WOAH Asia Regional Training on Swine Disease Diagnosis

Verification and Validation of Diagnostic Methods for Animal Disease

China Animal Disease Control Center

National /WOAH Reference Laboratory for PRRS

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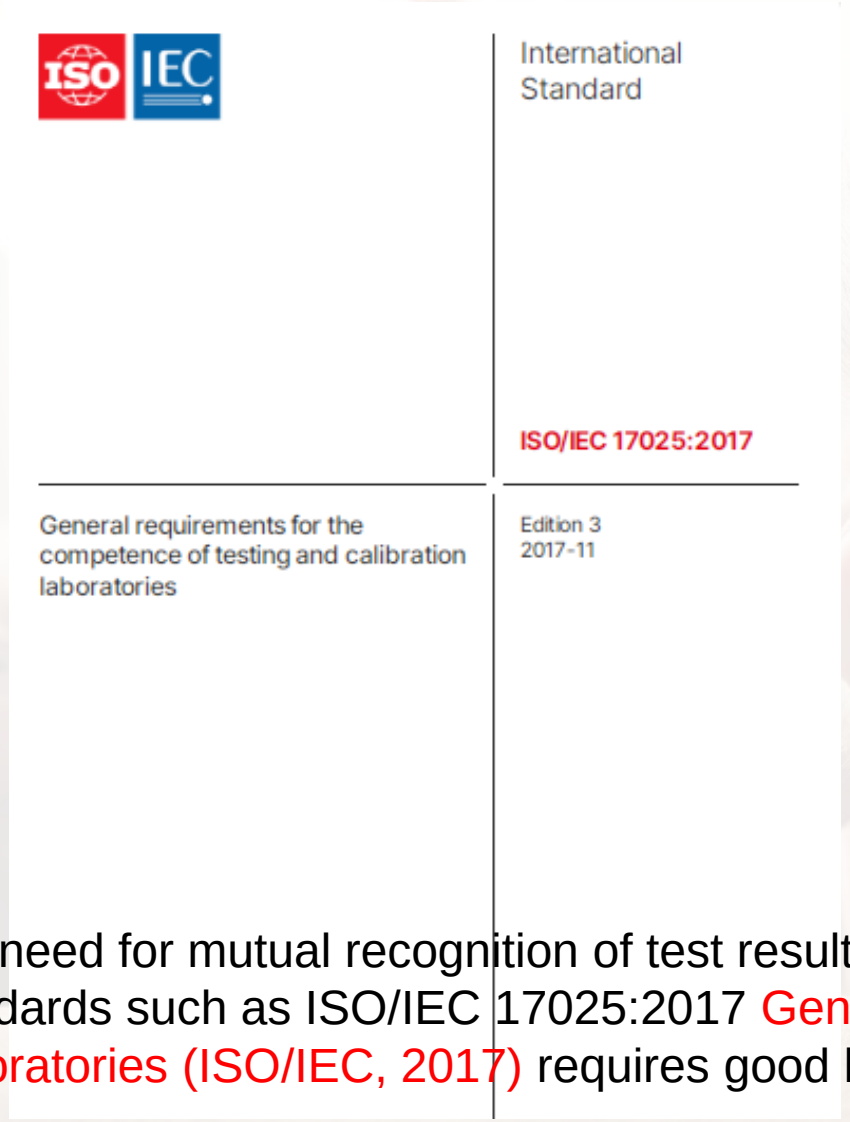
30th Jul, 2024

Valid laboratory results are essential for diagnosis, surveillance and trade.



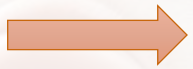
ISO IEC

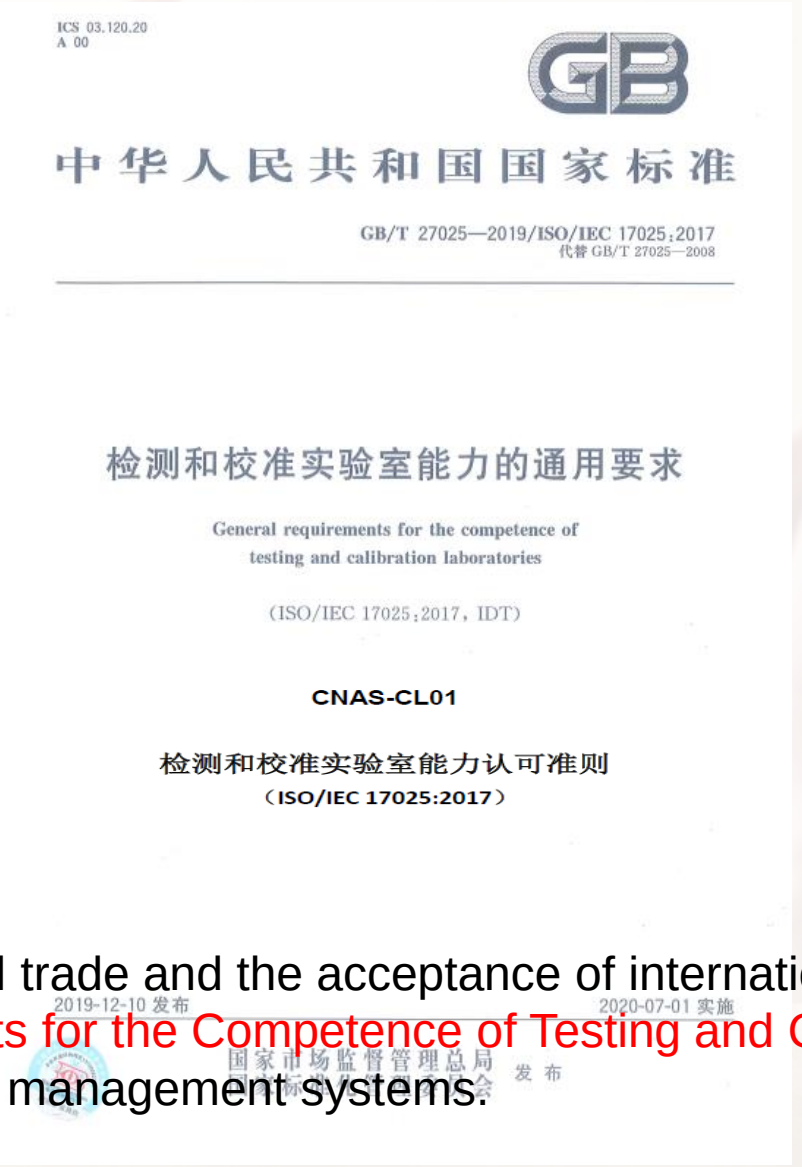
International Standard

ISO/IEC 17025:2017

General requirements for the competence of testing and calibration laboratories

Edition 3
2017-11





ICS 03.120.20
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GB

中华人民共和国国家标准

GB/T 27025—2019/ISO/IEC 17025:2017
代替 GB/T 27025—2008

检测和校准实验室能力的通用要求

General requirements for the competence of testing and calibration laboratories

(ISO/IEC 17025:2017, IDT)

CNAS-CL01

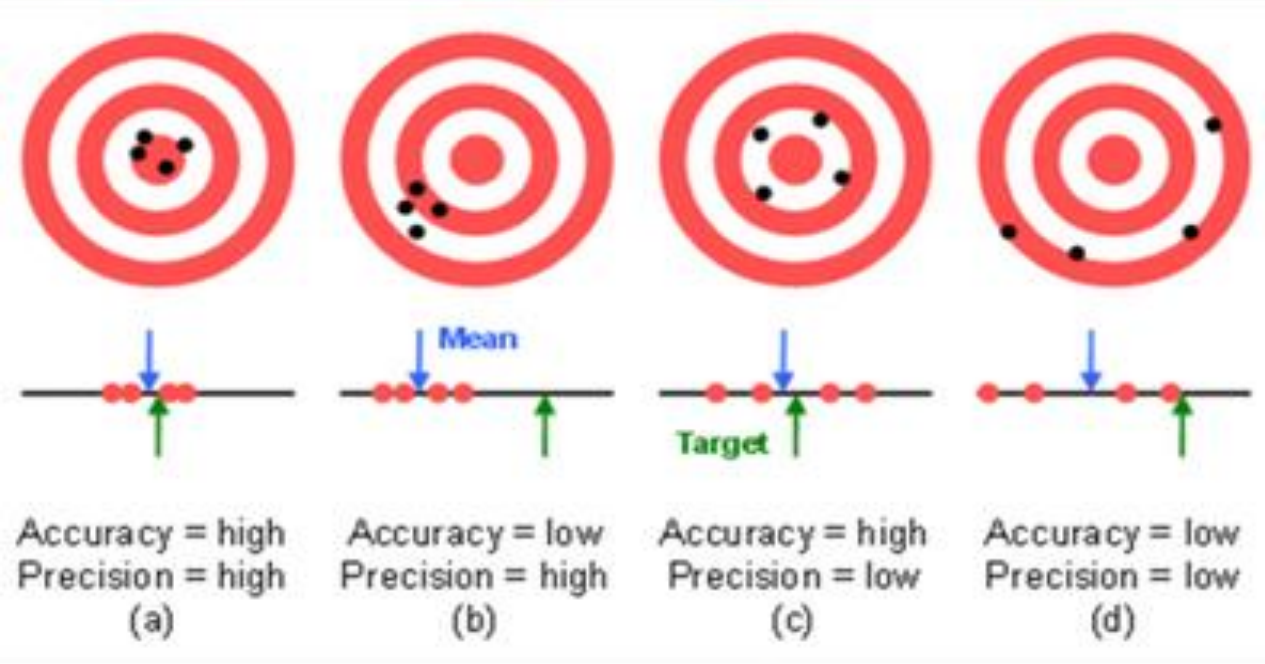
检测和校准实验室能力认可准则
(ISO/IEC 17025:2017)

2019-12-10 发布　　2020-07-01 实施

国家市场监督管理总局
国家标准化管理委员会　发布

The need for mutual recognition of test results for international trade and the acceptance of international standards such as ISO/IEC 17025:2017 General Requirements for the Competence of Testing and Calibration Laboratories (ISO/IEC, 2017) requires good laboratory quality management systems.

What is valid laboratory results?



- **Accuracy**

  Relates to its ability to give a true measure of the animal disease according to its own epidemic situation. An accurate test will not over- or under-estimate the true value.

  Accuracy= trueness

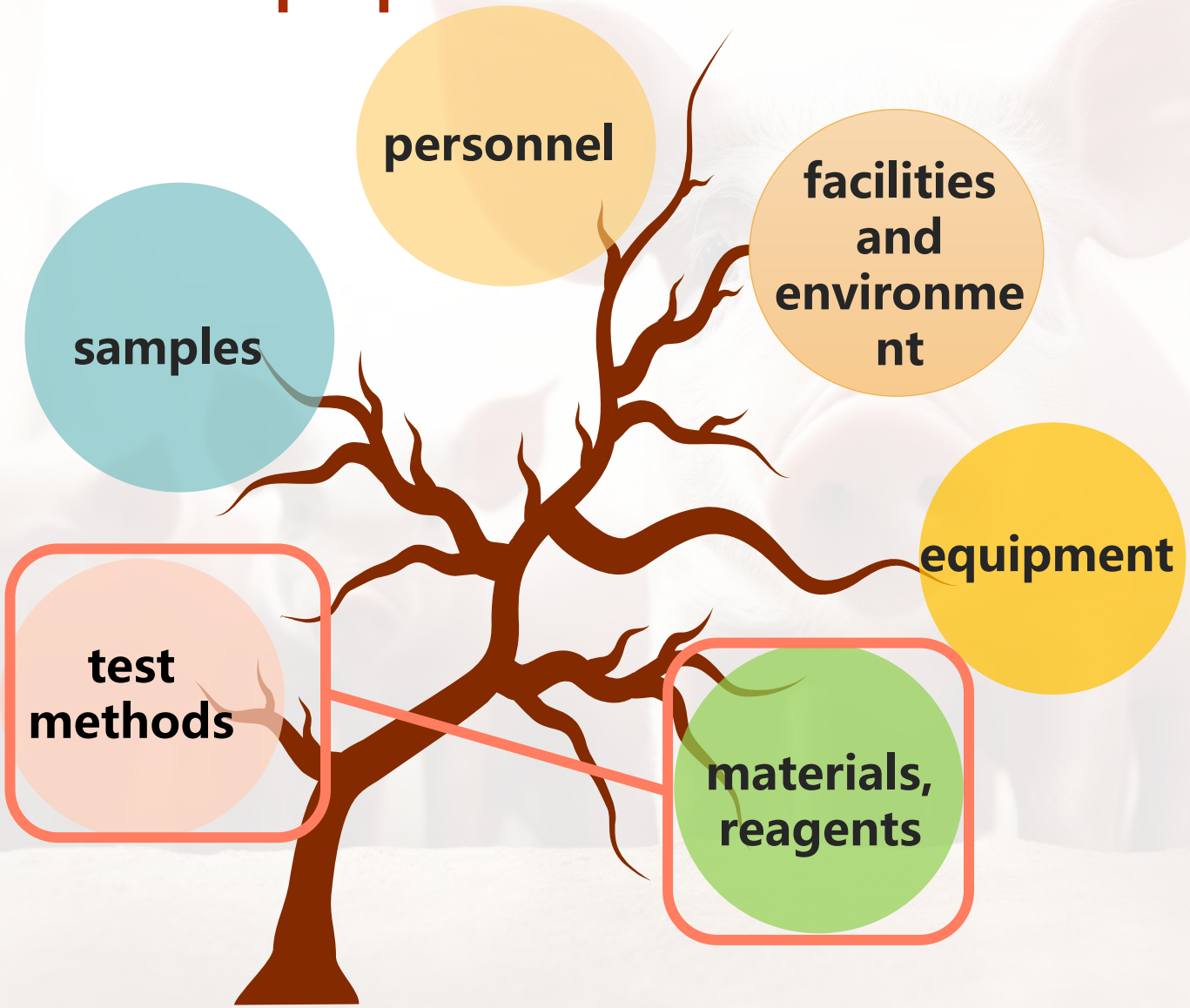
- **Precision**

  Relates to how consistent the results of the test are. If a test always gives the same value, it is said to be precise.

  Precision= repeatability

ISO/IEC 17025 requires the use of appropriate test methods and has requirements for their selection, development, and validation to show fitness for purpose.

——from chapter 1.1.5 Quality Management



Verification

provision of objective evidence that a given item fulfils specified requirements

——from ISO/IEC 17025 3.8

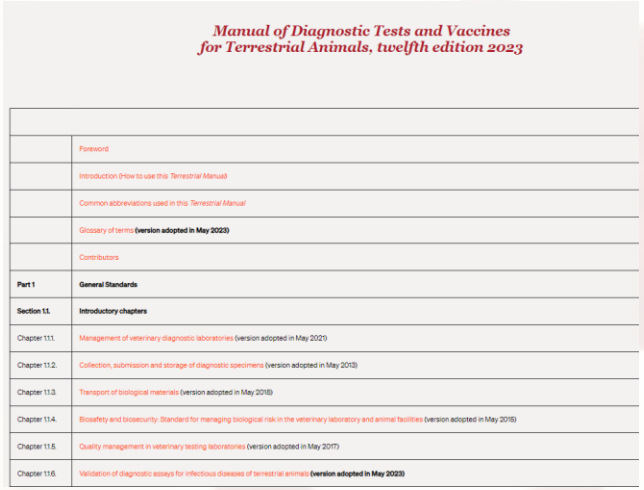
Validation

verification, where the specified requirements are adequate for an intended use

——from ISO/IEC 17025 3.9

Verification of test methods → Standard method

- International standard


Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, twelfth edition 2023

	Foreword
	Introduction (How to use this *Terrestrial Manual*)
	Common abbreviations used in this *Terrestrial Manual*
	Glossary of terms (version adopted in May 2023)
	Contributors
Part 1	General Standards
Section 1.1.	Introductory chapters
Chapter 1.1.1.	Management of veterinary diagnostic laboratories (version adopted in May 2021)
Chapter 1.1.2.	Collection, submission and storage of diagnostic specimens (version adopted in May 2013)
Chapter 1.1.3.	Transport of biological materials (version adopted in May 2018)
Chapter 1.1.4.	Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities (version adopted in May 2016)
Chapter 1.1.5.	Quality management in veterinary testing laboratories (version adopted in May 2017)
Chapter 1.1.6.	Validation of diagnostic assays for infectious diseases of terrestrial animals (version adopted in May 2023)

- National standard


GB

中华人民共和国国家标准

GB/T 18648—2020

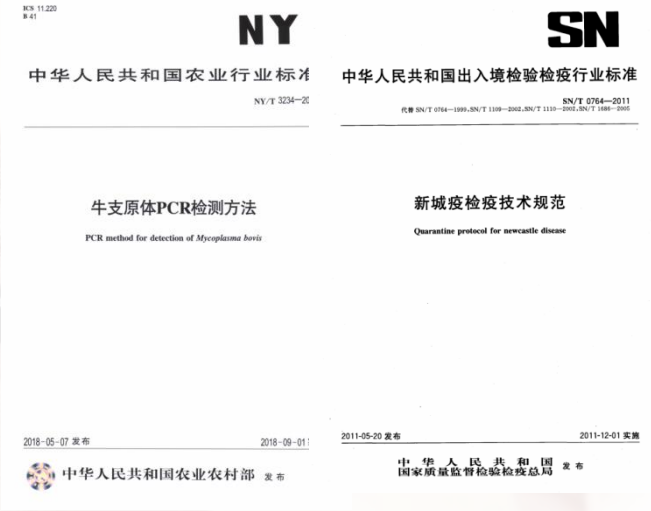
非洲猪瘟诊断技术

Diagnostic techniques for African swine fever

2020-12-14 发布　2020-12-14 实施

国家市场监督管理总局
国家标准化管理委员会　发布

- Industry standard


NY

中华人民共和国农业行业标准

牛支原体PCR检测方法

PCR method for detection of *Mycoplasma bovis*

2018-05-07 发布　2018-09-01 实施

中华人民共和国农业农村部　发布

SN

中华人民共和国出入境检验检疫行业标准

SN/T 0764—2011

新城疫检疫技术规范

Quarantine protocol for newcastle disease

2011-05-20 发布　2011-12-01 实施

中华人民共和国
国家质量监督检验检疫总局　发布

- Group standard


团　体　标　准

T/CVMA 37-2020

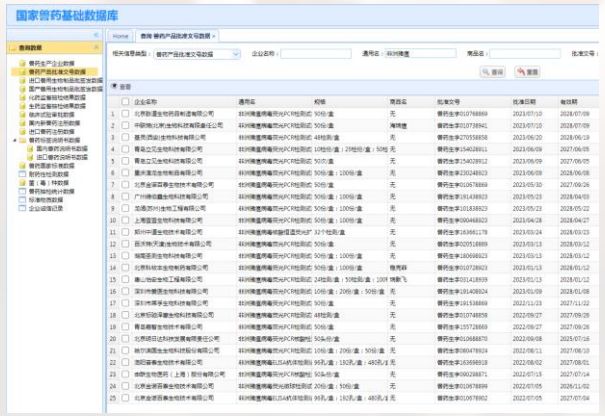
种鸡场鸡白痢和鸡伤寒监测控制技术指南

Technical guidelines for surveillance and control of Pullorum disease and Fowl typhoid in breeding chicken farms

2020-12-22 发布　2020-12-22 实施

中国兽医协会　发布

- Compliant reagent: obtain the production number of veterinary drugs from MARA


国家兽药基础数据库

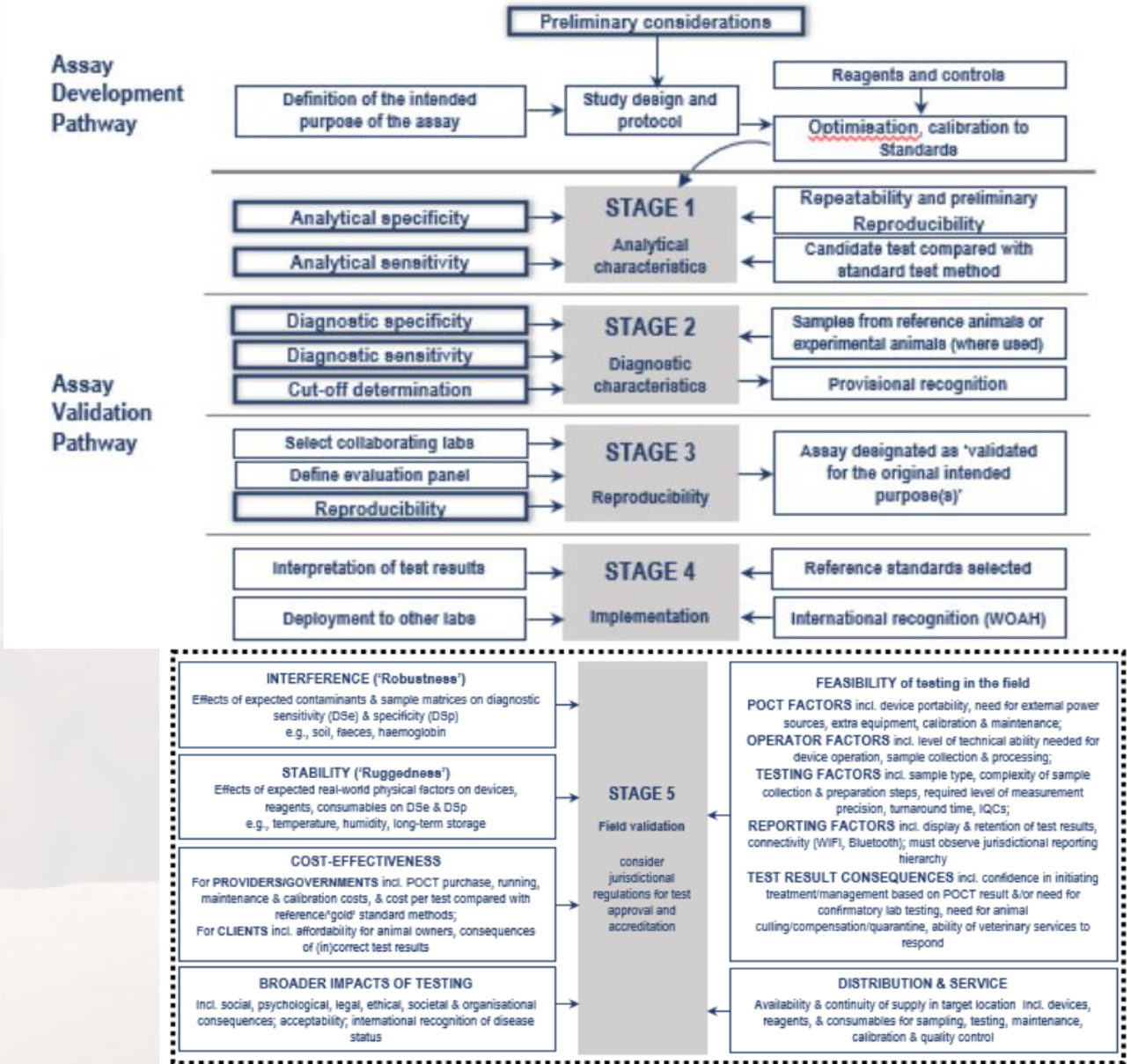
ISO/IEC 17025 7.2.1.5: The laboratory shall verify that it can properly perform methods before introducing them by ensuring that it can achieve the required performance.

Validation of test methods →

- Non-standard methods, laboratory-developed methods, standard methods used beyond the intended scope, or other modified standard methods

WOAH

Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, thirteenth edition 2024



Chapter	Content
1.1.6	Validation of diagnostic assays for infectious diseases of terrestrial animals
2.2.1.	Development and optimisation of antibody detection assays
2.2.2	Development and optimisation of antigen detection assays
2.2.3	Development and optimisation of nucleic acid detection assays
2.2.4	Measurement uncertainty
2.2.5	Statistical approaches to validation
2.2.6	Selection and use of reference samples and panels

Validation parameters

——from chapter 1.1.6 Validation of diagnostic assays for infectious diseases of terrestrial animals

Accuracy

Analytical characteristics	Diagnostic characteristics	Reproducibility
• Analytical sensitivity • Analytical specificity	• Diagnostic sensitivity • Diagnostic specificity	• Repeatability • Reproducibility

Precision

1 Analytical characteristics
- Analytical sensitivity
- Analytical specificity
- Repeatability

2 Diagnostic characteristics

3 Reproducibility

4 Implementation

Analytical Sensitivity

- **Analytical sensitivity （ASe）** -Also known as the limit of detection (LOD)
  - is the estimated amount of analyte in a specified matrix that would produce a positive result at a certain confidence level (usually 95%)

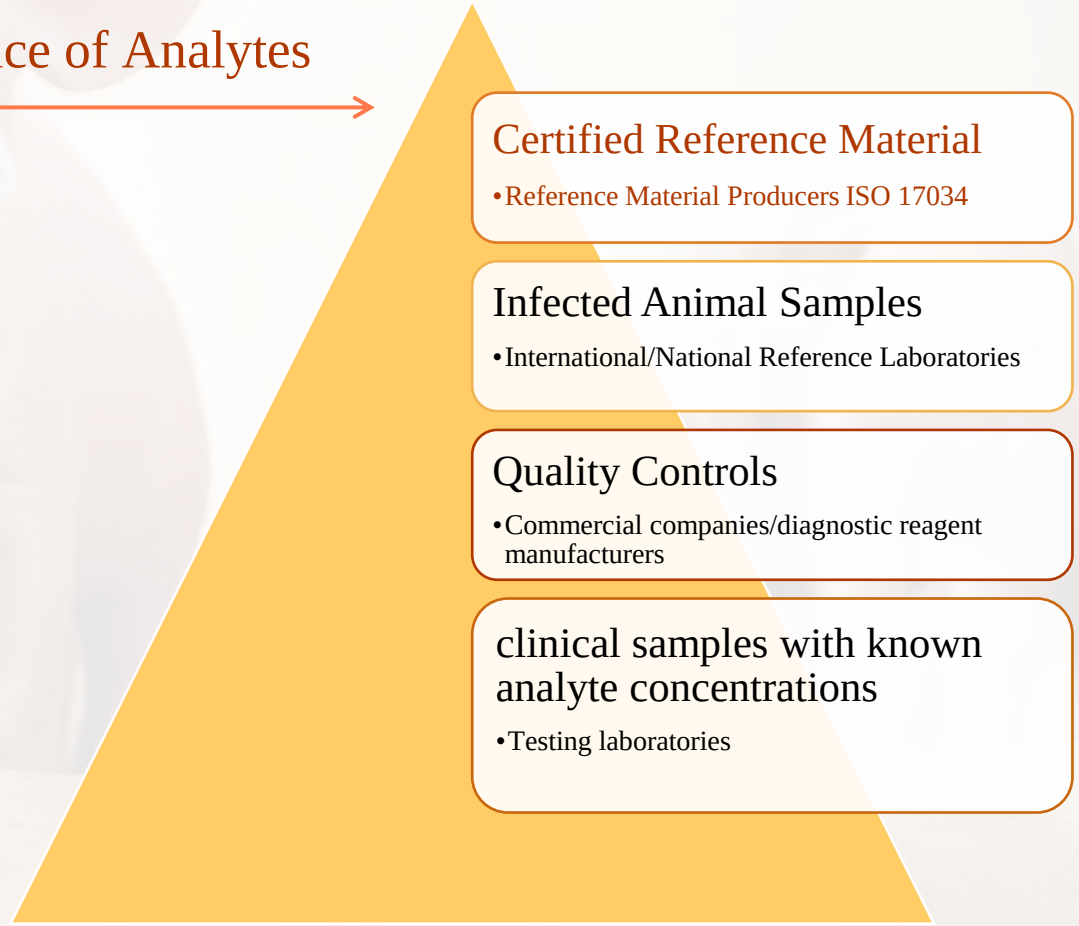
Stage 1: Using a series of dilutions of the analyte in the matrix. Setting 3 replicates for each dilution. Accepting the last dilution showing 100% response as the LOD.

- **Direct detection methods**: Represented by the number of pathogen gene copies, infectious dose, total number of colonies, etc.
- **Indirect detection methods:** Represented by antibody titer.

Stage 2: Using narrower intervals in the dilution scheme focusing on the region between the 100% and 0%, with at least 20 replicates for each dilution. Accepting the last dilution showing 90%-99% response as the LOD (usually 95%).

The more replicates at each dilution, the more precise the estimation of LOD.

Choice of Analytes



Analytical Sensitivity

- Molecular assays: In stage 1, use a tenfold serial dilution with the same matrix. In stage 2, select specific dilutions within the region determined in stage 1, performing at least 20 repetitions. If there are 20 detections, at least 18 must detect the target nucleic acid.

Number of copies	Replicates	ASFV (FAM) C_T	C_T mean	sd	Result
10^7	1	13.18	**13.21**	0.03	positive
	1	13.23			positive
	1	13.23			positive
10^6	2	16.25	**16.18**	0.10	positive
	2	16.22			positive
	2	16.07			positive
10^5	3	19.59	**19.44**	0.27	positive
	3	19.13			positive
	3	19.61			positive
10^4	4	22.23	**22.64**	0.36	positive
	4	22.82			positive
	4	22.88			positive
10^3	5	26.17	**26.05**	0.41	positive
	5	26.39			positive
	5	25.60			positive
10^2	6	28.67	**29.28**	0.54	positive
	6	29.70			positive
	6	29.46			positive
10^1	7	32.32	**33.74**	1.63	positive
	7	35.52			positive
	7	33.38			positive
10^0	8	No C_T	**No C_T**	-	negative
	8	No C_T			negative
	8	No C_T			negative
10^{-1}	9	No C_T	**No C_T**	-	negative
	9	No C_T			negative
	9	No C_T			negative

重复	6 copies/μL	3 copies/μL	2 copies/μL
1	34.96	34.19	Un
2	35.51	34.44	39.10
3	35.11	34.95	Un
4	35.41	36.07	Un
5	37.19	36.34	Un
6	36.07	36.46	Un
7	35.83	35.63	39.65
8	35.85	Un	36.05
9	34.87	Un	Un
10	35.23	Un	39.22
11	34.66	37.57	Un
12	36.2	Un	Un
13	36.2	35.06	Un
14	37.05	36.45	39.06
15	35.32	Un	37.86
16	35.6	37.17	Un
17	35.64	37.53	Un
18	35.83	35.51	37.49
19	37.90	34.79	Un
20	35.87	36.02	Un

The ideal LOD of Molecular assays should be 500copies/mL~1000copies/mL

Analytical Sensitivity

- Serological assays: In stage 1, use a log2 serial dilution with the same matrix (negative serum). In stage 2, select specific dilutions within the the region determined in stage 1, performing at least 20 repetitions.

LOD of commercial kit is very different.

Brucellosis Reference Matieral Concentration of Cattle Serum	Indirect ELISA	Competitive ELISA	Tube Agglutination Test	Rose Bengal Plate Agglutination Test
250IU	+	+	+	+
125IU	+	+	+	+
62.5IU	+	+	+	+
31.25IU	+	+	-	+
15.6IU	+	+	-	-
7.8IU	+	+	-	-
3.9IU	+	+	-	-
1.95IU	+	+	-	-
1IU	-	-	-	-
0.5IU	-	-	-	-

Brucellosis Reference Matieral Concentration of Sheep Serum	Indirect ELISA	Competitive ELISA	Tube Agglutination Test	Rose Bengal Plate Agglutination Test
400IU	+	+	+	+
200IU	+	+	+	+
100IU	+	+	+	+
50IU	+	+	+	+
25IU	+	+	-	+
12.5IU	+	+	-	-
6.25IU	-	-	-	-
3.13IU	-	-	-	-
1.56IU	-	-	-	-
0.78IU	-	-	-	-

According to (EC) No 535/2002: The OIE Standard Serum for Cattle (OIEISS, previously known as the WHO Second International Standard Anti-Brucella abortus Serum; WHO, 1953) is used to calibrate the LOD of various Brucellosis testing methods.

ELISA 1.67-6.67IU/mL **RBT 18.18-22.22IU/mL** **SAT 30IU/mL** SAT is generally regarded as being unsatisfactory for the purposes of international trade. **CF 20IU/mL**

Analytical Sensitivity

☐ Analytical specificity （ASp） -Also known as: Cross-reactivity

-The ability to distinguish the target analyte in a sample (such as pathogens, antibodies, genomic sequence) from non-target analytes (including matrix components)

Selectivity

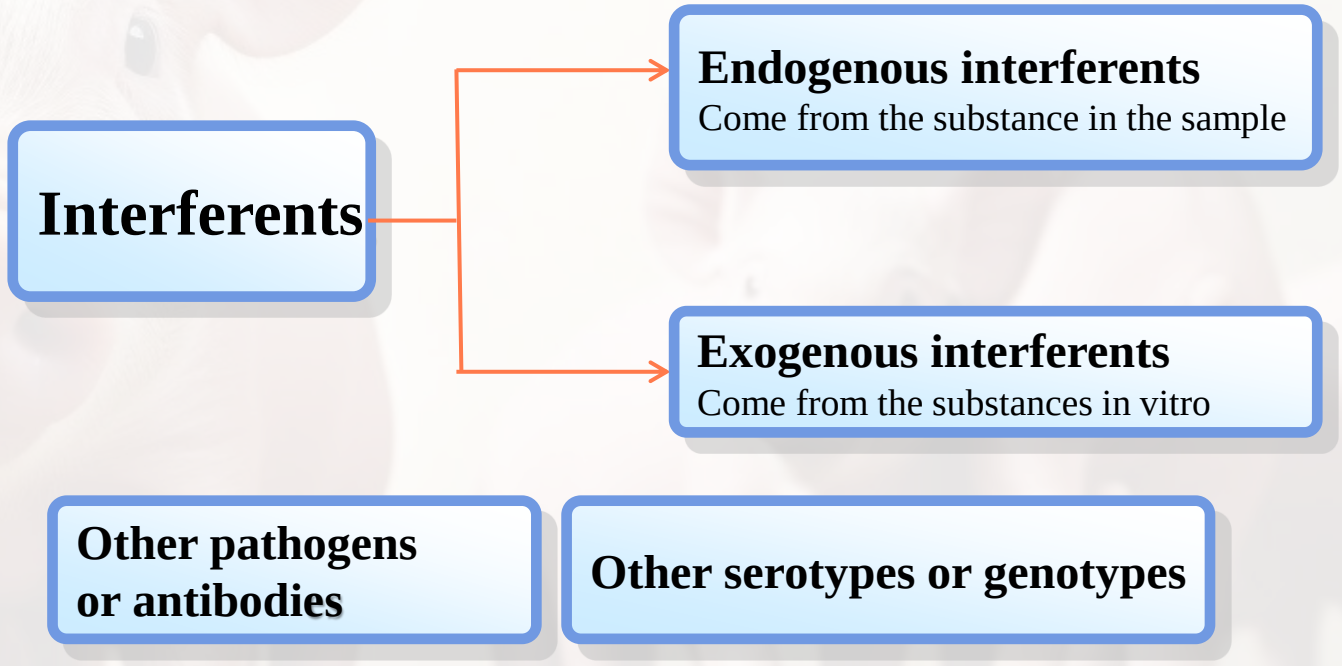
- Interferents, such as matrix components (e.g. inhibitors of enzymes in the reaction mix)
- Degradation products (e.g. toxic factors)
- Non-specific binding of reactants to a solid phase (e.g. conjugate of an ELISA adsorbed to well of microtiter plate)
- Antibodies to vaccination that may be confused with antibodies to active infection.

Exclusivity

- an analyte or genomic sequence that is unique to a targeted organism, not other known organisms that are potentially cross-reactive

Inclusivity

- Specific strains or serotypes of a species within the target analyte, several species of a genus, or a similar grouping of closely related organisms or antibodies thereto



Analytical Sensitivity

Endogenous interferents	Come from the substance in the sample • Blood: heme, triglycerides, IgG • Urine: urea • Feces: plant polysaccharides, cholate • Tissue: protease, collagen • Milk: protease, calcium ions • Drugs: antibiotics, antiviral drugs, hormone drugs
Exogenous interferents	Come from the substances in vitro, such as sample additives or substances during sample collection and processing • Test supplies: serum separation gel, sample collection tube, glove powder • Anticoagulant: heparin, EDTA • Organic solvents: isopropyl alcohol, glycerin, PEG • Disinfectant: alcohols, aldehydes, phenols, peroxides, strong acids, strong bases • Detergent: SDS • Soil: humus • Dye: indigo

Diagnosis and Treatment Plan on COVID-19 (Ninth Edition)

For etiological and serological examinations, it is noted that "due to the positive judgment value of the reagent itself, or the presence of interfering substances in the body (such as rheumatoid factors, heterophilic antibodies, complement, lysozyme, etc.), or specimen reasons (such as hemolysis of the specimen, bacterial contamination of the specimen, excessive storage time of the specimen, incomplete coagulation of the specimen, etc.), false positive results may occur in antibody testing.

Analytical Sensitivity

Endogenous interferents: faeces /sperm /autolyzed tissues typically contain a higher concentration of inhibitors for nucleic acid analysis

编号 NO.	样品类型	核酸提取	Kit 1	Kit 2	Kit 3	Kit 4
1	ASFV标准物质	不提取NO Extraction	25.69	25.72	25.90	25.69
3	肉 meat	提取Extraction	25.88	25.62	27.89	26.94
4	肺脏 lung	提取Extraction	26.49	25.11	25.94	25.89
5	脾脏spleen	提取Extraction	26.83	26.65	26.06	26.01
6	**肝脏liver**	提取Extraction	26.22	**28.41**	**29.84**	**28.50**
7	大肠cecum	提取Extraction	25.90	25.56	25.33	25.54
8	小肠duodenum	提取Extraction	26.89	26.88	26.46	26.75
9	**肾脏kidney**	提取Extraction	26.04	**27.89**	**28.75**	**27.95**
10	淋巴结lymph node	提取Extraction	**27.14**	25.38	26.90	27.08
11	扁桃体tonsil	提取Extraction	26.25	26.37	25.69	26.16
12	饲料feed	提取Extraction	24.73	24.23	26.10	25.97
13	饺子陷dumpling	提取Extraction	25.03	**27.60**	**28.39**	**28.62**
14	肉肠sausage	提取Extraction	25.40	25.19	**28.29**	26.21
15	**猪精液sperm**	提取Extraction	**26.99**	**27.46**	**29.64**	**29.57**
16	**EDTA抗凝血blood**	提取Extraction	**28.94**	**30.56**	**32.56**	**30.81**
17	**血浆 plasma**	提取Extraction	**28.81**	**29.26**	**29.96**	**29.56**
18	血清serum	提取Extraction	25.95	25.72	27.87	26.89
19	**唾液oral fluid**	提取Extraction	**27.53**	**30.94**	26.34	26.51
	平均值average		26.53	26.96	27.77	27.35

表 3　不同基质添加对提取效率的影响

基质	荧光 RT-PCR Ct 均值		数字 RT-PCR 核酸含量均值 /（copies/μL）	
	B	C	B	C
TE	26.29	25.59	3 480	3 681
抗凝血 blood	27.2	25.45	808	4 512
血清 serum	26.83	25.39	1 510	4 401
血浆 plasma	26.96	25.90	1 620	2 898
猪精液sperm	28.24	31.65	1 078	84
淋巴结Lymph node	26.47	25.88	3 500	2 835
扁桃体 tonsil	26.56	26.03	3 090	3 780
肺脏 lung	26.9	26.35	2 620	3 762
唾液 oral fluid	26.97	25.52	1 640	3 879
饲料feed	26.94	25.71	2 080	4 392

Cited from Xu Qi et al. Comparison of five nucleic acid extraction kits using a porcine reproductive and respiratory syndrome virus (PRRSV) nucleic acid standard. [J]. China Animal Quarantine，2020,37（12）：120-123

Diagnostic sensitivity and Diagnostic specificity

- Diagnostic sensitivity （DSe）： percentage of known infected animals that test positive in an assay

$$DSe = Pr(T+ \mid D+) = TP/(TP+FN)$$

- Diagnostic specificity （DSp）： percentage of known uninfected animals that test negative in an assay

$$DSp = Pr(T- \mid D-) = TN/(TN+FP)$$

DSe & DSp represent the ability to accurately distinguish between animals based on their disease status.

They are the foundation for estimating the positive predictive value(PPV) and the negative predictive value(NPV).

	Disease Status – Infected (D+)	Disease Status – Health (D-)	Total
Test Result-Positive (T+)	True positive	False positive	T+
Test Result-Negative (T-)	False negative	True negative	T-
Total	D+	D-	

predictive values are a function of its DSe and DSp and the local prevalence of infection

- a PPV of 0.9 means that an animal testing positive has 90% chance of being indeed infected and 10% probability of being a false positive.

Diagnostic sensitivity and Diagnostic specificity

- **The accuracy of diagnostic sensitivity and specificity depends on the quality and quantity of the sample panels.**
- DSe and DSp are not fixed values but a Beta distribution (binomial process).

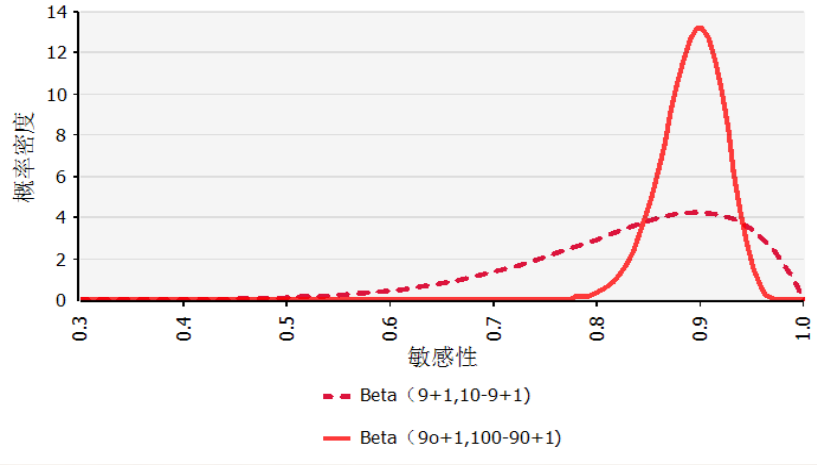
		Number of reference samples required*	
		Known positive (279)	Known negative (95)
Test results	Positive	270 TP	FP 7
	Negative	FN 9	TN 88
		Diagnostic sensitivity* TP/(TP + FN) **96.8%** (94.0 – 98.5%)**	Diagnostic specificity* TN/(TN + FP) **92.6%** (85.4 – 97.0%)**

**95% exact binomial confidence limits for DSe and DSp calculated values

WOAH chapter1.1.6,

Beta Distribution: If two of the three parameters n, x, and p are known, the third can be estimated.

Application	n	x
Diagnostic sensitivity	Number of infected reference animals	Number of infected animals that test positive
Diagnostic specificity	Number of uninfected reference animals	Number of uninfected animals that test negative
Prevalence	Number of animals or herds	Number of infected animals or herds



Diagnostic sensitivity and Diagnostic specificity

- The quantity of the sample panels: The ideal number of sample panels to obtain DSe and DSp is based on the expected value, confidence level, and acceptable error.
- As the sample size grows, the confidence interval becomes more refined, enhancing precision and diminishing the impact of random error.

Table 1. *Theoretical number of samples from animals of known infection status required for establishing diagnostic sensitivity (DSe) and specificity (DSp) estimates depending on likely value of DSe or DSp and desired error margin and confidence*

Estimated DSe or DSp	**2%** error allowed in estimate of DSe and DSp			**5%** error allowed in estimate of DSe and DSp		
	Confidence			Confidence		
	90%	95%	99%	90%	95%	99%
90%	610	864	1493	98	138	239
92%	466	707	1221	75	113	195
94%	382	542	935	61	87	150
95%	372	456	788	60	73	126
96%	260	369	637	42	59	102
97%	197	279	483	32	45	77
98%	133	188	325	21	30	52
99%	67	95	164	11	15	26

The expected DSe is 97%, and DSp is 99%

Diagnostic sensitivity and Diagnostic specificity

- The quality of sample panels for obtaining DSe and DSp

Covering animal types, age, gender, breed, infection stage (early/late infection, fluctuation pattern), vaccination history, and disease history

Reference sample banks e.g. from Ref. Labs	Experimental challenge trials	Identified by a gold standard test	Field populations with animals of unknown infection status
• Negative samples – It can usually be obtained from countries or zones that have eradicated or have never had this disease. • Positive samples (more difficult) – It can be obtained from experimental challenge trials or identified by a gold standard test.	• experimentally infected animals • experimentally vaccinated animals • The strain of organism, dose, and route of administration to experimental animals are examples of variables	• Virus isolation, and sequencing • IFA/IPMA/Virus neutralization test	• Latent class models(LCM) used for data analysis: Bayesian latent class model

- major genotypes/serotypes and different type of sanples (e.g. whole blood, serum, plasma, oral fluid, feces, etc.) should be considered.

Appropriate levels of DSe and DSp are contingent upon the intended purpose

Purpose	appropriate DSe and DSp
Historical freedom (with or without vaccination)	high DSp, high PPV
Contribute to the eradication of disease or elimination of infection from defined populations	
Certify freedom from infection or agent in individual animals or products for trade/movement purposes	high DSe, high NPV
Confirm diagnosis of suspect or clinical cases	high DSp, high PPV
Estimate prevalence of infection or exposure to facilitate risk analysis	a screening test with high DSe, then a confirmatory test with high DSp
Determine immune status	high DSp, high PPV

During animal disease outbreaks, the true prevalence is high.

- The NPV is less likely to be low, which means there is a higher risk of false-negative results and potential missed detections. Hence, it is essential to select methods and reagents with high DSe and high NPV.

As control measures are implemented, the true prevalence gradually decreases.

- The PPV may be less reliable, leading to an increased risk of false-positive results and unnecessary culling. Consequently, it is important to opt for methods and reagents with high DSp and high PPV.

Reproducibility

Repeatability

Comparing the consistency of within-batch and between-batches measurements by using the same method in the same laboratory.

- **Within-batch Repeatability: At least 3 samples (usually 5 samples, including at least strong positive, weak positive, borderline, and negative), with at least 5 repetitions for each sample.**
- **Between-batches Repeatability: At least 3 samples (usually 5 samples), tested at different times by at least 2 operators, with at least 20 measurements conducted.**
- **Calculate the standard deviations, coefficient of variation CV**

It is difficult to get different batches of test reagent.

Reproducibility

Comparing the consistency of test results in different laboratories.

- **At least 3 laboratories test the same panel of samples containing at least 20 samples.**
- **The preferred choice is laboratories located in different regions or countries, using the same methods, reagents, quality control, etc.**

Sample panel example of real-time PCR for detecting nucleic Acid of ASFV

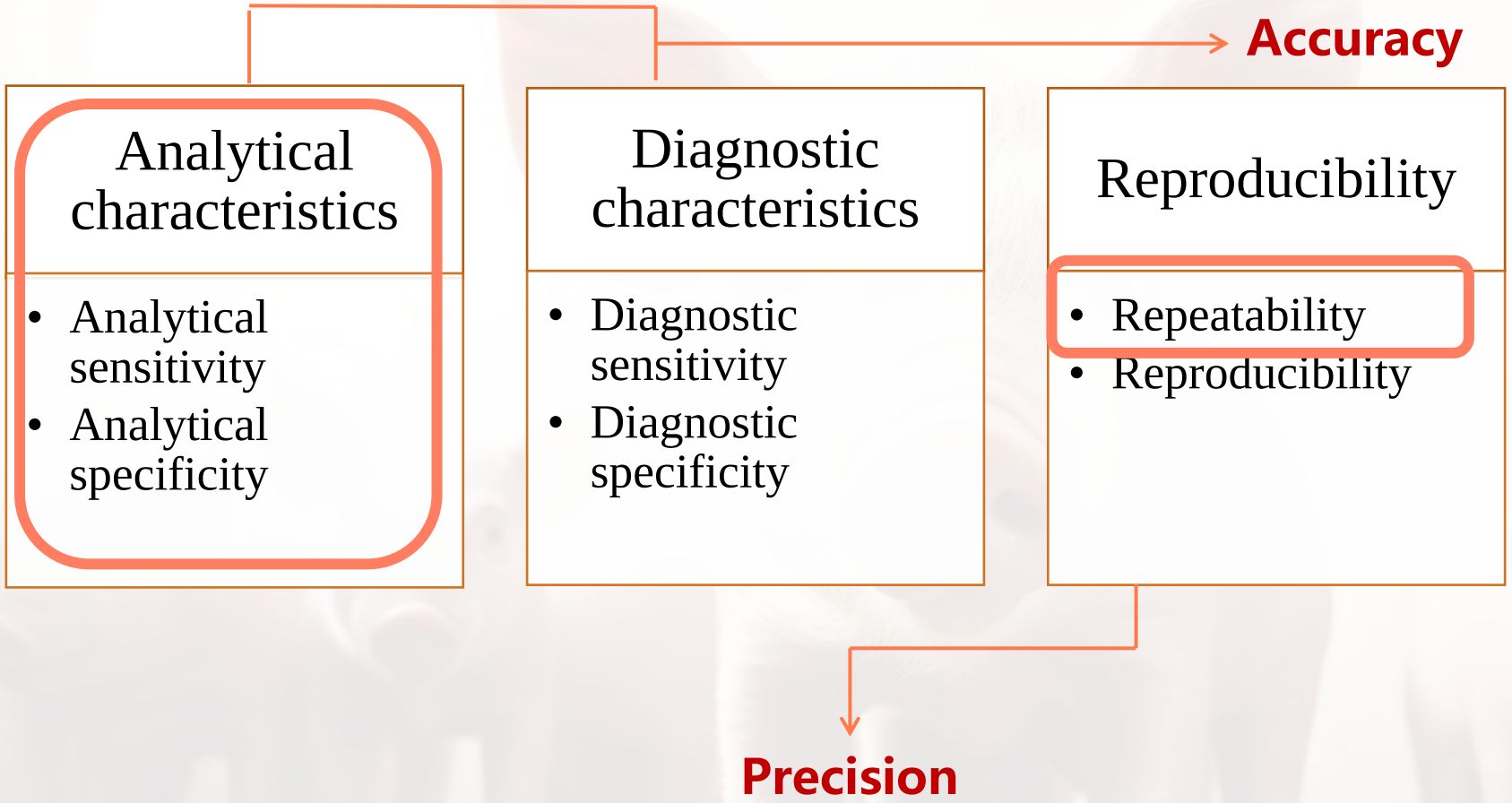
Parameters	Sample Panel	number of samples	Optimization	Sample Verification
Analytical Sensitivity	Positive whole blood tenfold serial dilutions from 1:10 to $1:10^5$ (three replicates for each dilution) Ct 22.33-35.74	15	Adding reference materials into the matrix 10^4-10^0copies/mL	Determined as positive/negative by QPCR at the national reference laboratory
Diagnostic Sensitivity	Positive samples (tissue/whole blood, clinical samples, experimentally infected pig samples)	40	Increasing the number of samples with varying virus titers, including early and late stage experimentally infected pig samples, and try to cover various sample types (feces, environmental samples)	
Diagnostic Specificity/Analytical Specificity	Negative samples (tissue, whole blood)	14	Increasing the number of samples, including both endogenous and exogenous Interferents (such as saliva, environmental swabs, pork, triglycerides, hemoglobin and heparin, feces, soil, feed, etc.)	
Diagnostic Specificity/Analytical Specificity	Cell cultures of PRRSV and other common pig disease viruses	6		
Within-Batch and Between-Batch Repeatability	Strong positive whole blood (3 replicates)	3	Adding reference materials into the matrix 10^3-10^1copies/mL increasing the number of repetitions	
Total		78		

If it is a typing test, different genotype samples are also needed: P72, CD2v, MGF, I177L

Sample panel example of ELISA for detecting antibody of ASFV

Parameters	Sample Panel	number of samples	Optimization	Sample Verification
Analytical Sensitivity	Positive serum (from three pigs immunized with virus) twofold serial dilutions from 1:2 to 1:256 (three replicates for each dilution)	24	It is not advisable to use a single serum sample; a mixture of serum from early and late stages of infection should be recommended, targeting different virus proteins (e.g. p30/p72/p54)	Determined as positive /negative by IFA at the national reference laboratory
Diagnostic Sensitivity	Positive serum (from experimental infected pigs at 9 days, 14 days and 28 days/infected serum after immunization/ clinical positive serum)	39	Increase the number of samples, including weak positive serum or positive serum whose determination results are close to cut-off	
Diagnostic Specificity	Negative serum (clinical negative serum/ SPF pig serum)	20	Increase the number of samples, including complex background serum	
Analytical Specificity	Positive serum of PRRSV and other common pig disease viruses	5	Increase the number of samples and include exogenous Interferents (such as triglycerides, hemoglobin, and heparin)	
Within-Batch and Between-Batch Repeatability	Strong positive serum (three replicates)	3	Add reference materials intto the negative serum, including weak positive serum or positive serum whose determination results are close to cut-off increasing the number of repetitions	
Total		91		

Verification parameters



ISO/IEC 17025 7.2.1.5: The laboratory shall verify that it can properly perform methods before introducing them by ensuring that it can achieve the required performance.

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Prior to the analysis of clinical specimens, the laboratory should conduct essential performance verification on the detection system, which comprises nucleic acid extraction reagents, extraction equipment, amplification reagents, and amplification instruments. The performance parameters to be verified should include, at a minimum, repeatability and the limit of detection.

Thank You for your attention!