results: example of the TPS on ToBRFV	Wednesday 7/04, 2pm
Videos	Reporting TPS results	To be confirmed/announced

Outline



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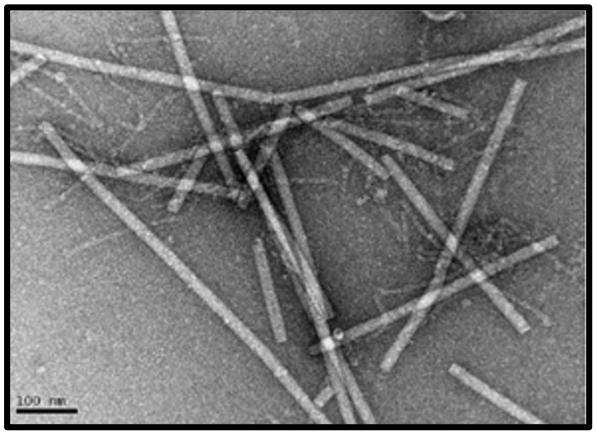
Tomato brown rugose fruit virus (ToBRFV)

Taxonomy

Family: *Virgoviridae*

Genus: *Tobamovirus*

+ssRNA genome



Luria et al., 2017 - PlosOne

Transmission

- Seeds
- Contact
- *Bombus terrestris*

Survival

ToBRFV can survive outside of the host on inert and biological surfaces as well as in soil for months without losing their virulence.

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Hosts

- *Solanum lycopersicum*
- *Capsicum annuum*

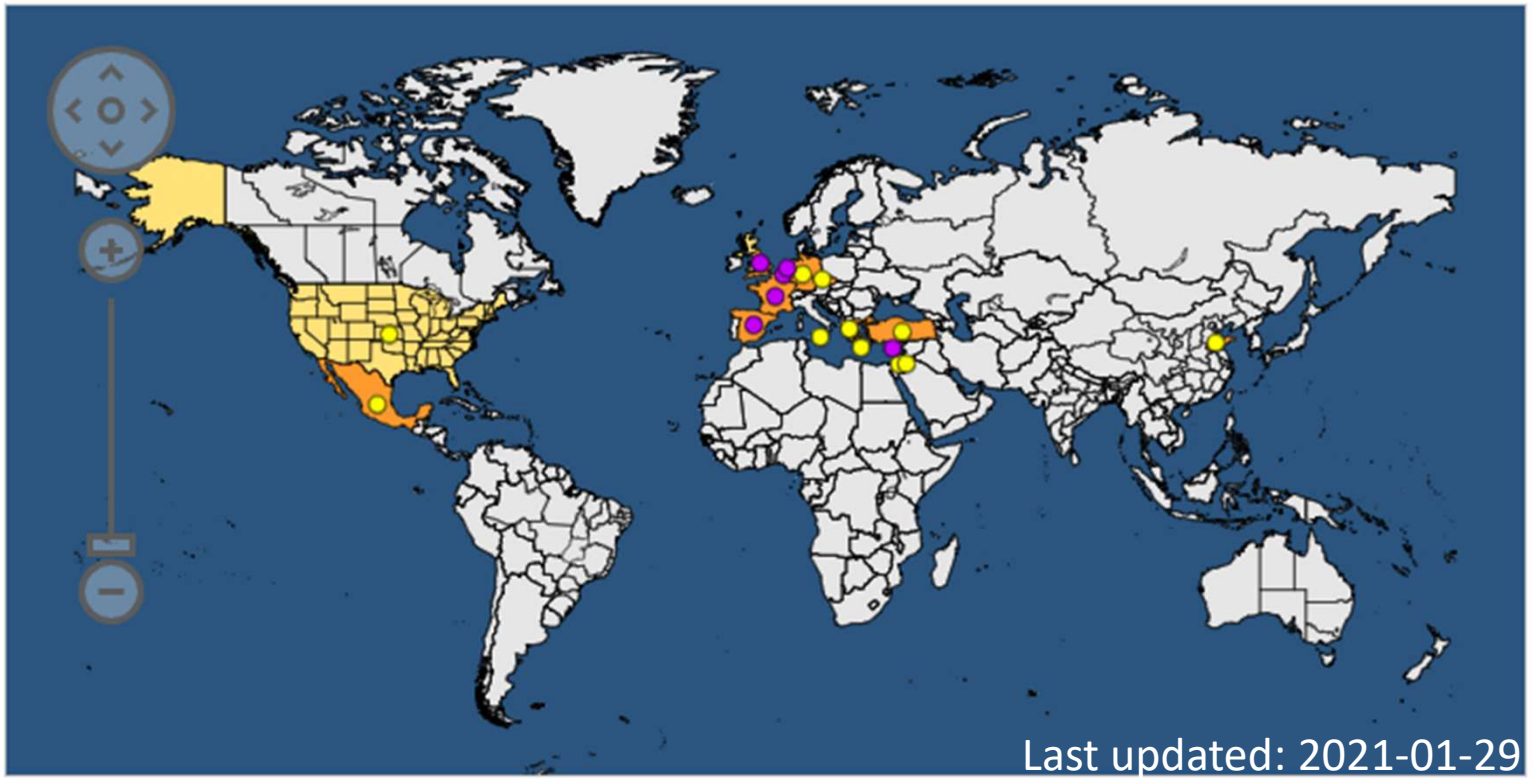
Symptoms

Leaves: mosaic, discoloration, deformation.

Fruits: size reduction, discoloration, brown rugosity, malformation: unmarketable.

Reduction of plant vigour

ToBRFV distribution



- First reports in Israel and Jordan in 2014, rapid spread worldwide.
- January 2019: EPPO Alert list - COMMISSION IMPLEMENTING REGULATION (EU) 2019/1615
- August 2020: EPPO A2 list - COMMISSION IMPLEMENTING REGULATION (EU) 2020/1191

ToBRFV resistance

In tomato ToBRFV has overcome the long-lasting resistance genes currently used for protection against several tobamoviruses (Tm-1, Tm-2/Tm-22).

No commercial varieties are currently known to be fully resistant to ToBRFV.

In pepper the L1, L3 and L4 genes/alleles were shown to provide complete resistance in pepper to ToBRFV (local hypersensitive response prevents the systemic spread of the virus to other parts of the plants).

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QUESTIONS

- *Do you have any expertise in ToBRFV testing?*

- *How many samples do you test per year for ToBRFV?*

Diagnosis - PM7/76

Detection = Assessment of presence / absence of a target pest.

- signs and/or symptoms associated with the pest
- part(s) of the plant in which the pest may be found
- likely occurrence of the pest associated with developmental stages of the host, climatic conditions and seasonality
- methods for detecting the pest in the commodity (e.g. visual, lens)
- methods for extracting and recovering the pest from the plants
- methods for indicating the presence of the pest in asymptomatic plant material or other materials (e.g. soil, water)
- viability of the pest

Identification = Assessment of (target) species or strains.

Questions?

- guidelines on methods that used either alone or in combination lead to the identification of the pest (several methods mentioned in a Standard)
- morphological methods, methods based on biological, biochemical and molecular properties

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Diagnosis - PM7/76

Method = Reliable diagnosis of regulated pests may be achieved by a single method or a combination of methods

- bioassays
- biochemical
- fingerprint
- isolation / extraction
- molecular, morphological and morphometric
- pathogenicity assessment
- serological methods.

capabilities of laboratories, circumstances of use

Test = Application of a method to a specific pest and specific matrix

Questions?

- basic requirements: repeatable and reproducible results
- performance characteristics (validation studies) and associated uncertainties
- experience of laboratories with the test (years and n. of samples)
- information obtained in verification studies

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QUESTIONS

- *Do you think DAS-ELISA is a method or a test?*

ToBRFV diagnosis

Emerging pathogen!

GUIDELINES

- no EPPO standard;
- no validated diagnostic test.

MAIN OBSTACLES

- limited validation data available and not always homogeneous;
- protocols involving long and time-consuming methodologies.

Scope of testing

Selection of different methods for different pests!

The scope relies on current diagnostic needs for each pest.

QUESTIONS

- *Which **characteristics** do you think it is important to identify in the **definition of scope** in a TPS?*

Scope of testing

Selection of different methods for different pests!

The scope relies on current diagnostic needs for each pest.

For every method, identify:

SAMPLE TYPE: DNA, sample spiked with pest,…
MATRIX: seeds, leaves,…
SUITABLE FOR: Symptomatic or asymptomatic samples
PURPOSE: Detection or identification
CONTROLS: NIC, NAC, PAC, PIC, IC, etc
NUMBER OF SAMPLES
NUMBER OF LABS

In the frame of the scope definition, also consider the **availability of plant material** and the **expertise of TPS organize**r.

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Scope of testing - ToBRFV

Detection of ToBRFV in symptomatic and asymptomatic leaves and fruits in tomato and pepper.

	DAS-ELISA	RT-PCR	REAL-TIME PCR	OTHER METHODS APPLICABLE FOR ON-SITE:
SAMPLE TYPE	freeze dried plant material	freeze dried plant material	freeze dried plant material	freeze dried plant material
MATRIX	leaves and fruits	leaves and fruits	leaves and fruits	leaves
SUITABLE FOR	symptomatic / asymptomatic	symptomatic / asymptomatic	symptomatic / asymptomatic	symptomatic / asymptomatic
PURPOSE	detection	detection	detection	early warning

SEEDS - Euphresco 2019-A-327

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Test selection

New Disease Reports **(2019) 39, 1. http://dx.doi.org/10.5197/j.2044-0588.2019.039.001**

New Disease Reports

First report of *Tomato brown rugose fruit virus* infecting tomato in Germany

W. Menzel [1]*, D. Knierim [1], S. Winter [1], J. Hamacher [2] and M. Heupel [3]

KEYWORDS!

H ARTICLE

A New Israeli *Tobamovirus* Isolate Infects Tomato Plants Harboring *Tm-2*^{2} Resistance Genes

Neta Luria[1✪], Elisheva Smith[1✪], Victoria Reingold[1], Ilana Bekelman[1], Moshe Lapidot[2], Ilan Levin[2], Nadav Elad[3], Yehudit Tam[1], Noa Sela[1], Ahmad Abu-Ras[4], Nadav Ezra[4], Ami Haberman[4], Liron Yitzhak[1,5], Oded Lachman[1], Aviv Dombrovsky[1]*

Criteria for test selection

PM7/98 ✚ VALITEST

- criteria of performance;
- descriptors (qualitative or quantitative, absolute or relative, e.g. value, %, yes/no, high/low);
- targets;
- relative weights (may be different for lab or on-site testing).

QUESTIONS

- *Which **criteria** do you think are important for **selection of test** for the particular **scope**?*

- *Explain your choices*

Criteria for test selection - PM7/98

- Analytical sensitivity
- Analytical specificity
    - Inclusivity
    - Exclusivity
- Selectivity
- Repeatability
- Reproducibility
- Robustness

Questions?

Criteria for test selection - PM7/98

- How do you assess these performance criteria?

Virology and phytoplasmology

Table A7. Virology and phytoplasmology (see also the instructions for the use of the tables). This table covers viruses, viroids and phytoplasmas

Molecular methods, e.g. PCR, real-time PCR, LAMP This step also includes methods for extraction of RNA/DNA from the matrix.	
Analytical sensitivity (relative sensitivity)	Because the concentration of viruses, viroids and phytoplasmas is never known, determine the maximum dilution of RNA/DNA detected. Perform at least three experiments with serial dilutions of infected sample in the healthy sample selected. If consistent results are not obtained after three series, additional series should be prepared and tested.
Analytical specificity	Inclusivity: analyse a range of variants/strains of the target covering genetic diversity, different geographic origin and hosts. Exclusivity: analyse relevant non-targets, in particular those that might be present in the matrix. The concentration of nucleic acid should be high enough to maximize the possibility of cross-reaction but remain realistic. *For both inclusivity and exclusivity, the test results can be supported by 'in silico' comparison of primer/probe sequences to sequences in genomic libraries.*
Selectivity	Determine whether variations of the matrix (e.g. by using different cultivars of the host plant) affect the test performance.
Repeatability	Analyse at least three replicates of sample extracts with a low (relative) concentration. If consistent results are not obtained, additional replicates should be prepared and tested.
Reproducibility	As for repeatability, but with different operator(s) if possible, on different days and with different equipment when relevant.

Criteria for test selection - Valitest

Criteria	Descriptor (%, number, text)	Target	Relative Weight (lab)	Relative Weight (on-site)
Validation data (prior to preliminary studies)				
Is the target (gene/protein) appropriately selected?	Yes/No	Yes	High	High
Available validation data	Yes/No	No	Low	Low
Validation data available for selected matrix	Yes/No	No	Low	Low
Analytical sensitivity (LOD) (pure culture or DNA diluted in water)	Conc. (absolute value if possible or relative conc. or low/medium/high)	NA	NA	NA
Sensitivity in plant material (selected matrix)	Conc. (absolute value if possible or relative conc. or low/medium/high)	High	High	Medium
Diagnostic sensitivity (comparison of different tests)	%	100%	High	Medium
Analytical specificity	Level	High	High	Medium
a) Exclusivity (Non-target organism): False positives	Level	0%	High	Medium
b) Inclusivity (Target organisms): False negatives	Level	0%	High	Medium
Selectivity	Presence of cross reactions with matrix	No	High	Medium
Repeatability (near LOD)	Level	High	High	High
Reproducibility / robustness	%	100%	High	High
Results of interlaboratory comparisons available	Yes/No	No	Low	Low

Criteria for test selection - Valitest

Criteria	Descriptor (%, number, text)	Target	Relative Weight (lab)	Relative Weight (on-site)
Validation data (after preliminary studies)				
Analytical sensitivity (LOD) (pure culture or DNA diluted in water)	Conc. (absolute value if possible or relative conc. or low/medium/high)	NA	NA	NA
Sensitivity in plant material (selected matrix)	Conc. (absolute value if possible or relative conc. or low/medium/high)	High	High	Medium
Diagnostic sensitivity (comparison of different tests)	%	100%	High	Medium
Analytical specificity	Level	High	High	Medium
a) Exclusivity (Non-target organism): False positives	Level	0%	High	Medium
b) Inclusivity (Target organisms): False negatives	Level	0%	High	Medium
Selectivity	Presence of cross reactions with matrix	No	High	Medium
Repeatability (near LOD)	Level	High	High	High
Reproducibility / robustness	%	100%	High	High

Criteria for test selection - Valitest

If some tests show similar performance, other criteria are taken into account regarding:

- Applicability
- Protocols
- Chemicals
- Equipment

Criteria	Descriptor (%, number, text)	Target	Relative Weight (lab)	Relative Weight (on-site)
APPLICABILITY				
Applicability in different matrixes	Level	Medium	Medium	Medium
Amount of material which is included in one sample	Amount of plant units tested	25 plants/sample	Medium	Medium
Standardized preparation of the reaction (e.g. ready to use reagents)	Yes/No	Yes	Medium	High
Availability and relevance of controls (in the case of kits)	Yes/No	Yes	High	High

Criteria for test selection - Valitest

Criteria	Descriptor (%, number, text)	Target	Relative Weight (lab)	Relative Weight (on-site)
PROTOCOLS				
Available detailed protocols	Yes/No	Yes	Medium	Medium
Simple test procedure	Yes/No	Yes	Medium	Medium
Simplicity of data analysis	Yes/No	Yes	High	High
User-friendly test	Yes/No	Yes	Medium	High
Time needed to complete analysis (less than one hour/ one day/ several days)	Duration in time unit	one day/less than one hour	Medium	High
Easy to multiplex?	Yes/No	Yes	Medium	NA
Database/library dependent (yes/ no) (for example fatty acids profiling, sequencing...)	NA	NA	NA	NA

Criteria for test selection - Valitest

Criteria	Descriptor (%, number, text)	Target	Relative Weight (lab)	Relative Weight (on-site)
CHEMICALS				
Stability of chemicals at ambient temperature	Yes/No	Yes	Low	High
EQUIPMENT				
No equipment/ instrument needed (relevant only for on-site tests)	Yes/No	Yes	NA	High
Test not exclusively developed for a specific instrument	Yes/No	Yes	High	High
Cost of obligatory equipment/ instruments (up to 10.000 EUR/ 10.000-50.000 EUR/ more than 50.000 EUR?)	Cost in euro	10.000 to 50.000 EUR	Low	Low

30 min BREAK!

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Selection of tests for TPS: the steps

- Collection and analysis of available data
- First selection of tests
- Preliminary studies
- Selection of the final tests

Available tests for ToBRFV detection

Tests:

- published shortly before TPS (poorly checked and screened by users, may still contain severe drawbacks);
- long and time-consuming methodologies;
- performed on different matrixes from those selected in the scope of validation.

Validation data:

- not always available;
- often not homogeneous from different sources (slightly different reagents, instruments, approaches); *
- direct comparison not possible even for the same test;

Time and budget constraints!

A pre-selection of the tests by TPS organizers was required

*Note: plenty of protocols and a variety of reagents, instruments and approaches represent a resource in plant health diagnosis!

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Overview of available tests for ToBRFV detection

DAS-ELISA kits

- Agdia
- DSMZ
- Loewe

RT-PCR tests

- Alkowni et al., 2019
- Levitzky et al., 2019
- Ling et al., 2019
- Luria et al., 2017
- Panno et al., 2019a
- Rodriguez-Mendoza et al., 2019
- Loewe kit (primers as Rodriguez-Mendoza)

Overview of available tests for ToBRFV detection

Real Time RT-PCR tests

- ISHI-Veg, 2019
- Menzel and Winter, 2019
- Panno et al., 2019b

Lateral flow kit

- Agdia

14 tests available

Available tests for ToBRFV detection

Method	Test	Comments
DAS-ELISA	Agdia, DSMZ, Loewe	Information provided by companies on cross reactions with other tobamoviruses
RT-PCR	Levitzky et al., 2019	two-step, amplicon size 811 bp No target tested: none *In silico* (Blast-NCBI) tested TMV, ToMMV
	Luria et al., 2017	two-step, amplicon size 1052 bp No-target tested: none
	Alkowni et al., 2019	two-step, amplicon size 563 bp No-target tested: TMV, ToMV, TMMV, TMGMV, PMMV, ORSV, CFMMV
	Rodriguez-Mendoza et al., 2019	two-step, amplicon size 475 bp Same primers as in Loewe kit
	Commercial kit (Loewe)	two-step, amplicon size 475 bp No-target tested: CGMMV, KGMMV, ORSV; PaMMV, PMMoV, RMV, TMGMV, TMV, ToMV, YMoV, ZGMMV, BPeMV, others
	Ling et al., 2019	one-step, amplicon size 482 bp No-target tested: ToMV, ToMMV
	Panno et al., 2019a	one-step, amplicon size 457 bp No-target tested: none
LATERAL FLOW	Commercial kits (Agdia)	No-target tested: TMV
Real Time RT-PCR	ISHI-Veg, 2019	No-target tested: none
	Menzel and Winter, 2019	No-target tested: TMV, RMV, ToMV, BPeMV, TMGMV, ORSV, CGMMV, PaMMoV, SFBV, SHMV, TVCV, CapMMV, YMoV, PChV, ObPV, Vigaviridae, others
	Panno et al., 2019b	No-target tested: CGMV, PaMMV, PMMV, TMGMV, TMV, ToMMV, ToMV, ZGMV

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QUESTIONS

- *Based on these information, which tests would you select for preliminary study and why?*
- *Ask for details or other information you may need.*

Test selection for preliminary study

Method	Test		Comments
DAS-ELISA	Agdia, DSMZ, Loewe	X	Information provided by companies on cross reactions with other tobamoviruses
RT-PCR	Levitzky et al., 2019	X	two-step, amplicon size 811 bp No target tested: none *In silico* (Blast-NCBI) tested TMV, ToMMV
	Luria et al., 2017	X	two-step, amplicon size 1052 bp No-target tested: none
	Alkowni et al., 2019	✓	two-step, amplicon size 563 bp No-target tested: TMV, ToMV, TMMV, TMGMV, PMMV, ORSV, CFMMV
	Rodriguez-Mendoza et al., 2019	X	two-step, amplicon size 475 bp Same primers as in Loewe kit
	Commercial kit (Loewe)	✓	two-step, amplicon size 475 bp No-target tested: CGMMV, KGMMV, ORSV; PaMMV, PMMoV, RMV, TMGMV, TMV, ToMV, YMoV, ZGMMV, BPeMV, others
	Ling et al., 2019		one-step, amplicon size 482 bp
Real Time RT-PCR	ISHI-Veg, 2019	✓	No-target tested: none
	Menzel and Winter, 2019	✓	No-target tested: TMV, RMV, ToMV, BPeMV, TMGMV, ORSV, CGMMV, PaMMoV, SFBV, SHMV, TVCV, CapMMV, YMoV, PChV, ObPV, Vigaviridae, others
	Panno et al., 2019b	✓	No-target tested: CGMV, PaMMV, PMMV, TMGMV, TMV, ToMMV, ToMV, ZGMV

14 tests available → 8 tests selected for preliminary study

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Preliminary study

Analytical sensitivity and analytical specificity

		conventional RT-PCR				real-time RT-PCR			lateral flow
		Alkowni et al., (ALK)	Loewe (Rodriguez-Mendoza et al.) LOE	Ling et al.,	Panno et al., a	ISHI-Veg (ISH)	Menzel and Winter (M&W)	Panno et al., b (PAN)	Agdia
inclusivity	Sicily isolate	+	+	+	+	+	+	+	not tested
	Piemonte isolate	+	+	+	+	+	+	+	not tested
	PV-1236	+	+	+	+	+	+	+	not tested
	PV-1241	+	+	+	+	+	+	+	not tested
exclusivity	ToMV PV-0141	-	-	-	-	-	-	-	not tested
	TMV PV-1252	-	-	+	+	-	-	-	not tested
	PMMoV PV-0165	-	-	+	-	-	-	-	not tested
	BPeMV PV-0170	-	-	-	-	-	-	-	not tested
	TMGMV PV-0124	-	-	-	-	-	-	-	not tested
analytical sensitivity (tomato)	10^{0}	+	+	not tested	not tested	+	+	+	+
	10^{-1}	+	+	not tested	not tested	+	+	+	-
	10^{-2}	+	+	not tested	not tested	+	+	+	not tested
	10^{-3}	+	+	not tested	not tested	+	+	+	not tested
	10^{-4}	-	+	not tested	not tested	+	+	+	not tested
	10^{-5}	-	+	not tested	not tested	+	+	+	not tested
	10^{-6}	-	-	not tested	not tested	+	+	+	not tested
	10^{-7}	-	-	not tested	not tested	+	+	+	not tested
	10^{-8}	-	-	not tested	not tested	+/-	+/-	+/-	not tested
	10^{-9}	-	-	not tested	not tested	-	-	-	not tested
	10^{-10}	-	-	not tested	not tested	-	-	-	not tested
analytical sensitivity (pepper*)	10^{0}	+	+	not tested	not tested	+	+	+	-
	10^{-1}	+	+	not tested	not tested	+	+	+	not tested
	10^{-2}	-	+	not tested	not tested	+	+	+	not tested
	10^{-3}	-	+	not tested	not tested	+	+	+	not tested
	10^{-4}	-	-	not tested	not tested	+	+	+	not tested
	10^{-5}	-	-	not tested	not tested	+/-	+/-	+/-	not tested
	10^{-6}	-	-	not tested	not tested	-	-	-	not tested
	10^{-7}	-	-	not tested	not tested	-	-	-	not tested
	10^{-8}	-	-	not tested	not tested	-	-	-	not tested
	10^{-9}	-	-	not tested	not tested	-	-	-	not tested
	10^{-10}	-	-	not tested	not tested	-	-	-	not tested

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Preliminary study
Repeatability and reproducibility

TEST REPEATED:

- at LOD level (from analytical sensitivity);
- with different operators and moments (reproducibility);
- with different master mixes (robustness).

All tests gave repeatable, reproducible and robust results, giving similar performance in all tested conditions.

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QUESTIONS

- *Based on this preliminary study, which test would you select for TPS and why?*

Preliminary study

Analytical sensitivity and analytical specificity

		conventional RT-PCR				real-time RT-PCR			lateral flow
		Alkowni et al., (ALK)	Loewe (Rodriguez-Mendoza et al.) LOE	Ling[illegible]t al.,	Pann[illegible]et al., a	ISHI-Veg (ISH)	Menzel and Winter (M&W)	Panno et al., b (PAN)	A[illegible]ia
inclusivity	Sicily isolate	+	+	+	+	+	+	+	not tested
	Piemonte isolate	+	+	+	+	+	+	+	not tested
	PV-1236	+	+	+	+	+	+	+	not tested
	PV-1241	+	+	+	+	+	+	+	not tested
exclusivity	ToMV PV-0141	-	-	-	-	-	-	-	not tested
	TMV PV-1252	-	-	+	+	-	-	-	not tested
	PMMoV PV-0165	-	-	+	-	-	-	-	not tested
	BPeMV PV-0170	-	-	-	-	-	-	-	not tested
	TMGMV PV-0124	-	-	-	-	-	-	-	not tested
analytical sensitivity (tomato)	10^{0}	+	+	not tested	not tested	+	+	+	+
	10^{-1}	+	+	not tested	not tested	+	+	+	-
	10^{-2}	+	+	not tested	not tested	+	+	+	not tested
	10^{-3}	+	+	not tested	not tested	+	+	+	not tested
	10^{-4}	-	+	not tested	not tested	+	+	+	not tested
	10^{-5}	-	+	not tested	not tested	+	+	+	not tested
	10^{-6}	-	-	not tested	not tested	+	+	+	not tested
	10^{-7}	-	-	not tested	not tested	+	+	+	not tested
	10^{-8}	-	-	not tested	not tested	+/-	+/-	+/-	not tested
	10^{-9}	-	-	not tested	not tested	-	-	-	not tested
	10^{-10}	-	-	not tested	not tested	-	-	-	not tested
analytical sensitivity (pepper*)	10^{0}	+	+	not tested	not tested	+	+	+	-
	10^{-1}	+	+	not tested	not tested	+	+	+	not tested
	10^{-2}	-	+	not tested	not tested	+	+	+	not tested
	10^{-3}	-	+	not tested	not tested	+	+	+	not tested
	10^{-4}	-	-	not tested	not tested	+	+	+	not tested
	10^{-5}	-	-	not tested	not tested	+/-	+/-	+/-	not tested
	10^{-6}	-	-	not tested	not tested	-	-	-	not tested
	10^{-7}	-	-	not tested	not tested	-	-	-	not tested
	10^{-8}	-	-	not tested	not tested	-	-	-	not tested
	10^{-9}	-	-	not tested	not tested	-	-	-	not tested
	10^{-10}	-	-	not tested	not tested	-	-	-	not tested

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Final results of test selection

Method	Test		Comments
DAS-ELISA	Agdia, DSMZ, Loewe	X	Information provided by companies on cross reactions with other tobamoviruses
RT-PCR	Levitzky et al., 2019	X	two-step, amplicon size 811 bp No target tested: none *In silico* (Blast-NCBI) tested TMV, ToMMV
	Luria et al., 2017	X	two-step, amplicon size 1052 bp No-target tested: none
	Alkowni et al., 2019	✓	two-step, amplicon size 563 bp No-target tested: TMV, ToMV, TMMV, TMGMV, PMMV, ORSV, CFMMV
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	Ling et al., 2019	X	one-step, amplicon size 482 bp No-target tested: ToMV, ToMMV, TMV, PMMoV
Real Time RT-PCR	Menzel and Winter, 2019	✓	PaMMoV, SFBV, SHMV, TVCV, CapMMV, YMoV, PChV, ObPV, Vigaviridae, others
	Panno et al., 2019b	✓	No-target tested: CGMV, PaMMV, PMMV, TMGMV, TMV, ToMMV, ToMV, ZGMV

14 tests available → 8 tests selected for preliminary study → 5 tests selected for TPS

Selection of participants

NUMBER OF PARTICIPANTS

Maximum number of participants for a TPS:

- not stated in EPPO PM7/122;
- in Valitest, it was set to 25 participants, except particular situations

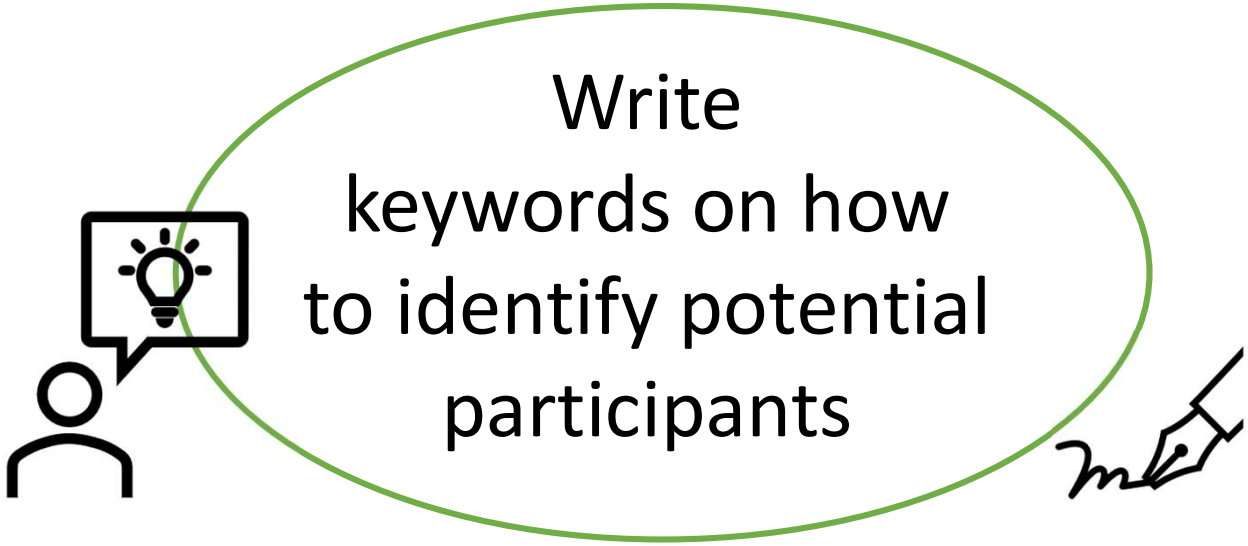
Minimum number of participants for a TPS:

- 10 participants (PM7/122) if smaller, resulting parameter estimates subject to an increased uncertainty (possible bias of confidence intervals) .

The number of participants will depend on:

- number of laboratories performing the test(s) proposed;
- availability of the matrix/pest to be tested;
- time and budget constraints.

Selection of participants



IDENTIFICATION OF POTENTIAL PARTICIPANTS

- Professional network
- Databases/surveys (e.g. EPPO database on diagnostic expertise)
- Previous participation in a TPS or PT
- Members of an existing consortium
- Website

Invitation letter - PM7/122

- Test organism and matrix (scope of the tps).
- Description of the methods.
- Possible phytosanitary requirements.
- A time schedule.
- Equipment and other facilities required.
- Chemicals involved (for evaluation of national/local safety and transport regulations).
- Information about whether the report/results will be made public and to which extent.

A TPS participant information form must be filled including relevant qualification criteria the participants have to reach.

Criteria for selection of participants

LEADED DISCUSSION

- Which criteria do you consider essential for selection of participants to your TPS?

- ✓ Hint: For a TPS, it is critical that participants can provide evidence that they are competent to undertake the test in question.

Criteria for selection of participants

Criteria	Descriptor	Target
TPS time schedule compatible with participant's availability and participant has committed to perform the test and deliver results in the time frame defined	Yes/No	Yes
Ability/willing to perform all the tests	Yes/No	Yes at least for the same method
Technical expertise for the pest group (e.g. virology, bacteriology, etc.)	nb of years or validation data submitted to EPPO database or other publications	>1 year; advantage if validation data submitted/published
Expertise in the use of the method ELISA	nb of years or nb of samples or validation data submitted to EPPO database or other publications	>1 year or > 30 samples; advantage if validation data submitted/published
Expertise in the use of the method RT-PCR	nb of years or nb of samples or validation data submitted to EPPO database or other publications	>1 year or > 30 samples; advantage if validation data submitted/published
Expertise in the use of the method Real Time PCR	nb of years or nb of samples or validation data submitted to EPPO database or other publications	>1 year or > 30 samples; advantage if validation data submitted/published
Authorized to work with the specific pest (viable pest/ inactivated pest/ DNA/ RNA)	Yes/No	Yes
Possibility to obtain an import document or Letter of Authority (EU countries)	Yes/No	Yes
Possibility to obtain an import document or Letter of Authority (EU countries) within 4 weeks to receive samples containing the specific pest (only necessary when viable pests are sent)	Yes/No	Yes
Previous participation in TPS or PT	Yes /No	Yes
Available equipment:		
- ELISA: Plate reader (company/model of instrument, wavelength of filters)	NA	NA
- (RT-)PCR: thermal cycler / gel electrophoresis system / gel imaging system (company/model of instrument)	Yes/No	Yes with appropriate characteristics
- Real Time (RT-)PCR: Thermal cycler (company/model of instrument)	Yes/No	Yes (should be compatible with TaqMan Universal PCR Master Mix)
a) channels available (FAM, VIC,...)	Wavelength filter	FAM/BHQ1
b) for multiplexing (instrument with at least two channels)	NA	NA
Constraints for delivery?	Yes/No (if yes explanations)	No
Any problems or limitations with delivery on dry ice?	Yes/No (if yes explanations)	Preferably No
Traceability in place / QA in place	Yes/No	Yes
- ISO17025 accreditation	Yes/No	Yes

Valitest

Participant contract

Registration in the conditions of participation of the contract:

- Evaluate all the tests per method
- Apply the detailed test protocol
- Regulatory requirements
- Availability to receive the samples
- Communication of difficulties
- Provide results within deadline
- No information on samples/results to other participants / third parties
- Participant anonymization (coding)
- Prevent the risk of unintentional release of plant pests
- Use samples for the project only
- Destroy samples after results submission

Selection of participants - ToBRFV TPS

96 invitation letters sent to potential participants

- Valitest partners;
- Euphresco 2019-A-327 partners (synergistic project);
- EPPO database on diagnostic expertise;
- diagnostic laboratories, either private laboratories at commercial companies or laboratories at public institutions.

37 applications have been received. Among these, only one laboratory did not pass one of the exclusion criteria because it does not have the possibility of performing molecular analysis. Among the other 36 laboratories, only **34 finally made the full registration to the TPS**.

Panel of samples

TPS test items = **reference material**

Guide 30:2015 — **ISO**

any material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process.

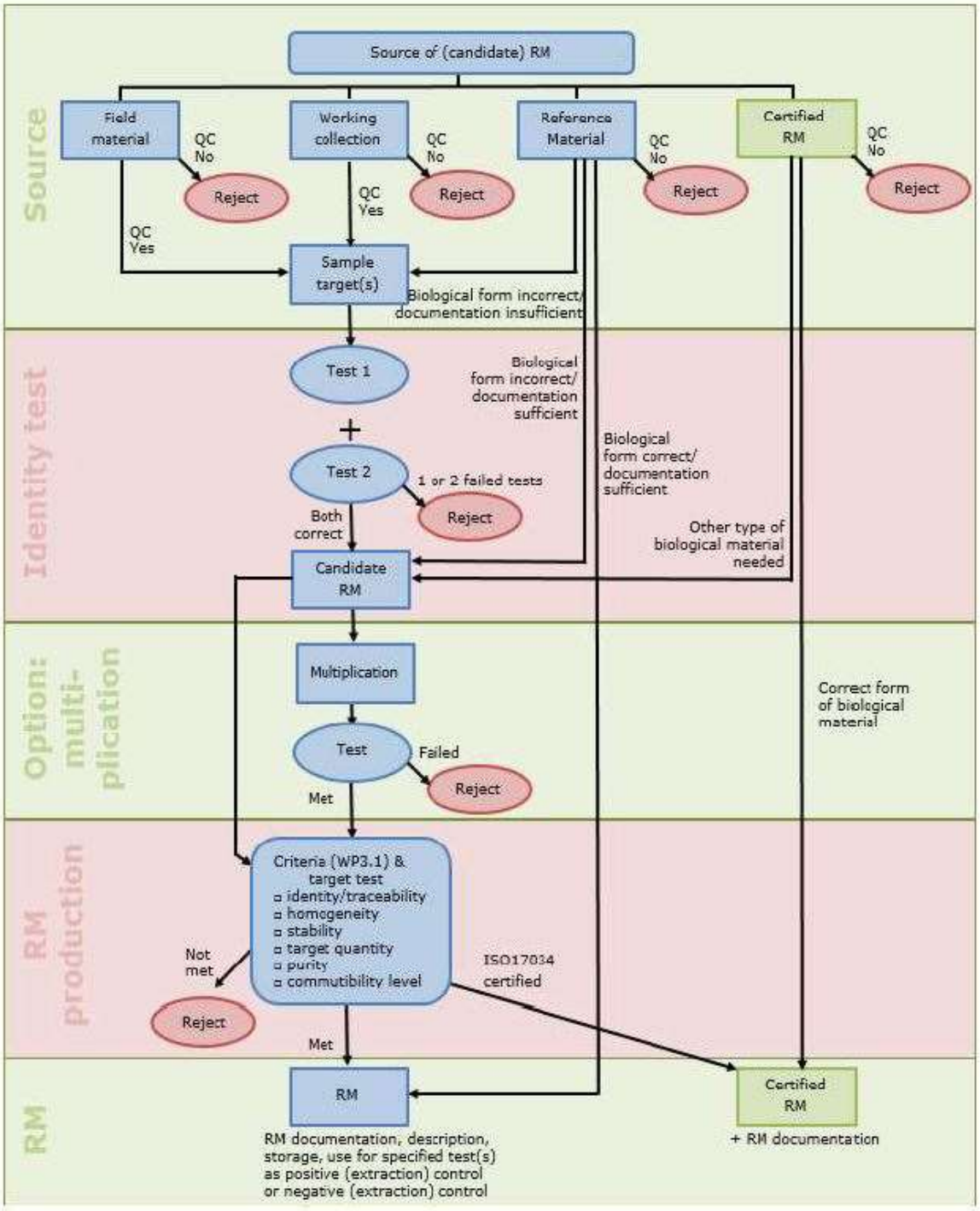
EPPO — PM7/76

material appropriate to the test and diagnosis being performed such as live cultures, infested plant material, DNA/RNA preparations, images of a diagnostic quality or mounted specimens.

- Valitest WP3 Quality assurance for reference materials for validation purposes
  Leader : Prof. Dr René van der Vlugt (WUR)

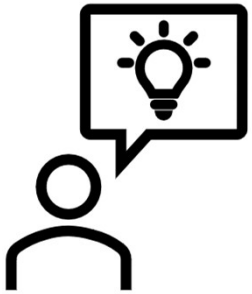
RM should contribute to results respecting FAIR principles

Valitest



Process for RM production: Valitest D3.3 «Standard Operating Procedures (SOP) finalised for the production of the reference materials»

Write keywords to describe a reference material



List of descriptors for reference material

Descriptor	Value	Minimum criterion (yes/no)
Intended use	should be defined (in this case it equals preparation of RM for the scope of the individual test or TPS)	
Identity	identified to the level of internationally recognized diagnostic protocols (mention tests and outcome)	
Traceability	traceability to a specimen from a reference culture collection	
	traceability to a specimen from a working culture collection	
	traceability provided for the target pest and matrix used (the latter if relevant)	
Commutability level	□ naturally infested plant material	
	□ artificially infested plant material	
	□ spiked plant material	
	□ purified organisms	
	□ total nucleic acids from a sample (target organism in background)	
	□ purified nucleic acids	
	□ synthetic nucleic acids	
Homogeneity	homogenous Provide test & test results	
Stability	stable	
	stability - short term	
	stability - long term	
Assigned value	absolute concentration known	
	level of concentration known (high/medium/low)	
	qualitative status known (above LOD level)	
	originating from plants with known health status with a recent test result (a given period of time depends on the plant-pest combination and previous experience)	
Purity	absence of non-targets	
	absence of interfering non-targets	
	known ratio of target vs. non-target interfering with the test - high	
	known ratio of target vs. non-target interfering with the test - medium	
	known ratio of target vs. non-target interfering with the test - low	

Descriptor	Value	Minimum criterion (yes/no)	Description
Intended use	should be defined (in this case it equals preparation of RM for the scope of the individual test or TPS)	**yes**	**TPS ToBRFV, leaves and fruits, molecular and serological tests**
Identity	identified to the level of internationally recognized diagnostic protocols (mention tests and outcome)	**yes**	**Serological and molecular tests (references), Sanger and HTS sequencing**
Traceability	traceability to a specimen from a reference culture collection	no	
	traceability to a specimen from a working culture collection	no	
	traceability provided for the target pest and matrix used (the latter if relevant)	**yes**	**ToB-SIC21-25/19 CREA-DC collection**
Commutability level	□ naturally infested plant material	**yes**	**X**
	□ artificially infested plant material	**yes**	**X**
	□ spiked plant material	**yes**	**X**
	□ purified organisms	no	
	□ total nucleic acids from a sample (target organism in background)	**yes**	**X**
	□ purified nucleic acids	no	
	□ synthetic nucleic acids	no	
Homogeneity	homogenous Provide test & test results	**yes**	**10 samples in duplicate M&W 2019 100% concordant**
Stability	stable	**yes**	**3 random samples**
	stability - short term	**yes**	**2 months room T**
	stability - long term	no	
Assigned value	absolute concentration known	no	
	level of concentration known (high/medium/low)	**yes**	**high**
	qualitative status known (above LOD level)	**yes**	**yes**
	originating from plants with known health status with a recent test result (a given period of time depends on the plant-pest combination and previous experience)	**yes**	**yes**
Purity	absence of non-targets	**yes**	**yes**
	absence of interfering non-targets	no	
	known ratio of target vs. non-target interfering with the test - high	no	
	known ratio of target vs. non-target interfering with the test - medium	no	
	known ratio of target vs. non-target interfering with the test - low	no	

Panel of samples

Number of samples will depend on:

- scope of the TPS, performance criteria to be assessed, requirements of statistical analysis (include replicates? dilution points?)
- time and budget constraints
- availability of the pest and the matrix (season, location)

Panel of samples

ID	REPETITION	TRACEABILITY	TYPE OF SAMPLE	PLANT	DILUTION
SAMPLE 1	2	-	Healthy	*S. lycopersicum*	
SAMPLE 2	2	-	Healthy	*C. annuum*	
SAMPLE 3	3	ToB-SIC 21/19	Serial dilution	*S. lycopersicum*	10^{-8}
SAMPLE 4	3				10^{-6}
SAMPLE 5	3				10^{-4}
SAMPLE 6	3				10^{-2}
SAMPLE 7	2				10^{0}
SAMPLE 8	2	ToB-SIC 23/19	Low concentration	*S. lycopersicum*	10^{-6}
SAMPLE 9	2	ToB-SIC 25/19	Medium concentration	*S. lycopersicum*	10^{-4}
TOBRFV-M-NIC	1	-	Healthy	*S. lycopersicum*	10^{0}
TOBRFV-M-PIC	1	ToB-SIC 24/19	PIC	*S. lycopersicum* (fruit)	10^{0}
TOBRFV-M-PAC	1	ToB-SIC 22/19	PAC	*S. lycopersicum*	10^{-2}

25 test items → 22 blind samples
3 controls (PIC, PAC, NIC) + primers mix
primers and probe mix
molecular grade water

Valitest

Homogeneity and stability – PM7/122

- The organizer should take care that the samples prepared and used for the interlaboratory comparison are as homogenous and as stable as possible, because this can affect the evaluation of the test performance.

- Samples which are demonstrated to be not sufficiently homogeneous or stable respectively during the homogeneity and the stability testing, should not be used to evaluate the performance of the test in a TPS.

- The assessment of homogeneity and stability should be performed by the same laboratory (generally the organizer) using the same analytical method and measuring the same characteristic of the samples.

Valitest

Homogeneity testing – PM7/122

- **When:** after the samples have been packaged in the final form and before distribution to participants.
- **How:** PM7/122 provides details for homogeneity testing for the different types of material: DNA/RNA samples, ground or mixed freeze dried material, naturally infested material etc.
- **How many:** theoretically (ISO 13528), a minimum of 10 randomly chosen samples (for each pest/matrix/infestation level, including negative samples) in duplicate; or the square root (rounded up) of the total number of samples. This is not always feasible because of the multiple pest/matrix/infestation level combinations. Therefore, number of samples included in the homogeneity testing may be reduced (data are available from previous homogeneity testing or according to the expertise of the organizer). The choice of the number of samples should be documented.

Stability testing – PM7/122

- **When:** after the deadline for performing analyses by the participants;
- **How:** as for homogeneity testing;
- **How many**: 3 randomly chosen samples (for each pest/ matrix/infestation level including negative samples) in duplicate (ISO 13528);
- Stability of provided reagents.

Questions?

Valitest

Homogeneity testing

- 9 randomly chosen test items + PIC and NIC
- 3 technical repetitions
- Real Time RT-PCR (Menzel and Winter, 2019)

Stability testing

- after 20 weeks of storage at room temperature
- PAC (RNA mixture from different extractions from the same plant) maintained 20 weeks at -20 °C, then stored at room temperature for 3 days (to simulate extreme conditions of shipping)

Homogeneity and stability

SAMPLES ID	*1 week Pos/All*	*20 weeks Pos/All*	*Av. Cq ± St.Dev.*
Sample 1	0/9	0/9	>38
Sample 2	0/9	0/9	Neg
Sample 3	9/9	9/9	37.40 ± 0.61
Sample 4	9/9	9/9	30.04 ± 0.98
Sample 5	9/9	9/9	23.31 ± 0.87
Sample 6	9/9	9/9	15.89 ± 0.77
Sample 7	9/9	9/9	9.71 ± 0.825
Sample 8	9/9	9/9	34.24 ± 0.86
Sample 9	9/9	9/9	27.13 ± 0.57
NIC	0/9	0/9	>38
PIC	9/9	9/9	13.88 ± 0.48
PAC	3/3	6/6	18.54 ± 0.74

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1 h BREAK!

Valitest

Assigned reference values

PM7/122

The organizer has to define/establish assigned values for samples, i.e. value attributed to a particular property of an interlaboratory test sample.

In the plant health field, assigned values correspond to the expected results of the test:

- pest present / absent;
- concentration of the pest.

In some cases it could be "undetermined“ (e.g., near the LOD).

Assigned reference values

VALITEST D3.1

"While it may not be always feasible or indeed necessary, it is possible to provide information on the quantity of the target pest in the reference material."

LEVEL

- Different levels of the quantity descriptions were defined:
- **Known amount** = absolute quantification of the target pest and/or its components (e.g. DNA copy numbers).
- **Level of concentration (high/medium/low) known** (as determined through use of at least one semi-quantitative or quantitative test)
- **Qualitative status known** (positive/negative above the determined limit of detection using at least one test)
- **Consensus values from participants in proficiency test.** Rules for definition of these values should be defined: statistical methods, outliers' effect
- Originating from **plants with known health status.**

Valitest

LEADED DISCUSSION

ID	REPETITION	TRACEABILITY	TYPE OF SAMPLE	PLANT	DILUTION	EXPECTED OUTCOME (QUALITATIVE TESTS)	EXPECTED OUTCOME (QUANTITATIVE TESTS)
SAMPLE 1	2	-	Healthy	*S. lycopersicum*			
SAMPLE 2	2	-	Healthy	*C. annuum*			
SAMPLE 3	3	ToB-SIC 21/19	Serial dilution	*S. lycopersicum*	10^{-8}		
SAMPLE 4	3				10^{-6}		
SAMPLE 5	3				10^{-4}		
SAMPLE 6	3				10^{-2}		
SAMPLE 7	2				10^{0}		
SAMPLE 8	2	ToB-SIC 23/19	Low concentration	*S. lycopersicum*	10^{-6}		
SAMPLE 9	2	ToB-SIC 25/19	Medium concentration	*S. lycopersicum*	10^{-4}		
TOBRFV-M-NIC	1	-	Healthy	*S. lycopersicum*	10^{0}		
TOBRFV-M-PIC	1	ToB-SIC 24/19	PIC	*S. lycopersicum* (fruit)	10^{0}		
TOBRFV-M-PAC	1	ToB-SIC 22/19	PAC	*S. lycopersicum*	10^{-2}		

LEADED DISCUSSION

ID	REPETITION	TRACEABILITY	TYPE OF SAMPLE	PLANT	DILUTION	EXPECTED OUTCOME (QUALITATIVE TESTS)	EXPECTED OUTCOME (QUANTITATIVE TESTS)
SAMPLE 1	2	-	Healthy	*S. lycopersicum*			
SAMPLE 2	2	-	Healthy	*C. annuum*			
SAMPLE 3	3				10^{-8}		
SAMPLE 4	3				10^{-6}		
SAMPLE 5	3				10^{-4}		
SAMPLE 6	3				10^{-2}		
SAMPLE 7	2				10^{0}		
SAMPLE 8	2						
SAMPLE 9	2	ToB-SIC 25/19	Medium concentration	*S. lycopersicum*	10^{-4}		
TOBRFV-M-NIC	1	-	Healthy	*S. lycopersicum*	10^{0}		
TOBRFV-M-PIC	1	ToB-SIC 24/19	PIC	*S. lycopersicum* (fruit)	10^{0}		
TOBRFV-M-PAC	1	ToB-SIC 22/19	PAC	*S. lycopersicum*	10^{-2}		

Reference Values Assignment: based on the true health status of the plant of origin

LOD for tomato samples in RT PCR: 10^{-4}
LOD for tomato samples in Real Time RT PCR: 10^{-7}

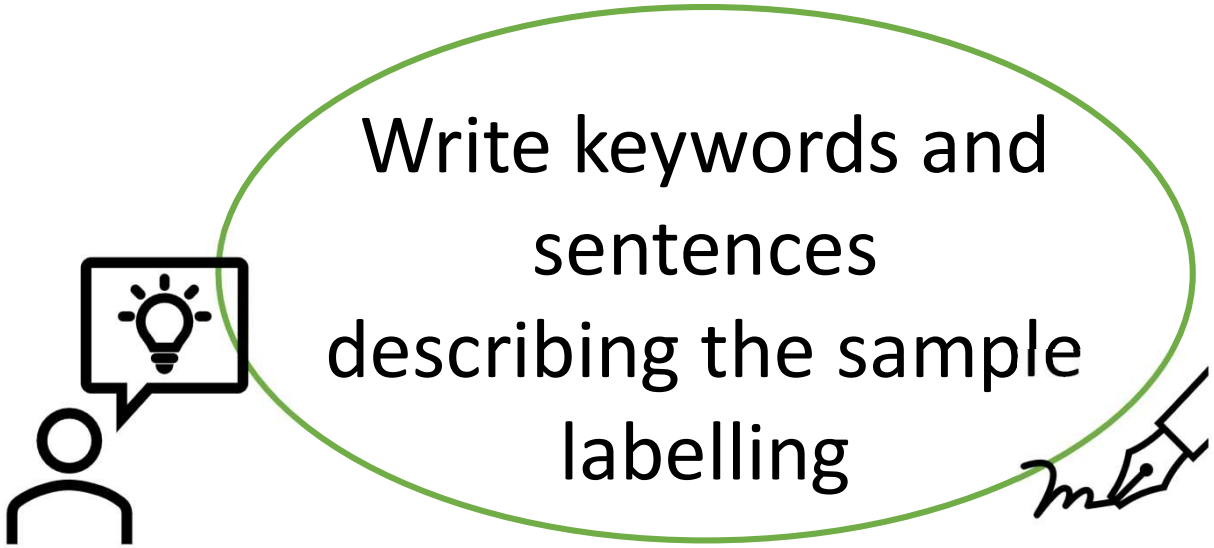
Assigned reference values

ID	REPETITION	TRACEABILITY	TYPE OF SAMPLE	PLANT	DILUTION	EXPECTED OUTCOME (QUALITATIVE TESTS)	EXPECTED OUTCOME (QUANTITATIVE TESTS)
SAMPLE 1	2	-	Healthy	*S. lycopersicum*		Neg	Neg
SAMPLE 2	2	-	Healthy	*C. annuum*		Neg	Neg
SAMPLE 3	3	ToB-SIC 21/19	Serial dilution	*S. lycopersicum*	10^{-8}	Neg	Pos (below LOD)
SAMPLE 4	3				10^{-6}	Pos (below LOD)	Pos
SAMPLE 5	3				10^{-4}	Pos (LOD)	Pos
SAMPLE 6	3				10^{-2}	Pos	Pos
SAMPLE 7	2				10^{0}	Pos	Pos
SAMPLE 8	2	ToB-SIC 23/19	Low concentration	*S. lycopersicum*	10^{-6}	Pos (below LOD)	Pos
SAMPLE 9	2	ToB-SIC 25/19	Medium concentration	*S. lycopersicum*	10^{-4}	Pos (LOD)	Pos
TOBRFV-M-NIC	1	-	Healthy	*S. lycopersicum*	10^{0}	Neg	Neg
TOBRFV-M-PIC	1	ToB-SIC 24/19	PIC	*S. lycopersicum* (fruit)	10^{0}	Pos	Pos
TOBRFV-M-PAC	1	ToB-SIC 22/19	PAC	*S. lycopersicum*	10^{-2}	Pos	Pos

Randomization and distribution of samples

CODES & LABELS

LEADED DISCUSSION



Randomization and distribution of samples

CODES & LABELS

CLEAR

BLIND

RANDOM

CONFIDENTIAL

Labels for samples	Clear label: legible through the TPS
Random assignement	Labels for labs
Confidential identity	Blind test

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Randomization and distribution of samples

CODES & LABELS

ID	L1; L23	L2; L24	L3; L25	L4; L26	L5; L27	L6; L28	L7; L28	L8; 29	L9; L30	L10; L31
ToBRFV-M-1	1	22	21	20	19	18	17	16	15	14
ToBRFV-M-2	9	8	7	6	5	4	3	2	1	22
ToBRFV-M-3	17	16	15	14	13	12	11	10	9	8
ToBRFV-M-4	22	21	20	19	18	17	16	15	14	13
ToBRFV-M-5	2	1	22	21	20	19	18	17	16	15
ToBRFV-M-6	14	13	12	11	10	9	8	7	6	5
ToBRFV-M-7	18	17	16	15	14	13	12	11	10	9
ToBRFV-M-8	3	2	1	22	21	20	19	18	17	16
ToBRFV-M-9	13	12	11	10	9	8	7	6	5	4
ToBRFV-M-10	19	18	17	16	15	14	13	12	11	10
ToBRFV-M-11	4	3	2	1	22	21	20	19	18	17
ToBRFV-M-12	12	11	10	9	8	7	6	5	4	3
ToBRFV-M-13	20	19	18	17	16	15	14	13	12	11
ToBRFV-M-14	5	4	3	2	1	22	21	20	19	18

Example of item decoders

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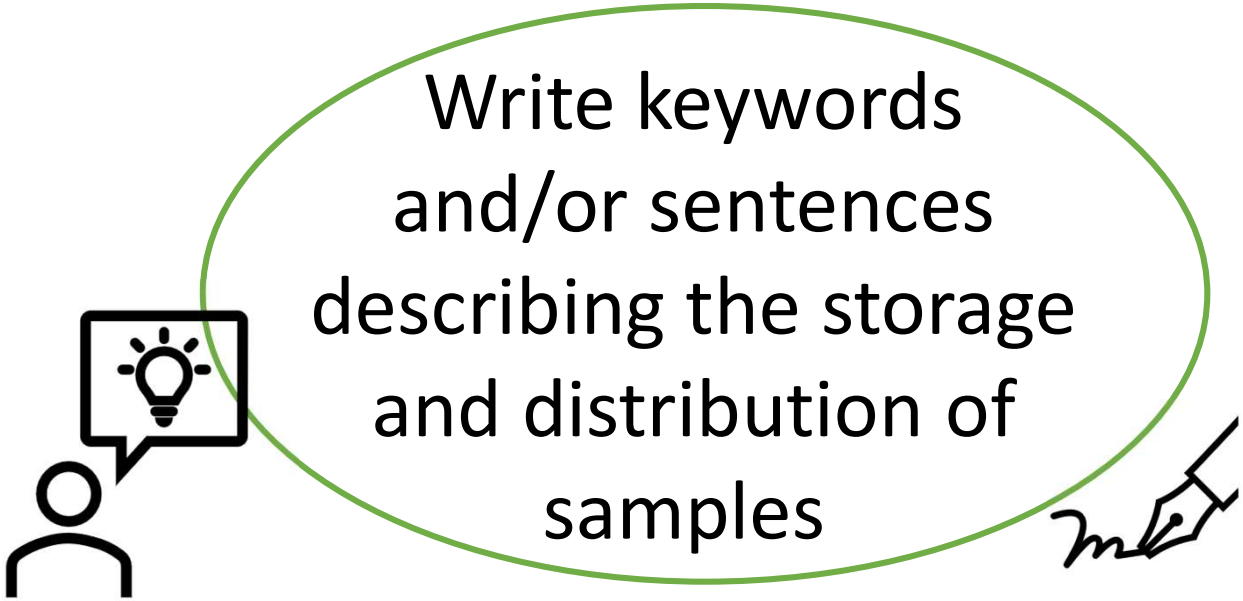
Randomization and distribution of samples

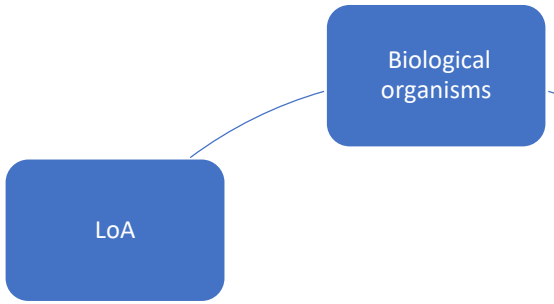
STORAGE & DISTRIBUTION

LEADED DISCUSSION



Randomization and distribution of samples

STORAGE & DISTRIBUTION



BIOSAFETY

OFFICIAL DOCUMENTS

- Countersigned LoA (if needed).
- Technical sheet describing the experimental protocols to be performed.
- Instruction sheet to be followed in each step of the TPS.
- Acknowledgment of samples receipt: a form to be returned to the organizer upon receiving the samples.
- Form for reporting the TPS results (on-line).

INT

REGUL

Valitest

Randomization and distribution of samples

STORAGE & DISTRIBUTION

1-2 DAYS

TROUBLES:

- Two-month delay due to Covid-19 pandemic;
- Window of 2-3 months for receiving the LoA;
- Shipping and customs troubles for extra-EU countries (New Zealand, Israel...);
- Material to be sent twice in a couple of countries: importance to have extra-material (samples and consumables).

Valitest

Consumables

Probes and primers were provided by the organizers as a mixture and dispatched with the panel of samples thus lowering the possibility of errors during preparations of reaction mixtures and lowering the required amount of work of the participants.

Provided consumables need to be tested for homogeneity and stability!

Valitest

Technical instructions

Technical sheet

provided with shipped samples

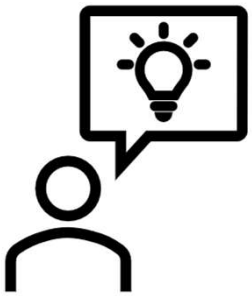
- description of tests and other useful information;
- description of panel and the scheme of TPS;
- detailed protocol of each test;
- list of controls.

Recommend maximum adherence to protocols?

QUESTIONS

- *Would you recommend maximum adherence to protocols in the technical instructions?*

Write keywords on information to be provided in technical sheet



Technical instructions

Technical sheet

provided with shipped samples

- description of tests and other useful information;
- description of panel and the scheme of TPS;
- detailed protocol of each test;
- list of controls.

Recommend maximum adherence to protocols?

PROS: avoid deviations

CONS: robustness not checked

Technical instructions

➢ Extraction

Appendix 5 – Technical sheet

APPENDIX 1 - Molecular tests – Extraction

Consumables
RNeasy Plant Mini kit (Qiagen cat no 74904)
96-100% ethanol

IMPORTANT: Perform a single extraction per sample.

Reprocessing of the samples for the molecular tests

Samples packaged in 3 mL bottles: these samples must be rehydrated with **0.5 mL of RNase free water** per bottle, pipette up and down for several times until the aliquot appears homogeneous. The obtained liquid must be used immediately for RNA extraction. Store the remainder at ≤ -18°C.

RNA extraction

RNA extraction should be done using RNA extraction kit according to the manufacturer's instructions.
The RNA extraction kit "RNeasy® Plant Mini kit" (Qiagen) can be used on 100 µl of the liquid obtained according to the instructions given in the previous paragraph. Follow the protocol provided with the Qiagen RNeasy® Plant Mini kit to extract RNA, starting with adding 380 µL buffer RLT (already added with β-mercaptoethanol) to the sap.
At the end of the extraction, elute once with 50 µL RNase Free Water.
The RNA thus extracted should be stored at a temperature ≤ –18 °C until its use as a template for the molecular tests.

Controls

Each time an extraction is performed, the following controls should be included:

- A negative isolation control (NIC). This consist of performing a nucleic acid extraction using a known 'blank' sample that does not include target nucleic acids (e.g. uninfected plant material or clean extraction buffer)
- A positive isolation control (PIC). This consists of performing a nucleic acid extraction using a known sample that includes target nucleic acids (e.g. infected plant material)

A negative control and a positive control packaged in 3 ml bottles are provided by the TPS organizer and have to be used as extraction controls.

← Consumables

← How to rehydrate samples

← Extraction procedure

← Controls provided and how to manage them

Valitest

Technical instructions

➢ Conventional RT-PCR

APPENDIX 2 - Molecular tests – conventional RT-PCR test adapted from *Alkowni et al., 2019 (Journal of Plant Pathology 101, pages 719–723).*

Consumables

The organizer will supply the primers (premixed at the right concentration with molecular grade water) and controls (PIC, NIC, PAC and NAC).
The participant has to use its own kits/reagents, disposables and equipment.
The preliminary studies conducted by the TPS organizer used the ONE-STEP RT-PCR kit (Qiagen, cat no. 210212),

Sequences of primers

Primers	5'-3'
ToBRFV-F	AAT GTC CAT GTT TGT TAC GCC
ToBRFV-R	CGA ATG TGA TTT AAA ACT GTG AAT

Experimental protocol

Reagent	*Final concentration*	*Volume (for one reaction)*
Primer Mix A	0.2 µM 0.2 µM	12.4 µl
One step RT-PCR Buffer	1X	4 µl
dNTPs	0.4 mM	0.8 µl
One step RT-PCR Enzymes	-	0.8 µl
RNA	-	2 µl
Final volume		20 µl

Amplification program:		
Reverse transcriptase	50°C 30 min	
Initial denaturation	95°C 15 min	
Denaturation	94°C 30 s	35 cycles
Annealing	58°C 30 s	
Extension	72°C 30 s	
final extension	72°C 10 min	

IMPORTANT: Each sample is to be analysed in duplicate (i.e. two PCR wells per sample).

Controls

Controls to be included PIC, NIC, PAC, NAC

← Consumables

← Primers

← Reaction mix

← Amplification program

← Replicates

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Technical instructions

➢ Conventional RT-PCR

Interpretation of results

The specific product for ToBRFV amplification will generate a single band at 563 bp.

Expected band

Verification of the controls:

- NIC and NAC must be negative.
- PIC and PAC must generate a single band at right length.

Controls results

If these conditions are met:

- A sample test will be positive if both the duplicate produces a band of the right length.
- A sample test will be negative if both the duplicate produces no band.

Any contradictory or unclear results should be repeated.

PCR		Result	Formulation
Well 1	Well 2		
+	+	**POSITIVE**	Positive sample test
+	-	**PCR to be carried out again.** If there is again at least one positive result out of two, then the test is interpreted as positive.	
-	-	**NEGATIVE**	Negative sample test

Test items results

Technical instructions

➢ Real Time RT-PCR

APPENDIX 4 - Molecular tests Real Time:
Test adapted from ISHI-Veg, 2019 (Prophyta Annual)

Consumables

The organizer will supply the primers and probes (premixed at the right concentration with molecular grade water) and controls (PIC, NIC, PAC and NAC).
The participant has to use its own kits/reagents, disposables and equipment.
The preliminary studies conducted by the TPS organizer used the TaqMan® RNA-to-Ct™ 1-Step Kit (Thermo Fisher scientific, cat no. 4392938) or iTaq™ Universal Probes One-Step Kit (BioRad, 1725141

Sequences of primers and probes

Primers and probes	5'-3'
CaTa28 Fw	GGT GGT GTC AGT GTC TGT TT
CaTa28 Pr	6FAM - AGA GAA TGG AGA GAG CGG ACG AGG - BHQ1
CaTa28 Rv	GCG TCC TTG GTA GTG ATG TT
CSP1325 Fw	CAT TTG AAA GTG CAT CCG GTT T
CSP1325 Pr	VIC - ATG GTC CTC TGC ACC TGC ATC TTG AGA - BHQ1
CSP1325 Rv	GTA CCA CGT GTG TTT GCA GAC A

Experimental protocol

Reagent	*Final concentration*	*Volume (for one reaction)*
Primers and probes Mix B	0.15 µM	7.5 µl
	0.1 µM	
	0.15 µM	
	0.15 µM	
	0.1 µM	
	0.15 µM	
2X master mix	1X	10 µl
RT enzyme mix	1X	0.5 µl
RNA	-	2 µl
Final volume		20 µl

IMPORTANT: Each sample is to be analysed in duplicate (i.e. two PCR wells per sample).

Controls

Controls to be included PIC, NIC, PAC, NAC

← Consumables

← Primers and probes

← Reaction mix

← Replicates

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Technical instructions

➢ Real Time RT-PCR

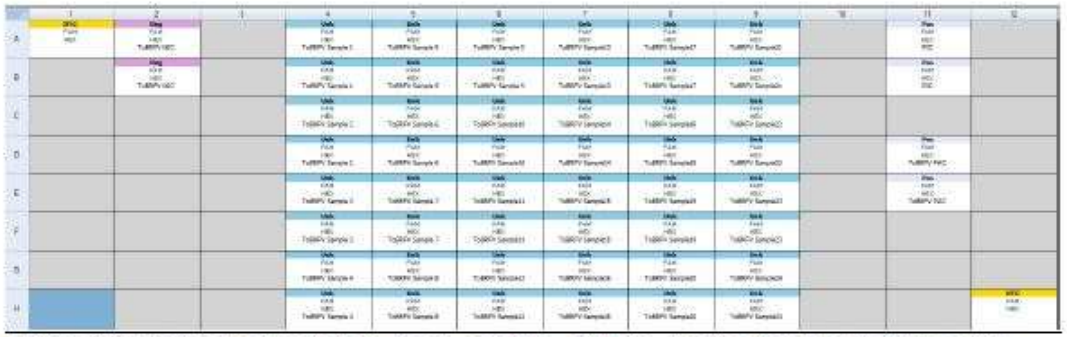

Figure 1-Schematic representation of an example of plate loading for the Real-time PCR amplification of ToBRFV

Example of plate loading

Amplification program:

Reverse transcriptase	48°C 15 min	
Initial denaturation	95°C 10 min	
Denaturation	95°C 15 s	40 cycles
Annealing	60°C 1 min	

Amplification program

Interpretation of results

The specific product for ToBRFV will generate two exponential amplification curves, the cycle cut off value for the target is set at 35.

Verification of the controls:

- NIC and NAC must be negative (Ct > 35).
- PIC and PAC must generate two exponential curves with Cts below or equal to 35.

If these conditions are met:

- A sample test will be positive if both the duplicates produce exponential amplification curves with Cts below or equal to 35.
- A sample test will be negative if both the duplicate produces no curves, no exponential curves or curves with Ct > 35.
- Any contradictory or unclear result should be repeated.

Controls results, cycle cutoff value

Test items results

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Collection of results

organizer

participants

DEADLINE

Reporting formats:

- dates of arrival of samples;
- conditions of samples at arrival;
- conditions of storage until testing;
- dates of starting and finishing of the test;
- source of consumables;
- results of testing;
- ease of use, equipment;
- deviations from protocols.

Collection of results

- **Invited potential participants: 96**
  - **Including the Valitest consortium and Euphresco participants**
- **Participants who responded: 37 (38%)**
- **Selected participants: 36 (97%)**

- **Participants who performed analysis: 34**

2 participants did not succeed in participating to the TPS due to COVID-19 emergency

- **Participants who submitted results: 34 (100%)**

QUESTIONS

- *Why do you think deadlines for providing results by participant laboratories are important?*

Analysis of results

1) Assessment of valid datasets

- analysis on data from controls (PIC, PAC, NIC, NAC);
- negative/positive results in strong disagreement from other laboratories;
- incomplete testing (e.g. no technical repetitions);
- outlying undetermined results (n. undetermined > average undetermined + 3 σ).

2) Analysis of results → performance of tests

- performed on valid datasets from previous step;
- rates of TP, TN, FP, FN;
- repeatability and reproducibility;
- diagnostic sensitivity (DSE) and diagnostic specificity (DSP);
- other parameters.

Practical training sessions	How to analyse the results of Test Performance Studies?	30th of March to 1st of April - 9 am to 4:15 pm (one one-day session to be chosen among the 3 proposed)	MORE INFORMATION	Register (limited to 60 participants)

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Analysis of controls

		NIC	PIC	PAC	NAC
Alk	Concordant %	100%	89%	93%	96%
	Non concordant %	0%	11%	7%	0%
	Untested	0	0	0	1
Loe	Concordant %	96%	92%	96%	100%
	Non concordant %	4%	8%	4%	0%
ISH	Concordant %	76%	100%	100%	85%
	Non concordant %	24%	0%	0%	12%
	Untested	0	0	0	1
	Av Cq ± sd	36.28 ± 3.27	15.32 ± 3.05	19.94 ± 2.75	38.72 ± 3.02
M&W	Concordant %	79%	100%	97%	94%
	Non concordant %	21%	0%	3%	3%
	Untested	0	0	0	1
	Av Cq ± sd	36.53 ± 3.17	15.68 ± 2.65	20.28 ± 3.43	39.23 ± 2.04
Pan	Concordant %	87%	97%	97%	93%
	Non concordant %	10%	0%	0%	0%
	Untested	0	0	0	1
	Av Cq ± sd	36.81 ± 3.10	18.05 ± 3.79	22.58 ± 2.15	39.92 ± 0.37
Total	Concordant	**131**	**145**	**146**	**141**
	Non concordant	**19**	**5**	**4**	**5**
	Concordant %	**87%**	**97%**	**97%**	**94%**
	Non concordant %	**13%**	**3%**	**3%**	**3%**
	Untested	**0**	**0**	**0**	**4**

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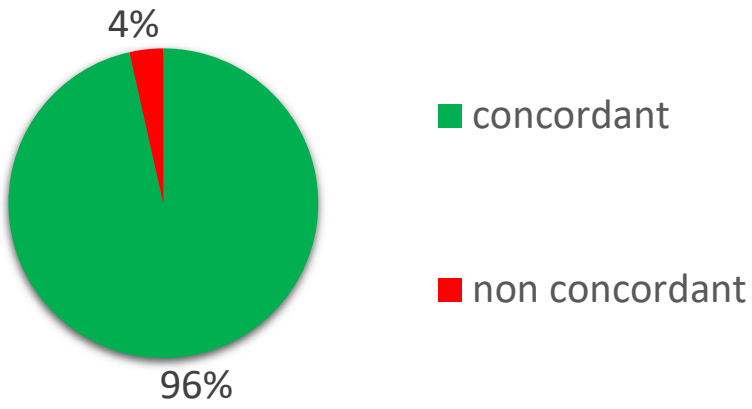
QUESTIONS

- *Which datasets do you think should be excluded from analysis of results based on the analysis of controls?*

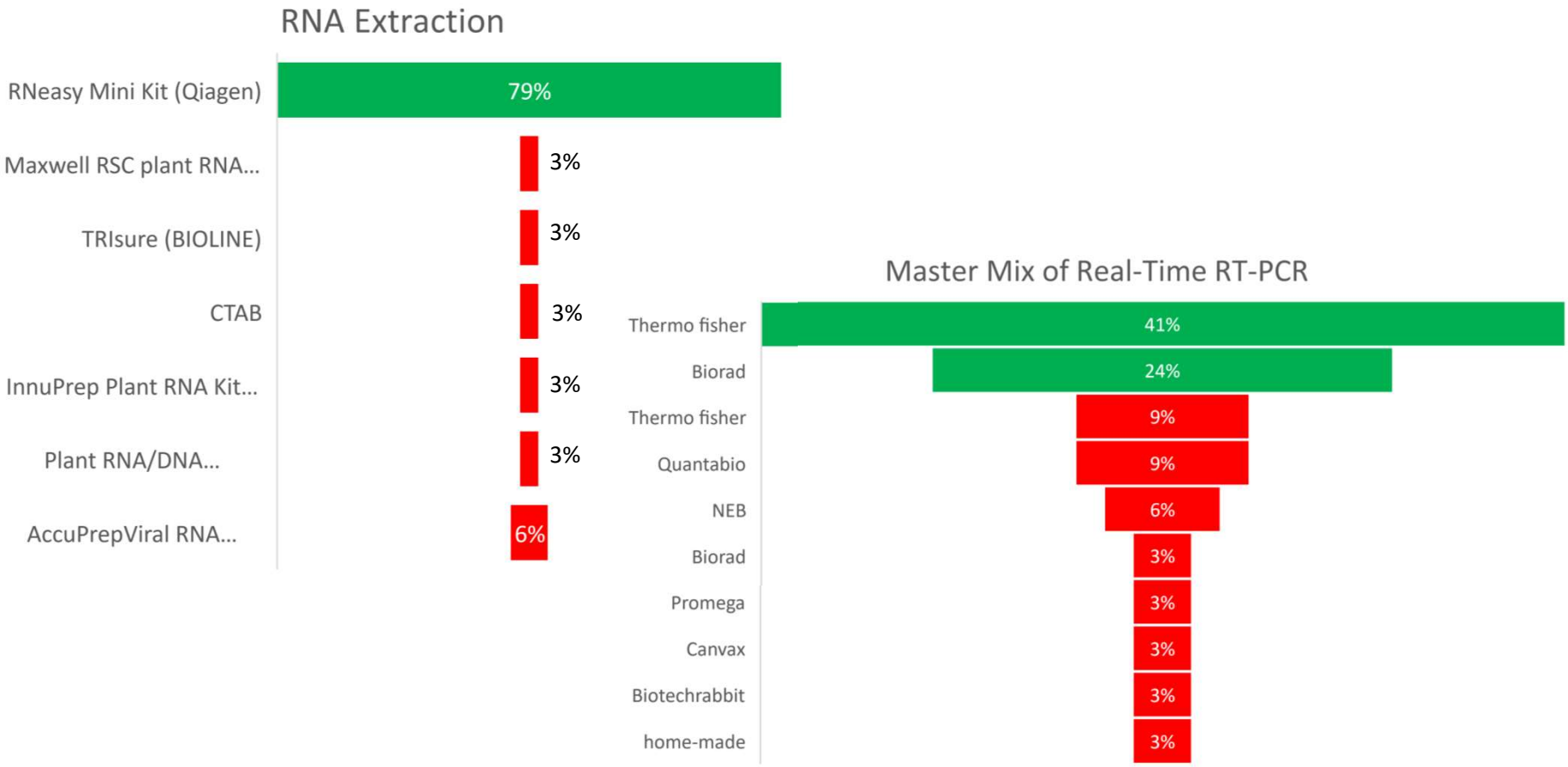
Valid datasets

- analysis on data from controls (PIC, PAC, NIC, NAC);
- negative/positive results in strong disagreement from other laboratories;
- incomplete testing (e.g. no technical repetitions);
- high number of undetermined results (n. undetermined > average undetermined + 3 σ).



Protocols	number of data set	number of valid data set	percentage
Alkowani et al., - ALK	27	22	81%
Loewe (Rodriguez-Mendoza et al.,) - Loe	26	21	81%
Conventional RT-PCR	**53**	**43**	81%
ISHI-Veg - ISH	34	24	71%
Menzel and Winter - M&W	34	25	74%
Panno et al., - PAN	30	22	73%
Real Time RT-PCR	**98**	**71**	72%
Total	**151**	**114**	75%

Evaluation of deviations



Should a dataset obtained with deviations from the test protocol be considered reliable for evaluating the performance of the test?

Is it the «same test»?

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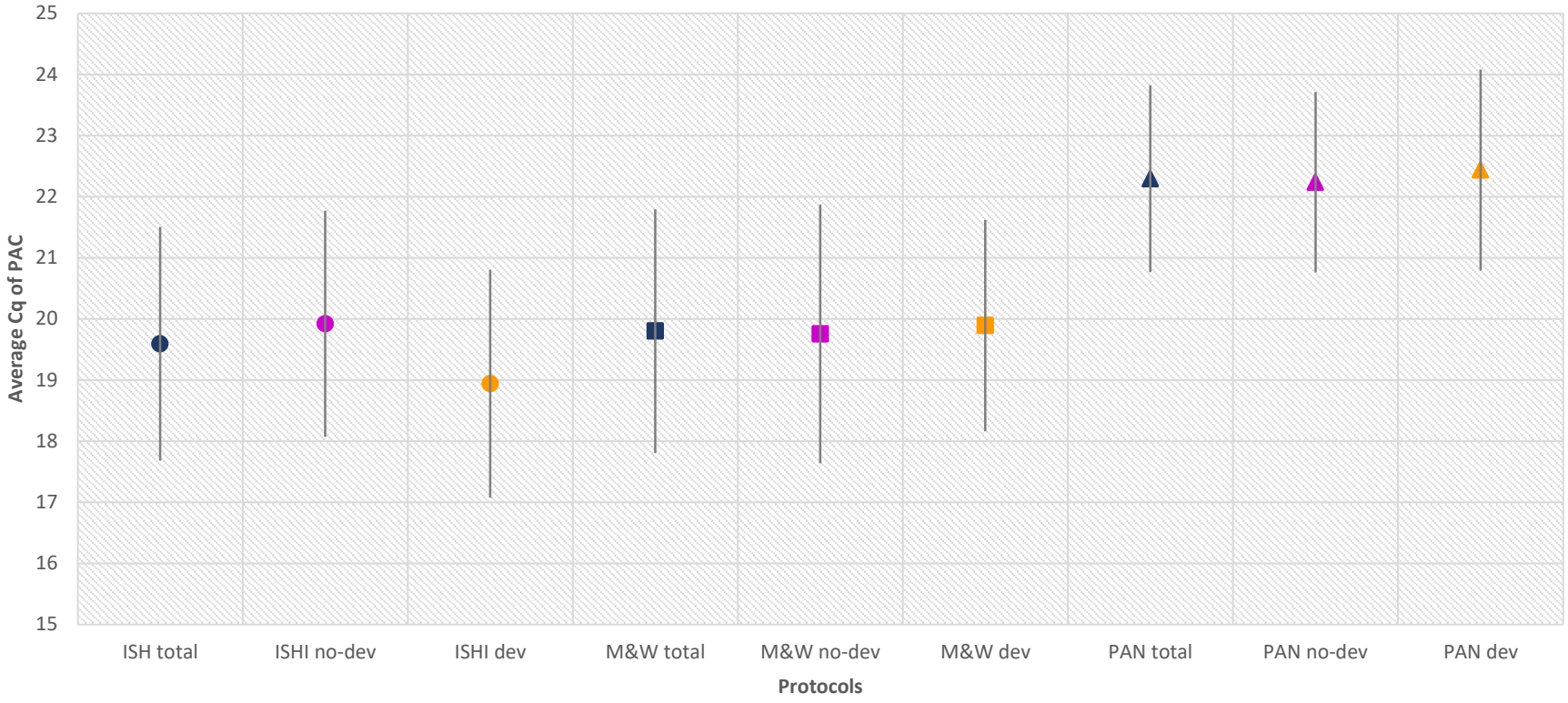
QUESTIONS

- *Which sample's results would you take into account in order to evaluate deviations in the Real Time RT-PCR master mix used?*

Evaluation of deviations

➢ Use of different Real Time RT-PCR master mixes



✓ Yes, it is the «same test»

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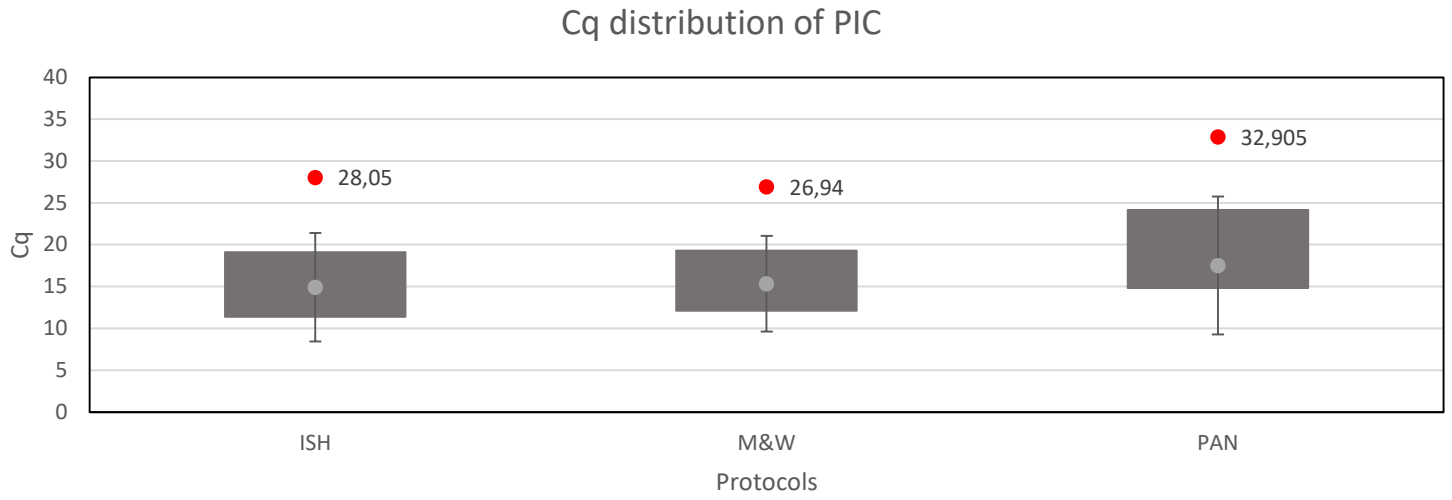
QUESTIONS

- *Which sample's results would you take into account in order to evaluate deviations in the extraction procedure?*

Evaluation of deviations

➢ Use of different RNA extraction kits

● = CTAB extraction



PIC Cq from CTAB extraction is an outlier with respect to the mean Cq distribution for all 3 Real Time RT-PCR protocols

× No, it is not the «same test»

Diagnostic parameters

Reference participant	Assigned value = Positive	Assigned value = Negative	Assigned value = Undetermined
Result obtained is Positive	TP = True Positive	FP = False Positive	TP = True Positive
Result obtained is Negative	FN = False Negative	TN = True Negative	FN = False Negative
Result obtained is Undetermined	FN = False Negative	FP = False Positive	TP = True Positive

$\%\ TP = \Sigma\ TP/N^{+} \times 100$ (concordant)

$\%\ TN = \Sigma\ TN/N^{-} \times 100$ (concordant)

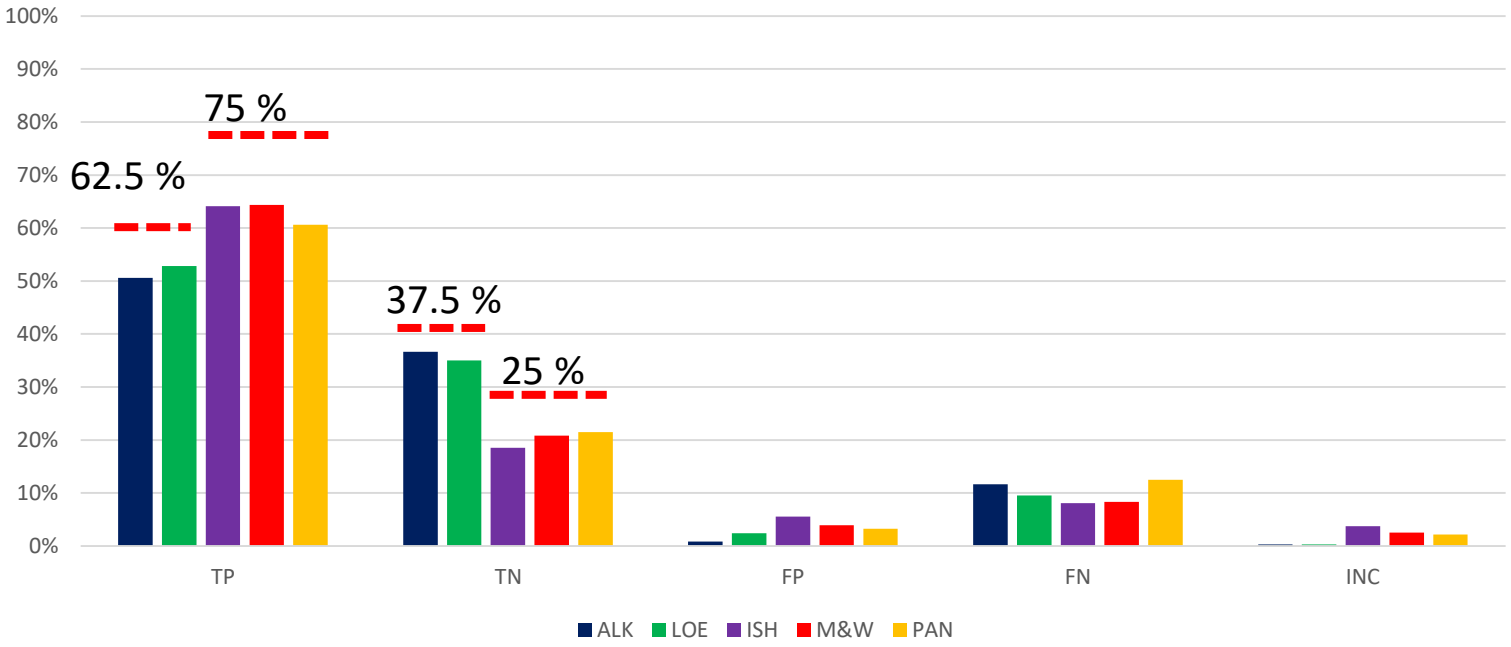
$\%\ FP = 1 - (\%\ TN) \times 100$ (non concordant)

$\%\ FN = 1 - (\%\ TP) \times 100$ (non concordant)

Questions?

Analysis of results

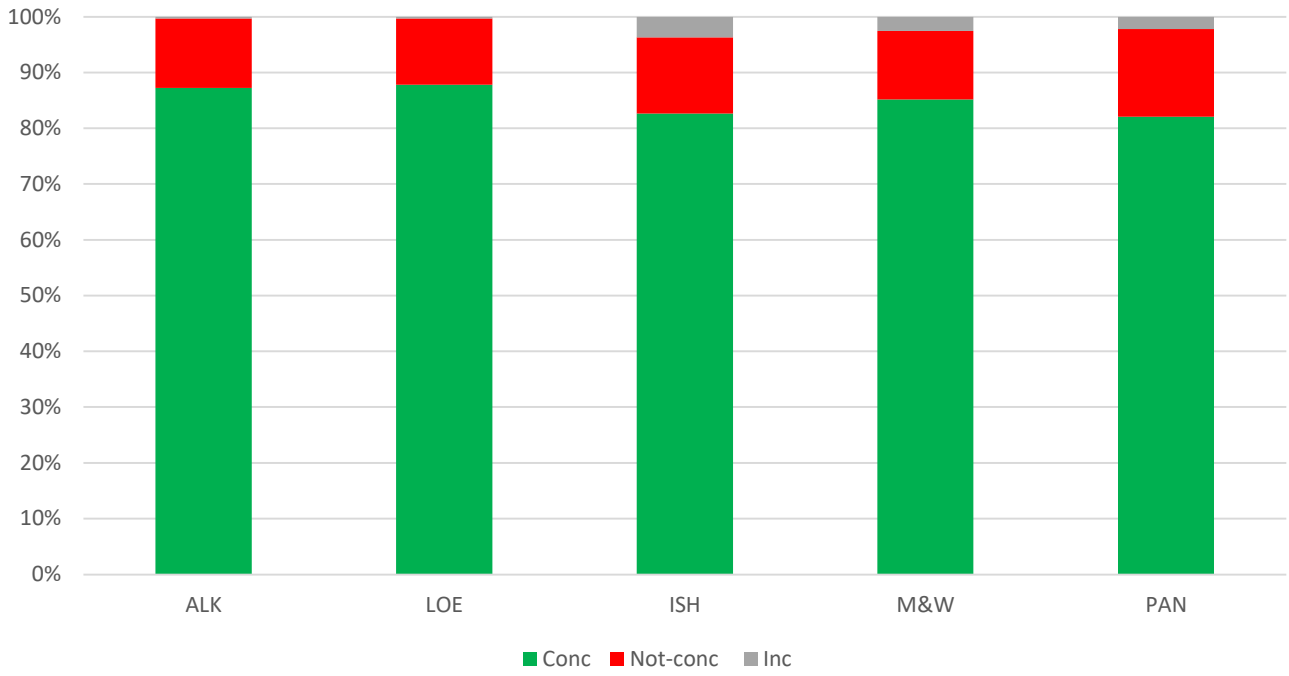
		ALK	LOE	ISH	M&W	PAN
Total data set		22	21	27	27	23
Total data points		352	337	432	432	368
TP %	TP/N %	51%	53%	64%	64%	61%
TN %	TN/N %	37%	35%	19%	21%	21%
FP %	FP/N %	1%	2%	6%	4%	3%
FN %	FN/N %	12%	9%	8%	8%	13%
INC %	INC/N %	0%	0%	4%	3%	2%



Dotted line = theoretical value of % TN and % TP according to the expected result

Analysis of results

		ALK	LOE	ISH	M&W	PAN
Concordant	TP+TN	307	296	357	368	302
Not-concordant	FP+FN	44	40	59	53	58
Concordant % (ASP)	TP+TN/N	87%	88%	83%	85%	82%
CI_{asp} 95%		66-100 %	73-100 %	67-98 %	68-100 %	59-100 %
Not-concordant %	FP+FN/N	13%	12%	14%	12%	16%



Diagnostic parameters

Diagnostic parameter	Description	Formula
Diagnostic sensitivity (DSE)	estimation of the ability of the method to detect the target	TP/TP+FN
Diagnostic specificity (DSP)	ability of the method not to detect non-targets	TN/TN+FP
Repeatability (DA)	agreement between replicates of a sample tested under the same conditions	Langdon et al., 2002
Reproducibility (CO)	agreement between measurements made on a subject under changing conditions	Langdon et al., 2002
Concordance odds ratio	variation between vs. variation within labs	DA (1-CO)/(CO(1-DA)
Analytical sensitivity (POD)	dilutions corresponding to a 50% or 95% probability of detection (LOD50 and LOD95)	binomial generalized linear model (bGLM)
Accuracy (ACC)	ability of the method to correctly evaluate the presence or absence of the pathogen	TP+TN/TP+TN+FP+FN

All diagnostic parameters have to be expressed with a confidence interval (CI) at $p < 0.05$ (95%)

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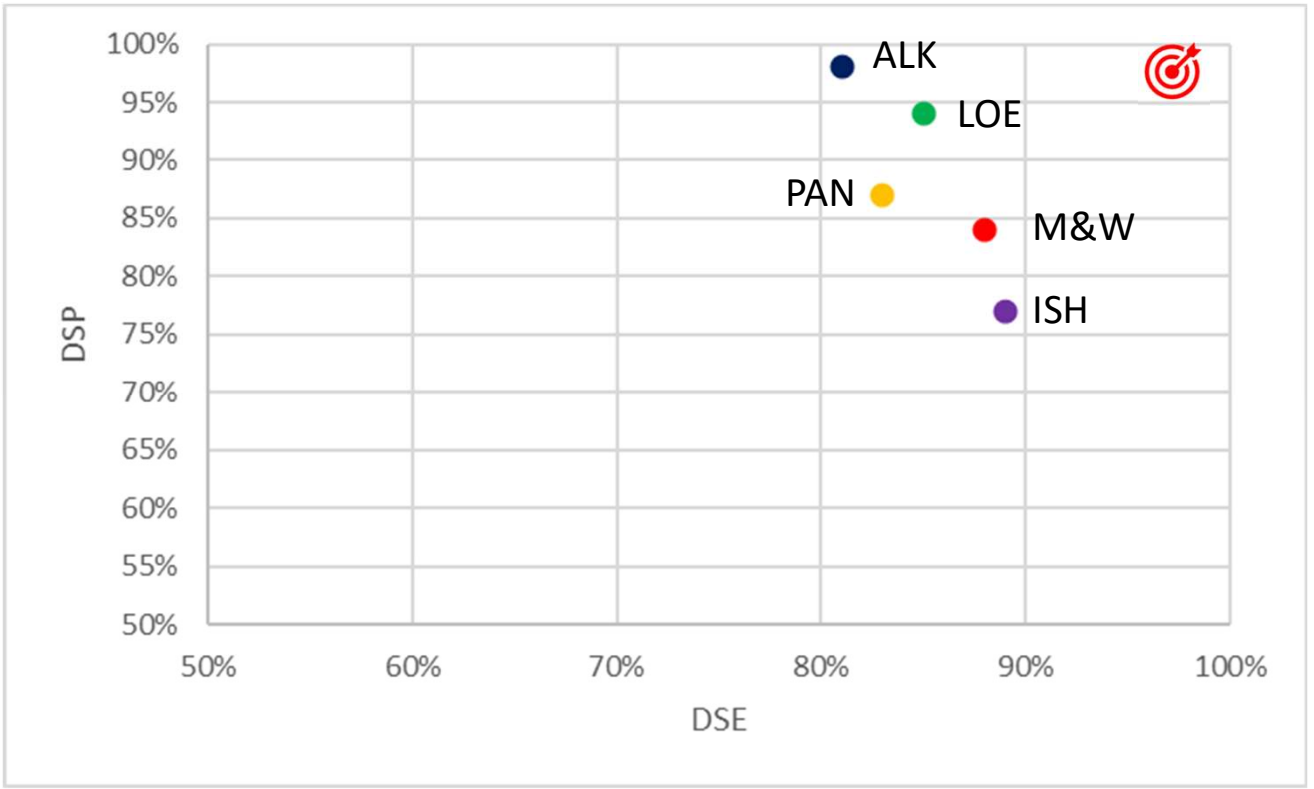
Analysis of results

Diagnostic parameter	Formula	Alk	Loe	ISH	M&W	Pan
Total data set		22	21	24	25	22
Total data points (= N)		351	336	368	389	344
Concordant	TP+TN	307	296	323	344	293
Not-concordant	FN+FP	44	40	45	45	51
Accuracy % (= ACC)	TP+TN/TP+TN+FP+FN	87%	88%	88%	88%	85%
CI_{RAC} 95 %		66-100 %	73-100 %	66-100%	66-100%	54-94 %
p-Value Fisher RAC		NS	NS	NS	NS	NS
Diagnostic sensitivity (=DSE)	TP/TP+FN	81%	85%	88%	88%	84%
CI_{DSE} 95 %		43-100 %	57-100%	59-100%	56-100 %	48-100 %
p-Value Fisher DSE		NS	NS	NS	NS	NS
Diagnostic specificity (=DSP)	TN/TN+FP	98%	93%	86%	89%	90%
CI_{DSP} 95 %		95-100 %	88-99 %	80-92 %	79-99 %	74-100 %
p-Value Fisher DSP		NS	NS	NS	NS	NS
Probability of detection (= POD)		3.4	3.3	4.6	5	4.2
Diagnostic odd ratio (= DOR)	(TP/FP)/(FN/TN)	0.101	0.377	1.255	0.941	0.591
Positive predictive value (= PPV)	TP/TP+FP	98%	96%	95%	96%	96%
Negative predictive value (= NPV)	TN/TN+FN	76%	79%	71%	72%	65%
Positive likelihood ratio (= PLR)	DSE/1-DSP	40.5	12.14	6.29	8.00	8.40
Negative likelihood ratio (=NLR)	DSP/1-DSE	5.16	6.20	7.17	7.42	5.63
Repeatability (= DA)	Langton et al., 2002	88%	81%	78%	79%	82%
CI_{DA} 95 %		78-98 %	68-94 %	65-90 %	66-92 %	72-91 %
Reproducibility (= CO)	Langton et al., 2002	87%	81%	71%	75%	80%
CI_{CO} 95 %		85-90 %	82-79 %	69-74 %	72-78 %	78-83 %
Concordance odd ratio (=COR)	DA(1-CO)/CO(1-DA)	1.05	1.02	1.19	1.13	1.02

Diagnostic sensitivity (DSE) and diagnostic specificity (DSP)

	ALK	LOE	ISH	M&W	PAN
Diagnostic sensitivity (DSE) TP/TP+FP	81%	85%	89%	88%	83%
CI_{DSE} 95%	43-100%	57-100%	63-100%	61-100%	48-100%
Diagnostic specificity (DSP) TN/TN+FN	98%	94%	77%	84%	87%
CI_{DSP} 95%	95-100 %	88-99%	74-80%	75-93%	74-99%



Repetability (DA)

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6		Sample 7		Sample 8		Sample 9		TOT
Alk	100%	95%	97%	72%	76%	100%	100%	91%	61%	88%
Loe	95%	79%	91%	50%	75%	100%	100%	91%	49%	81%
ISH	65%	62%	50%	73%	100%	100%	92%	51%	77%	74%
M&W	80%	67%	49%	73%	100%	100%	100%	53%	80%	78%
Pan	87%	68%	61%	66%	100%	100%	91%	73%	74%	80%

100%
90%
80%
70%
60%

Reproducibility (CO)

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6		Sample 7		Sample 8		Sample 9		TOT
Alk	100%	95%	97%	69%	76%	100%	100%	91%	57%	87%
Loe	95%	79%	91%	47%	75%	100%	100%	91%	49%	81%
ISH	65%	62%	49%	72%	100%	100%	89%	50%	77%	74%
M&W	80%	67%	49%	72%	100%	100%	100%	52%	79%	78%
Pan	87%	68%	60%	66%	100%	100%	90%	71%	73%	80%

100%
90%
80%
70%
60%

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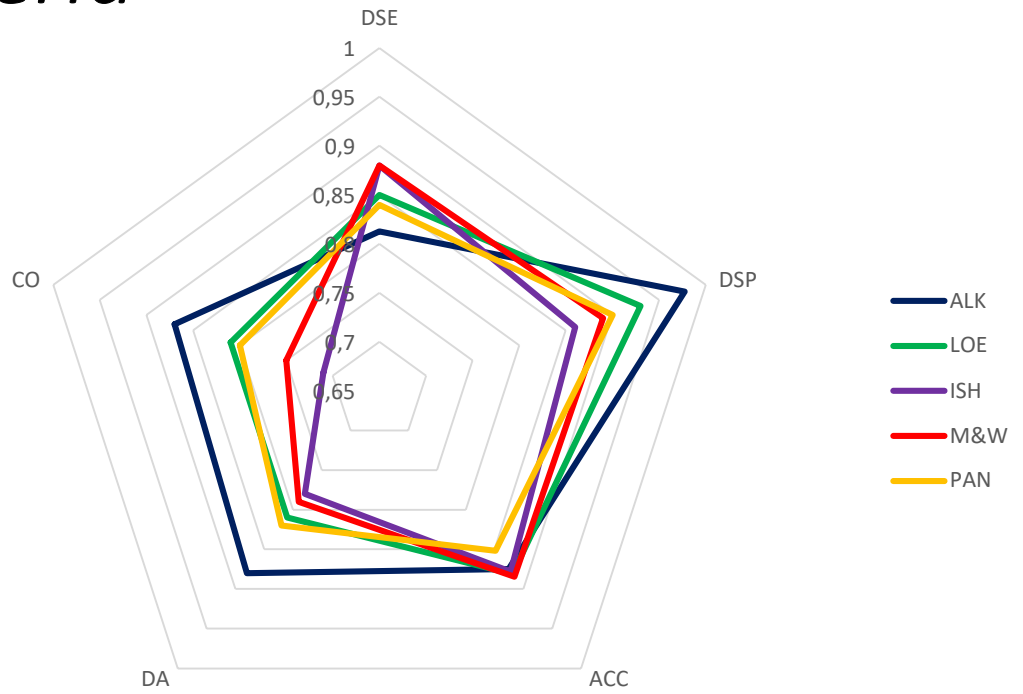
Concordance odd ratio (COR)

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Total
ALK	Inf	1	1	1.134657	1.026026	Inf	Inf	0.999378	1.158529	1.053098
LOE	1	0.99656	0.998776	1.122399	1.027581	Inf	Inf	0.999311	1.027325	1.024565
ISH	1.069354	0.982567	1.047353	1.5	Inf	Inf	Inf	1.00945	1.540385	1.191518
M&W	0.988142	0.995222	1.038865	1.317895	Inf	Inf	Inf	1.057897	1.415614	1.135606
PAN	0.988075	0.993357	1.034615	1.034271	Inf	Inf	1.02924	1.060002	1.023491	1.023845

Five main criteria



QUESTIONS

- *Based on all these results, do your think some tests show insufficient performance?*

Preparation of TPS report - PM7/122

- The reports should be clear and comprehensive and include data covering the aggregated results of all participants as well individual results. The data are anonymized but each laboratory should receive information allowing identification of its results.

- Reports should be made available to participants within planned timescales. The organizer should have a policy for the use of reports by individuals and organizations (results of a TPS might be for example the property of a research project).

Preparation of TPS report - PM7/122

TPS report should include:

- Name and contact details of the organizer, staff involved, date of issue, unique identification of report;
- clear description of the test samples used, including details of the sample preparation and homogeneity and stability testing;
- statistical data and summaries, including assigned values and range of acceptable results and graphical displays;
- procedures used to establish any assigned value;
- information about the design and implementation of the interlaboratory comparison;
- procedures used to statistically analyse the data;
- advice on the interpretation of the statistical analysis and comments or recommendations, based on the outcomes of the interlaboratory comparison.

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Thank you for your attention!

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