ISTA
Seed Quality Assurance

ISTA Secretariat
Richtiarkade 18, 8304 Wallisellen, Switzerland
Phone: +41 44 838 60 00 | Fax: +41 44 838 60 01
Email: ista.office@ista.ch
www.seedtest.org

Validation of seed health methods and organisation and analysis of interlaboratory comparative tests (CT)

Any hardcopy of this document is not controlled copy

CREATED	REVIEWED	APPROVED
07.09.2019	JULY 2019/ JULY 2021	30.08.2021
SEED HEALTH COMMITTEE	SEED HEALTH COMMITTEE	SEED HEALTH COMMITTEE CHAIR

Scope

In the life cycle of a method (Figure 1), characterization and validation are an essential part before routine use.

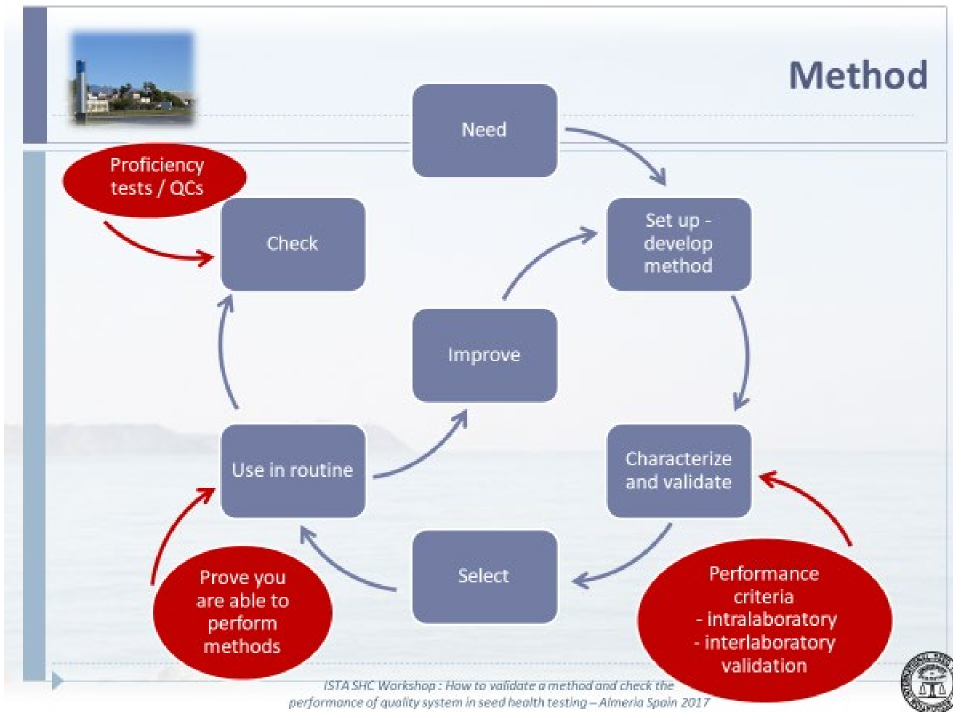

Fig 1: Life cycle of a method

Method validation is aimed for new methods, modifications of an existing method, comparison of a new method with an existing one. ISTA Seed Health Committee drafted these guidelines in collaboration with ISTA Stat Committee for tests organisers who want to validate Seed health methods by studying performance criteria and organise interlaboratory comparative tests (CT).

Different types of results are expected based on the class of pathogen or the method used to detect the pathogen.

- For detection of fungi, each seed may be tested individually on media or blotters and results are generally expressed as the number of infected seeds per the number of seeds tested or as a percentage infection. These are quantitative results.
- For detection of bacteria or viruses or some fungi or nematodes, seeds are tested using pools containing X number of seeds per subsample. Results are expressed as the number of infected subsamples (or positive subsamples) detected within a total number of subsamples tested. These are qualitative results.

Responsibility

The Seed Health Committee is responsible for validating methods and organising inter-laboratories tests for validation of methods and the evaluation of their results. Method validation and CT can also be organised by stakeholders and validation reports and method proposals submitted to ISTA.

Abbreviations

CT – Inter-laboratory (comparative) tests for validation of methods
StatCommittee – Statistic Committee
SHC – Seed Health Committee

Performance criteria

See below for seed samples used to determine performance criteria.

ANALYTICAL SENSITIVITY:

Definition: The limit of detection (LOD) is usually defined as the lowest quantity or concentration of a pest that can be reliably detected with a given analytical method.

The analytical sensitivity of an assay which is part of the method can in some cases be measured. An example is the use of PCR where analytical sensitivity of the assay can be measured by dilution of DNA and the analytical sensitivity of the method by spiking seed samples with different levels of pests. In all cases sufficient dilutions should be performed until one where the pest is not detected. In Figure 2 the analytical sensitivity will be 10^3 cfu/mL as at 10^2 cfu/mL the pest was not repeatably detected.

Analytical sensitivity should be determined on a minimum of 3 replicates, 5 preferably.

Concentration (cfu/mL)	0	10^1	10^2	10^3	10^4	10^5	10^6
Results rep 1	-	-	+	+	+	+	-
Results rep 2	-	-	-	+	+	+	-
Results rep 3	-	-	+	+	+	+	-

Fig 2: Determination of analytical sensitivity

Method	Qualitative by pools (1 to x pools)	Quantitative % or number	Type of data	Process	No of replicates	Analytical sensitivity of the assay	Analytical sensitivity of the method
Plating (bacteria)	x		No. of cfu	10 x dilution series of bacteria	3 for each dilution	in buffer for the media	by spiking seed extracts or samples
Grow out (bacteria, fungi)	x		No. of seeds	No. of seeds	3 for each level	NA	by spiking contaminated seeds in healthy seed samples
Serological (bacteria, viruses)	x		No. of cfu, dilution	10 x dilution series of bacteria or viruses	3 for each dilution	in buffer for the antisera	by spiking seed extracts or samples
Molecular (seed wash)	x		No. of cfu, dilution	10 x dilution series of bacteria or viruses	3 for each dilution	in buffer for the primers	by spiking seed extracts or samples
Plating (fungi)		x	No. of seeds	No. of seeds	3 for each level	NA	by spiking contaminated seeds in healthy seed samples
Seed wash (fungi, nematodes)	x		No. of spores, nematodes	No. of spores/nematodes	3 for each level	NA	by spiking spores/nematodes in healthy seed samples
Direct observation of seeds, embryos		x	No. of seeds	No. of seeds	3 for each level	NA	by spiking contaminated seeds in healthy seed samples

Table 1: example of data used to measure analytical sensitivity

A full measurement of analytical sensitivity is not necessary for confirmation of suspect isolates or symptomatic plantlets. In these cases, a measurement of minimum and maximum concentration to use will be necessary.

In the validation process, it is possible to measure analytical sensitivity before or after diagnostic sensitivity and specificity / analytical specificity has been evaluated (see p 4-5). Determination of analytical sensitivity can be performed in only one laboratory.

When comparing a new method to an existing one, the analytical sensitivity should be = or lower than that of the existing method.

ANALYTICAL SPECIFICITY:

Definition: ability to detect target pests (inclusivity) while not detecting closely related and other organisms or samples which do not contain the target (exclusivity).

Analytical specificity is to be evaluated for all tests that are part of a method when relevant (media, pathogenicity test, PCR step, morphological detection...)

Analytical specificity should be determined on a collection that covers genetic/morphological/aggressiveness diversity of targets and non-targets which can preferably be found on the same matrix/species. It is determined, when possible, on an isolate collection.

For targets, a min of 5, ideally 20 is tested and should represent diversity of the pest: geography, hosts, time of isolation…. For non-targets, isolates of same genus and/or host that reflects routine testing for the target species (if available) are used (min 5, ideally 20).

Depending on the method, it will be determined whether it is studied on a collection of isolates or on naturally/artificially infected seeds. In this case it is confirmed on a minimum number of 3 infected seed samples with different origins, background levels of saprotrophs (Table 2).

Table 2: examples of how to determine analytical specificity

Method	**Criteria validation**
Plating (bacteria)	Collection of isolates ideally 20 targets/20 non targets
Grow out (bacteria, fungi)	Collection of isolates ideally 20 targets/20 non targets and/or infected seeds
Serological (bacteria, viruses)	Collection of isolates ideally 20 targets/20 non targets
Molecular (seed wash)	Collection of isolates ideally 20 targets/20 non targets
Plating (fungi)	Collection of isolates ideally 20 targets/20 non targets Identification on media based on morphological characteristics (description)
Seed wash (fungi, nematodes)	Collection of isolates ideally 20 targets/20 non targets Identification on media based on morphological characteristics (description)
Direct observation of seeds, embryos	Collection of isolates ideally 20 targets/20 non targets Identification on media based on morphological characteristics (description)

Determination of analytical specificity can be performed in only one laboratory. A complementary measurement can be performed during CTs.

SELECTIVITY:
Definition: ability to without too much variation, detect the target pest of interest within different seed matrices either belonging to the same crop / plant species or to different plant species.

It is the responsibility of the organiser to determine what parameters are important to test for selectivity. For example, it could be variety or species.

It can be performed in a single lab on a minimum of 3 replicates per matrix on which the pest is detected. In every case, it should be performed on the whole method.

Pathogenicity tests are not of concern as a susceptible variety is described.

ROBUSTNESS:
Definition: ability to not vary according to small variations of parameters in the method.

It is the responsibility of the organiser to determine what parameters are important to test for robustness. For example, it should be parameters that could vary between labs as source of reagents, thermocyclers, substrate, light conditions…It can be performed in a single lab on a minimum of 3 replicates per parameter. It can be also demonstrated through reproducibility. In every case, it should be performed on the whole method.

DIAGNOSTIC SENSITIVITY AND SPECIFICITY

Definition: The ability of a method to produce neither false negatives (diagnostic sensitivity) nor false positives (diagnostic specificity). The combination of the two criteria corresponds to accuracy

Diagnostic sensitivity and diagnostic specificity are used on seed samples by comparison to an expected value. The diagnostic sensitivity allows to verify that there are no false negatives and diagnostic specificity that there are no false positives. Accuracy is the combination of the diagnostic sensitivity and diagnostic specificity.

Diagnostic sensitivity and specificity are evaluated by applying the whole method, including all steps (PCR, biotest...).

A minimum of 3 levels of contamination are tested:

- 1 healthy lot

- 1 infected lot (medium infection/contamination slightly above the detection limit)

- 1 highly infected/contaminated lot

The number of replicates for each level should be a minimum of 3 but can be increased especially for the medium level in order to guarantee the statistical analysis of the results.

The analysis can be carried out on naturally infected or artificially contaminated seeds if naturally infected seeds cannot be found.

The norm NF EN ISO 16140 will be used to determine the performance criteria of the method through diagnostic sensitivity, specificity and accuracy. The analysis consists of a comparison between the expected result (known samples, validated by homogeneity test, based on mean results, on reference method to be determined by organiser) and the obtained result in all participating laboratories. These comparisons record positive and negative agreement or positive and negative deviation (Table 3).

Table 3: positive and negative agreement or positive and negative deviation

	expected result + (target)	expected result - (non target)
Obtained result +	positive agreement +/+ (PA)	positive deviation -/+ (PD)
Obtained result -	negative deviation +/- (ND)	negative agreement -/- (NA)

This definition allows the calculation of sensitivity, specificity, accuracy and reproducibility according to the following mathematical formulas:

Sensitivity = $\Sigma PA/(\Sigma PA+\Sigma ND)x100$

Specificity = $\Sigma NA/(\Sigma NA+\Sigma PD)\times 100$

Accuracy = $(\Sigma NA+\Sigma PA)/(\Sigma PA+\Sigma NA+\Sigma PD+\Sigma ND)\ x100$

PA = positive agreement

ND = negative deviation

NA = negative agreement

PD = positive deviation

A diagnostic sensitivity of 100% shows that the method / laboratory always detects the target pathogen: there were no false negatives.

A diagnostic specificity of 100% shows that the method / laboratory does not give a positive result for a sample that does not contain the target pathogen: there were no false positives.

Accuracy is the synthesis of the two performance criteria. An accuracy of 100% shows that no false positive or negative results have been obtained.

Ideally it should be 100%, meaning that the method gives no false positive, and no false negative. The organiser determine the realistic threshold % below which the method is not validated. Generally, a performance criteria >95% is required. When comparing a new method to an existing one, the performance criteria should be = or > than the existing method.

For non homogeneous seed samples it is not possible to calculate diagnostic sensitivity or specificity. In this case, the organiser will determine if the obtained result on targets and non-targets corresponds to expected results, using seed calc for % infection and prob tool for expected number of infected subsamples.

REPEATABILITY:
Definition: accord between independent results for the same method in the same lab, by the same analyst, with the same equipment, on the same samples, during a short period.

Repeatability should be performed with samples that are just above the limit of detection when possible (collection of isolates, spiked seed samples) or at low levels of contamination (naturally contaminated seed lots) or at the recommended concentration (for confirmation tests like PCR or pathogenicity).

For collection of isolates, it can be combined with analytical specificity determination, considering that isolates are replicates of the targets and non-targets groups. In this case a minimum of 10 replicates should be used.

For seed samples, a minimum of 3 replicates at different levels of contamination should be used.

Repeatability can be measured at the same time as diagnostic sensitivity and specificity studies. CTs give information on repeatability in each participating lab.

REPRODUCIBILITY (CTS):
Definition: accord between independent results with the same samples and method in different conditions (analyst, equipment, lab).

Reproducibility should be performed with samples that are just above the limit of detection when possible (collection of isolates, spiked seed samples) or at low levels of contamination (naturally contaminated seed lots) or at the recommended concentration (for confirmation tests like PCR or pathogenicity).

Reproducibility can be measured at the same time as repeatability. It is performed as CTs.

Number of participants for CTs:

- For quantitative results, a minimum of 3 participants is required and should be 8 if possible
- For qualitative results a minimum of 3 participants are required

Samples:

See below for levels of contamination and characterization.

Test plan for CTs (to be submitted if submission to ISTA is at an early stage of the method validation process)**:**

A test plan should be provided by the organiser, describing the background, the performance criteria determined during validation studies, the detailed protocol/method, the number of participants, the type of samples (DNA, isolates, seeds…), how characterisation of samples is planned (homogeneity and stability), statistical analysis planned and templates for notation sheets.

A test plan should not indicate to participants the details about samples characteristics in term of number of targets/non-targets or levels of contamination.

Templates for notation sheets are provided in order to record all necessary information about lab, samples tested, raw results and final result per sample of the lab. Templates will need to be adapted depending of method used.

A blind coding of samples and participants should be planned to avoid collusion.

Statistical analysis:

- Results will need to be analysed without outliers (unexpected results, results too far from expected ones) if it is linked to a specific lab. Use side by side box plots (Fig 2 below), to look for outliers.

Statistical analysis for **qualitative results** (indicative to help the organiser analyse their results):

- Repeatability and reproducibility: Following the terminology used in the publication of Langton *et al*., 2002.
  - Repeatability and reproducibility are calculated on similar samples, so should be calculated for each level of infection or for target versus non-target organisms
  - Repeatability is called accordance. For each lab it is expressed as the probability that 2 samples give the same result, then the probability is averaged over all laboratories: count for each lab the numbers of pairs both + or – (i.e. number of accords) and number of possible pairs (total number of possible accords)
    - The number of pairing/accords between n items is : n (n-1) / 2
    - Accordance=number of accords/number of possible accords in one laboratory
    - See example Table 2
  - Reproducibility is called concordance. Count between laboratories the numbers of pairs both + or – (i.e. number of accords) and number of possible pairs (total number of possible accords)
    - The number of pairing / accords between n items is : n (n-1) / 2
    - Concordance=number of accords/number of possible accords between laboratories
    - See example Table 3
- Ideally repeatability and reproducibility should be 100%. The organiser should determine the realistic threshold % below which the method is not validated. Generally, a performance criteria >90% is required. When comparing a new method to an existing one, the performance criteria should be = or > than the existing method.

- Analysis depending on % of infection: qualitative results:
  - Useful for low contaminated samples where the number of positive subsamples which will be distributed is not precise, because distribution is not homogeneous.
  - In this case, homogeneity test will be used to give precision on determination of the expected value i.e. the % of infection of the sample. This % will be determined with seed calc (Fig. 3). Statistical analysis will be done on a probability to have from X to Y samples positives / total analysed (see example of Excel tool developed Fig. 4)

- For qualitative results (subsample identified as positive or negative), the counting of colonies or number of lesions or nematodes or Ct value for PCR… will be indicative (for a level of contamination for example). It is not expected to do statistical analysis on this aspect.

Statistical analysis for **quantitative results** (fungi: % contamination, number of contaminated seeds) (indicative to help the organiser analyze their results):

- Analysis of results
  - Box Plot can be used to confirm that the method is able to differentiate the different levels of contamination of seed samples
  - ANOVA on transformed / not transformed data will be an interim solution. A normal distribution is not compulsory, but it is important to have stable variance. It is used to determine if there is a lab or method effect.
  - GLMM (general linear modified model) targeted is better, ISTA will develop a statistical programme

- Repeatability and reproducibility:
  - ISTA ISO 5725 tool will be an interim solution.
  - GLMM targeted is better, ISTA will develop a statistical program

Report:

- For validation studies: see ISTA method validation programme
- Reports should include description of all performance criteria listed in these guidelines and the way they were obtained. The first parts of the report until Material and Methods part corresponds to the test plan. Reports should include the raw data in annex. The final validated protocol/method should be attached.

CHARACTERIZATION OF SEED SAMPLES

The seed samples will be used to show that the method performs on the matrix (seed species), on different lots that differ in their origin or saprophytic (saprotrophs) content and on different levels of contamination. Seed samples will be used to determine all performance criteria during the validation process.

Seed lots:

Seed lots used for method validation can be either naturally infected or artificially contaminated. When possible, naturally infected seed lots are preferred.

Artificial contamination can be achieved:

- For viruses by mixing seeds with ground contaminated tissues
- For bacteria by using a vacuum to infiltrate a bacterial suspension into individual seeds, (with or without damaging the teguments)
- For fungi, by immersing seeds in a spore suspension or plating seeds on a fungus colony under hydric restriction (use of Mannitol to decrease water availability)
- For nematodes by spiking seeds with suspension of nematodes

Stability of infection is lower in case of artificially contaminated seeds and should be checked carefully when artificial contamination is performed.

A pretest will be necessary to determine the level of infection, i.e., the expected results of subsamples.

Sample size: a minimum sample size should be indicated. The sample size will be dependant of crop and pest. It should be determined and justified by risk of transmission from seed to plant or environment, damage threshold, biology of the pest if known, or by experience on the pest-crop or similar methods.

Samples/subsamples: Subsamples will be prepared by the organisers to obtain consistent results. In comparative tests, no subsampling should be done in the participating laboratories. A good sampling procedure should be used and sufficient subsamples should be prepared at the beginning of the validation study to allow all steps to be performed: analytical sensitivity, diagnostic sensitivity and specificity, repeatability, reproducibility, homogeneity and stability tests. Samples should be prepared for all participants of the CT and 2 extra sets minimum in case of tests to be repeated or sent again.

Levels of contamination
Levels of contamination are determined based on pretests. In order to have a good estimation, it is advised to test a sufficient number of samples during the pretest.

- Healthy, no contamination (with saprotrophs if possible): all subsamples should be negative
- High infection level, all subsamples should be positive
- Low and/or medium contamination level: infection is above the detection limit of the method
    - For quantitative results, the organiser needs to be certain, based on pretests, that all low level's subsamples are infected (at least one infected seed in subsample analysed) and that infection is over the detection limit of the method. For example, a minimum of 5% infection for fungi is required to be sure that all subsamples tested will contain one infected seed.
    - For qualitative results, the organiser will perform homogeneity tests to check if all samples are positive either see just below.

For qualitative results, the organiser will determine the % of infection of the seed lot based on Seedcalc program provided in the STATCOM webpage (tools), and then determine the probability to find a number of infected samples over the number tested with the “probability tool for qualitative results” provided on the SHC webpage (tools). For example, in the fig below, during pretests, 8 subsamples of 1000 seeds were tested and 4 were positive. Seed calc allowed to determine that seeds were infected at 0.07% (Computed % in sample). In this case, for a 0.07% of contamination with 1000 seeds tested, if 3 subsamples are tested, at a probability >5%, it is possible to detect from 1 to 3 positive subsamples. In this example it would be useful to test more than 3 subsamples. If 6 subsamples were tested, at a probability >5%, it is possible to detect from 1 to 5 positive subsamples, which is more precise.

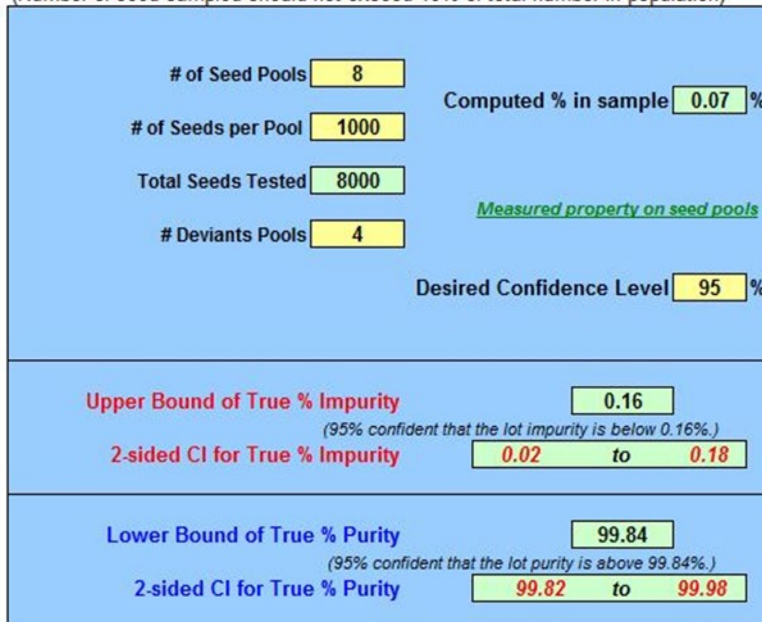

Fig 3: Use of seed calc to determine the % of infection of the seed lot (computed % in sample)

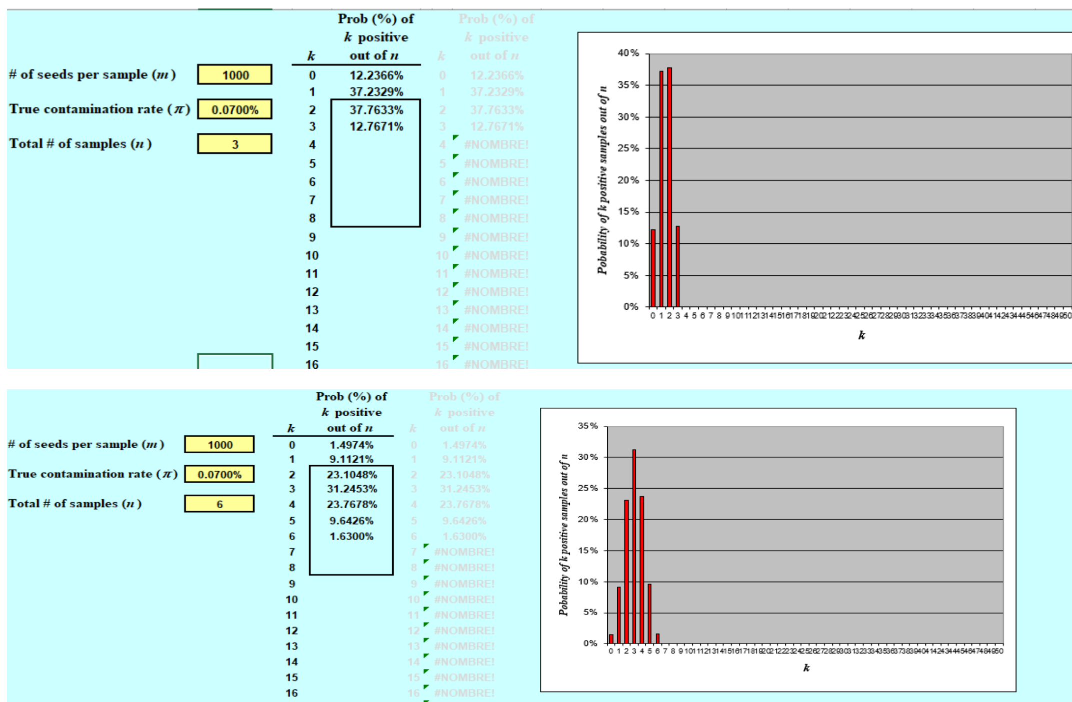

Fig 4: Use of probability tool to determine the number of expected positive samples out of total tested depending of % of infection

Number of subsamples per infection level:

- Same number of subsamples per infection level for fungi (i.e. 3 for healthy, 3 for low, 3 for high)
- Number depending on infection level when tested by pools (bacteria and viruses) with more focus on healthy and low/medium levels, i.e: more subsamples need to be tested at levels where there is more chance to have a false negative result
- A minimum of 3 subsamples per infection level for CT result will be reported

Size of subsample:

- For a new method: define the size of subsample which will depend on the detection limit of the method. The size of subsample used for validation studies will be the recommended one in the final method
- For modifications to existing methods, when a maximum subsample size is indicated in the method this subsample size should be generally used for validation process. Some modification of existing methods could consequentially change the subsample size, in this case, justification of modification of the subsample size should be given.

Homogeneity test of subsamples:

- Done to ensure a homogeneous distribution of pathogen in samples and enable an organiser to know if sample is acceptable, i.e. all subsamples will give the expected result.
- Done for each pathogen if multiple pathogens are included in the same validation.
- Completed using the method (final whole protocol) to be used in the validation
- Done by the organizing lab on 8-10 subsamples (per level per pathogen),
- Done when subsampling is completed, and subsamples are packed
- Done for methods giving a quantitative result (fungi, grow out tests): see proposed Excel file to determine if sample conforms to the required standard, i.e: is sufficiently homogeneous (Fig 1). This programme is under ISTA validation.

CALCULATION OF CONFORMITY FOR HOMOGENEITY TEST

Homogeneity test - seed health											
Sample size	400										
Subsample	1	2	3	4	5	6	7	8	9	10	
nb infected seeds	2	5	1	3	6	5	1	8	3	4	Mean
percentage of non infected seed	99.5	98.75	99.75	99.25	98.5	98.75	99.75	98	99.25	99	99.05
Sample Mean					99.05						
Tolerance					1.41						
H value					0.35						
Homogeneity check					OK						

Taille d'échantillonnage	400										
Echantillons	1	2	3	4	5	6	7	8	9	10	
nb. sem. infectées	51	52	41	53	36	42	46	29	40	45	Moyenne
pourcentage de semences saine	87,25	87	89,75	86,75	91	89,5	88,5	92,75	90	88,75	89,13
Moyenne					89,13						
Tolérance					1,41						
Valeur de H					9,28						
Réponse d'homogénéité					Out						

- An alternative is to use Hampels method to detect outliers: if no outliers are detected, the sample is considered as homogeneous. An example is given below.

MS Excel Hampels Outlier Test Example					
Lab	Lab Values (Xi)	| Xi - M |	Status		
1	12,75	1,875	OK	Median (M):	10,875
2	13,00	2,125	OK	MAD:	1,375
3	10,25	0,625	OK	5.2 X MAD	7,150
4	13,25	2,375	OK		
5	9,00	1,875	OK		
6	10,50	0,375	OK		
7	11,50	0,625	OK		
8	7,25	3,625	OK		
9	10,00	0,875	OK		
10	11,25	0,375	OK		

- For methods giving a qualitative result,
  - Homogenous distribution means that all subsamples tested in homogeneity test are positive for infected samples and negative for healthy samples
  - In some cases, for naturally infected seeds, contaminated at a low level, the distribution of the pathogen will not be homogeneous i.e: all subsamples tested will not have the expected positive result. In this case, the sample is not homogeneous, but homogeneity test can be used to determine the level of infection (in % using seed calc).
- Homogeneity test will be used to validate the expected results of subsamples
- Samples are valid for further analysis if homogeneous.
- In case of lack of homogeneity, it will be decision of the organiser to
  - Not use the samples in the validation process and choose another lot
  - Not use the subsamples in the statistical analysis
  - Use the subsamples in the statistical analysis, but take into account the lack of homogeneity in the interpretation of results

Stability test of subsamples:

- Done to be sure that the infection of the subsample does not change during the course of the validation. It is particularly important for artificially contaminated seeds where the level of contamination can decrease in time
- Done with the method (final protocol) used in the validation
- Done on a minimum of 3 subsamples (per level per pathogen) by the organiser
- Done just after the deadline for participants/or the last participant informed he started the test to complete the test.
- It must give the expected result (healthy for healthy subsamples, infected for infected seed samples).
- The same statistical tools as homogeneity test can be used to verify stability of samples
- If results show that the sample is not stable: this is an indication that unexpected or unpredicted results of validation may be due to problems with the sample and not to the lab or weaknesses in the method. In this case, it is the decision of the organiser to
  - Not use the subsamples in the statistical analysis
  - Use the subsamples in the statistical analysis, but take into account the lack of stability in the interpretation of results

References

- Association Française de Normalisation (AFNOR). 2003. Microbiologie des aliments-Protocole pour la validation des méthodes alternatives NF EN ISO 16140.www.afnor.org
- Langton, S.D., Chevennement, R., Nagelkerke, N. and Lombard, B. 2002. Analysing collaborative trials for qualitative microbiological methods: accordance and concordance. International Journal of Food Microbiology, 79, 175-181.

Annex

Annex 1: Fig 1: calculation of conformity for homogeneity test

Annex 2: Fig 2: Example of side by side box plot

Annex 3: Example of calculation of diagnostic sensitivity and specificity for a method

Annex 4: Example of calculation of diagnostic sensitivity and specificity for an assay

Annex 5: Table 2. Example of calculation of repeatability (CT for validation of Xap primers)

Annex 6: Table 3. Example of calculation of reproducibility (CT for validation of Xap pathogenicity test)

Distribution List

Seed Health Committee

Statistical Committee

Accreditation and Technical Department

Revision history

Version #	Changes
1.1	3-5 subsamples for stability tests has been changed to ”a minimum of 3 subsamples”
2.0	Layout change

ANNEX 1: FIG 1: CALCULATION OF CONFORMITY FOR HOMOGENEITY TEST

Homogeneity test - seed health											
Sample size	400										
Subsample	1	2	3	4	5	6	7	8	9	10	
nb infected seeds	2	5	1	3	6	5	1	8	3	4	Mean
percentage of non infected seed	99.5	98.75	99.75	99.25	98.5	98.75	99.75	98	99.25	99	99.05
Sample Mean					99.05						
Tolerance					1.41						
H value					0.35						
Homogeneity check					OK						

ANNEX 2: FIG 2: EXAMPLE OF SIDE BY SIDE BOX PLOT (FROM ISTA PT ON RICE 2011)

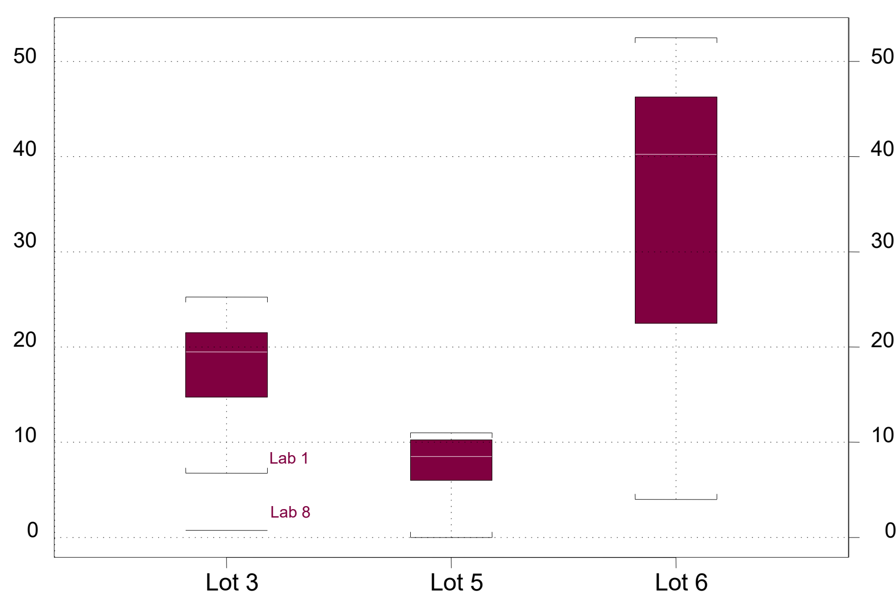

Figure 2 Participants average results of *Drechslera oryzae*

ANNEX 3: EXAMPLE OF CALCULATION OF DIAGNOSTIC SENSITIVITY AND SPECIFICITY FOR A METHOD

Qualitative analysis of the Blotter 2,4-D results (%)

			ISO 1640			Langton *et al.* 2002	
	Results expected +	Results expected -	Sensitivity	Specificity	Accuracy	Reproductibility	Repeatability
Results obtained +	48	1	100,00	95,83	98,61	95,50	97,70
Results obtained -	0	23					

ANNEX 4: EXAMPLE OF CALCULATION OF DIAGNOSTIC SENSITIVITY AND SPECIFICITY FOR AN ASSAY

Qualitative results obtained with Liu and PRI primers

			Nb of Strains	Accuracy
Liu primers.	*L. maculans*	Target	11	100,00
		Non Target	25	
	L. biglobosa	Target	9	100,00
		Non Target	27	
PRI primers	*L. maculans*	Target	11	100,00
		Non Target	25	
	L. biglobosa	Target	To be tested	
		Non Target	To be tested	

ANNEX 5: TABLE 2. EXAMPLE OF CALCULATION OF REPEATABILITY (CT FOR VALIDATION OF XAP PRIMERS)

		AUDY											
sample		test 1	test 2	test 3	test 4	test 5	test 6	nb +	nb -	nb positive accords	nb negative accords	nb possible accords	repeatability
35	non target	-	-	-	-	-	-	0	6	0	15	15	100
33	non target	-	-	-	-	-	-	0	6	0	15	15	100
31	non target	+	+	+	+	+	+	6	0	15	0	15	100
34	non target	-	-	-	-	-	-	0	6	0	15	15	100
32	non target	-	-	-	-	-	-	0	6	0	15	15	100
												Mean:	100

ANNEX 6: TABLE 3. EXAMPLE OF CALCULATION OF REPRODUCIBILITY (CT FOR VALIDATION OF XAP PATHOGENICITY TEST)

	Pathogenicity test on bean by dipping (+/-)								
Sample:	SNES	LSV	INRA	nb +	nb-	nb positive accord	nb negative accords	nb possible accords	reproducibility
1 target	+	-	+	2	1	1	0	3	0.333333333
2 target	+	+	+	3	0	3	0	3	1
3 target	+	+	+	3	0	3	0	3	1
4 target	+	+	+	3	0	3	0	3	1
5 target	+	+	+	3	0	3	0	3	1
6 target	+	+	+	3	0	3	0	3	1
7 target	+	+	+	3	0	3	0	3	1
8 target	+	+	+	3	0	3	0	3	1
10 target	+	+	+	3	0	3	0	3	1
11 target	+	+	+	3	0	3	0	3	1
12 target	+	+	+	3	0	3	0	3	1
13 target	+	+	+	3	0	3	0	3	1
14 target	+	+	+	3	0	3	0	3	1
15 target	+	+	+	3	0	3	0	3	1
16 target	+	+	+	3	0	3	0	3	1
17 target	+	+	+	3	0	3	0	3	1
18 target	+	+	+	3	0	3	0	3	1
19 target	+	+	+	3	0	3	0	3	1
20 target	+	+	+	3	0	3	0	3	1
21 target	+	+	+	3	0	3	0	3	1
22 target	+	+	+	3	0	3	0	3	1
23 target	+	+	+	3	0	3	0	3	1
25 target	+	-	+	2	1	1	0	3	0.333333333
26 target	+	+	+	3	0	3	0	3	1
27 target	+	+	+	3	0	3	0	3	1
28 target	+	+	+	3	0	3	0	3	1
29 target	+	+	+	3	0	3	0	3	1
30 target	+	+	+	3	0	3	0	3	1
total reproducibility in %						80	0	84	**95.24**