Guidelines for Development and Performance of PCR Assays in Veterinary Diagnostic Laboratories

Laboratory Technology Committee

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De Novo PCR assay Development
Guidelines for the Performance Evaluation Process

Development of novel PCR-based assays is often required of laboratories for a variety of applications. The guidelines below are meant to provide a template to assist the development process from initial assessment of need and intended use to implementation. No attempt has been made to provide performance benchmarks, as assays are required for a wide variety of needs, from detection of nucleic acid in clinical samples to typing of bacterial or viral isolates. It is the responsibility of the individual developing the assay to establish acceptable assay characteristics fit for the intended use of the assay. Use of these guidelines will facilitate evaluation of assay performance between labs and allow independent assessment and comparison of assay characteristics when published. This document is not intended to be a review of assay validation principles. A comprehensive discussion of assay validation can be found in references provided at the end of this document.

1. Assay Justification

1.1. Introduction
   1.1.a. Why assay is needed
   1.1.b. Literature review of topic/pathogen (check box), stored/archived
   1.1.c. Description of choice of target gene(s)/sequence(s)
   1.1.d. Choice of Gold Standard and Justification
   1.1.e. Description of Controls and Standards

1.2. Scope and Application (description of intended use)

1.3. Assay Design
   1.3.a. Conventional or real time detection
   1.3.b. Materials and equipment needed

1.4. Assay feasibility
   1.4.a. In silico evaluation
   - Appropriate primer and probe sites in target region
   - BLAST evaluation of primer sequences and primer specificity

2. Initial assessment of primer and probe sequences

2.1. Standard curve using 10-fold serial dilutions of target sequence
   2.1.a. During the feasibility stage
   i. It is recommended to test at least 2-5 primer pairs
   ii. Use buffer diluents to compare initial primer performance.
   iii. Assess performance with dilutions of target in nucleic acid from appropriate sample matrix at an early point in development.
   iv. Some labs use isolates, other labs use a synthetic construct. If using a synthetic construct, it is advised to test full length genomic length target at an early point in development in order to assess the effect of secondary structure.
   2.1.b. Include dilutions that facilitate determination of assay limits/analytical sensitivity and cover expected concentrations in clinical samples.
2.1.c. Dilutions should extend two 10-fold dilutions beyond detection limit. Smaller dilutions can be used to determine endpoint but many labs find little value added for the extra cost incurred.

3. Description of Assay Optimization

3.1. Optimization of reagents. If assay initially does not meet required performance characteristics, optimization of reaction conditions can be attempted. Suggested optimization adjustments:

3.1.a. Primer annealing temperature
3.1.b. Primer sequence/probe sequence corrections
3.1.c. Buffer adjustments (MgCl₂, BSA, etc), different commercial 2x kits
3.1.d. Cycling parameters

If the PCR conditions can be adjusted to meet assay performance requirements, then validation is continued as outlined below. If the assay performance is not acceptable, re-design of primers is required. Alternatively, if the laboratory is trying to develop conforming assays that all run with identical buffers, enzyme, and cycling conditions, then optimization is counter-productive and re-design should be pursued.

Once assay characteristics appear to be acceptable, feasibility should also include testing of the assay across expected strains and matrices (inclusivity) and also against near genetic neighbors and similar disease agents to ensure there are no cross reactions (exclusivity). Performing these steps during the feasibility stage will avoid spending time and money to characterize an assay that will ultimately fail to be useful.

4. Evaluation of Assay Performance:

A series of experiments using known concentrations of target sequence (for example virus titer, PCR amplicon, CFUs, copy number controls) in the most common sample matrix for that analyte is used to determine the assay characteristics described below. Concentrations of target should span the range expected in clinical samples and include 2 dilutions beyond the expected limit of detection. A minimum of three independent dilution series are tested using a unique master mix for each series and preferably tested on separate days. Concentrations should encompass the expected amount of analyte in field samples. If possible, include concentrations that exceed the upper and lower limits of detection. The results will be used to determine the following assay characteristics:

4.1. Range and Linearity

4.1.a. Linear regression analysis log concentration vs Ct.
4.1.b. Calculate slope, linearity (R²), and efficiency (\(10^{-1/\text{slope}}\)-1).

4.2. Limit of Detection (LOD)

Determination of LOD requires a known concentration of analyte. LOD determinations should be performed using nucleic acids derived from titered stocks of viruses or bacteria, artificial phage, amplicons or plasmid constructs containing the target sequence.
The target may be in sample matrix. A more precise LOD can be determined if copy numbers are calculated from purified single molecules of nucleic acid of known molecular weight. All options have advantages and disadvantages.

Limit of detection must be empirically determined for each pathogen strain or variant (e.g. BVDV 1a, 1b and 2). Approved reference strains or thoroughly characterized isolates or positive samples should be used.

4.3. Precision

Precision should be established through repetitively testing replicates of the target sequence at three concentrations, high, medium, and low. Guidelines developed for assay comparison can be used. A series of dilutions are prepared for a single reference strain sufficient for the entire series of experiments. Each concentration is tested 5 times within each of 6 independent runs.

4.3.a. Repeatability (within-run imprecision)

Analysis will be provided in the Methods Comparison publication.

4.3.b. Reproducibility (between-run imprecision)

Analysis will be provided in the Methods Comparison publication.

4.7. Diagnostic Performance

4.7.a. Test a series of samples previously tested by another method. It is recommended to test 30 positive samples with different concentrations of target and 30 negative samples.

4.7.b. Secondary assays to confirm specificity/target sequence amplification might be employed; they will only confirm specificity.

4.7.c. Refer to OIE guidelines for sample size determination for de Novo testing.

5. Conclusions Regarding Assay Validation

Does the assay function adequately for the purpose intended?

6. Ongoing assessment of assay function

It is prudent to perform in silico analysis of primer sequences to ensure that your assay can detect emerging isolates and sequence variants as needed according to the rate of change of your target as appropriate.

7. Re-validation

Note that changes in the validated assay such as chemistry, platform, species and matrix all require a methods comparison (Reising et al)
7. References:

Jacobsen, R (2010)
Principles and Methods of Validation of Diagnostic Assays for Infectious Diseases.
OIE Terrestrial Manual Chapter 1.1.4/5.

Burd, E.M. (2010)
Validation of Laboratory-Developed Molecular Assays for Infectious Diseases.
Clinical Microbiology Reviews 23:550-575.

Bustin, SA; Benes, V; Garson, JA; Hellemans, J; Huggett, J; Kubista, M; Mueller, R; Nolan, T; Pfaffl, MW; Shipley, GL; Vandesompele, J; and Wittwer, CT (2009)
The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments.
Clinical Chemistry 55 p. 611-22.

MIQE précis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments.

Primer sequence disclosure: a clarification of the MIQE guidelines.

Halling KC, Schrijver I, Persons DL. (2012)
Test verification and validation for molecular diagnostic assays.

Manuscript in preparation.

A practical approach to RT-qPCR-Publishing data that conform to the MIQE guidelines.
Methods 50(4):S1-5.
Determination of Ct cutoff values for negative samples

Establishment of Ct cutoff values for definition of negatives is an important consideration for the PCR assay development process. Many labs set an arbitrary cutoff of 40 or 45 for defining negative samples. Other labs use the linear range and limit of detection assay characteristics to determine the cutoff. Current published recommendations suggest establishing a cutoff based on the threshold values derived from lowest concentration of target sequence that can be detected 95% of the time. In practice, this often requires multiple repetitions at 2-fold dilutions around the target endpoint and more time and money than the precision is worth. In either case, a cutoff must be vetted by well characterized diagnostic samples (known positive and negative samples). A suspect range is determined by analysis of these well characterized diagnostic samples. In most labs 35-36 is the upper limit of positives; ≥37 marks the start of weak positive or suspect range. Confirmatory data should be obtained to support interpretation of CTs in the range of 37-40 or greater as positive.

Two examples of cutoff determination are described:

1) Method A:

Endpoint/cutoff values are determined for each assay after performing 12 replicates of the standard curve using known copy number targets. The last 10 fold dilution at which there is 100% detection is used to establish a cutoff pos/suspect point at 2 standard deviations above the average Ct at this dilution. An interpretive statement is included with suspect results. A suspect result is likely due to detection of very low amounts of target sequence in the sample and is not repeatable 100% of the time. Generally, this is followed up with repeated testing of 2 dilutions, including the endpoint concentration aliquoted into single use reactions in nucleic acid dilution solution during further testing of the assay using clinical samples to more robustly test the assay performance.

2) Method B uses the probit regression analysis as described in Burd et al, page 562.

Interpretation of the PCR result as well as the meaning of the Ct level depends upon the whole picture. Pertinent factors/questions include:
- is there clinical disease?
- Is the agent endemic or an exotic agent?
- Is it commonly found in animals or is it rarely diagnosed?
- Is the agent ubiquitous in the environment?
- Is the agent in a vaccine used on the premise?
Critical Issues for Conducting PCR Assays

The exquisite sensitivity of PCR assays requires the establishment of thorough and robust good laboratory practice (glp). PCR-associated glp initiates with proper design of the physical space and encompasses collection, transport and storage of samples; development and performance of assays; use of proper controls and consistent interpretation of assays. Stringent quality parameters are necessary to minimize contamination and to ensure consistent and reproducible results across different reagent lots, labs, and personnel. The following recommendations are provided primarily for BSL-2 laboratories; modifications for BSL-3 labs will be provided in specific sections as needed.

I. Facilities:
The location of work areas should be established, as much as possible, according to the appropriate building airflow direction. For example, the best scenario would be positive air pressure in the Master Mix preparation room.

Note: positive air pressure is not an option for BSL-3 sections

Designate a minimum of 3 dedicated work stations:

I.1. Clean Reagent Preparation Area
This area is for preparation of nucleic acid purification reagents and PCR master mix reagents only. No amplification targets (i.e. pathogens), plasmids, purified RNA or DNA, and no amplified products should be allowed. Only trained personnel may use this workstation. It is critical to have this area separated/dedicated as much as possible; at least in a hood. Special attention to disinfection and cleaning of this area is essential.

I.2. Specimen Preparation Area
This area is for sample receiving and processing, and nucleic acid extraction and purification only.

I.3. Target amplification/PCR Area
In this area the PCR instruments are located.

Ideally, work stations should be located in separate rooms. If needed (due to budget and space constraints), however, work stations may be located in one room. Multiple hoods with designated specific functions, i.e. master mix preparation, or nucleic acid purification reagent preparation, may be placed in the same room.

Additional designated work stations may be established:

I.4. Addition of purified nucleic acids to the PCR master mixes.
Many laboratories use desk top PCR hoods.

I.5. Gel electrophoresis
Many laboratories have a designated room; other laboratories take extra precautions to prevent aerosols such as freezing samples before opening tubes, and covering plates.

I.6. Preparation of amplification/extraction controls, standard curves, plasmids
There is strong agreement among laboratories that is very beneficial to have a separate area for preparation of positive controls, particularly from material with a very high pathogen/target concentration, i.e. cell culture supernatants, allantoic fluids, and high copy number plasmid preparations, to avoid contaminating areas designated for diagnostic samples processing.

I.7. nested PCR area

Some reference protocols still require the use of nested PCR. Some laboratories try to avoid the risk of contamination by not offering this service, other laboratories offer the service but are strictly designating the addition of the amplicon from the first step to the reagents for the nested step to a hood. In case of space constraints, this step might be carried out in the template-addition hood.

Additional measures may be employed to prevent contamination of the PCR workflow:

I.7.1. Establish a uni-directional physical workflow (pre-PCR to post-PCR) to avoid contamination between work stations

I.7.2. Change PPE for each designated work area

II. Room environment and equipment

II.1. Temperature and humidity

Monitor temperature and humidity to enable proper functionality of instruments. Liquid handling equipment, for example, is highly sensitive to changes in humidity. At this time, reference values for acceptable ranges of temperature and humidity values have not been established.

II.2. Monitor air and surface contamination in work stations

Establish schedules for monitoring: swab surfaces to check for surface contamination and leave open tubes over night to check for aerosol contamination. A monthly schedule is recommended. The PCR used may be the one that has the highest throughput in the lab. Analyze data to determine source and frequency of contamination.

II.3. Work stations, hoods, biosafety cabinets

Hoods should be used for master mix preparation as much as possible. Desktop PCR cabinets/workstations (with and without HEPA filters) provide enclosed and dedicated spaces for different tasks when individual rooms are not available. The blower may be turned on. Biosafety cabinets (BSL-2 hoods) may be used as well; they should be certified annually. Hoods ducted to the outside may also be used for small amounts of Trizol, Phenol, or beta-mercaptoethanol; check with applicable regulations.

II.4. Pipettors

Designate an individual set of pipettors for each work station. All pipettors should be calibrated annually (certificate needs to be on hand). Depending on the frequency of use, they should also undergo a functionality test (weight/volume check in the laboratory) at least once a year or as often as necessary (i.e. after a malfunction that has been corrected). Calibration may be performed by an external vendor. Functionality should be verified by a designated (i.e. internally certified) technician upon return of the pipettor(s) to the laboratory.

II.5. Thermocyclers:

II.5.1. Real-Time PCR Systems:
Read instrument manual for thorough understanding of system’s technology and guidelines. Perform maintenance, background checks, and calibrations as instructed by the manufacturer. For instruments under a manufacturer’s service contract, occasional background checks should still be performed by the laboratory. Monitor assay performance by use of appropriate controls for each assay. Three consecutive runs resulting in assay failures for more than one assay may indicate PCR system problems.

II.5.2. End-Point PCR Systems
Read instrument manual for thorough understanding of system’s technology and guidelines. Monitor assay performance by using appropriate controls for each assay. If desired or deemed necessary, perform system performance verification as follows:

- Perform serial (10x or 5x) dilution of an assay-specific amplification control.
- Perform PCR on all dilutions and analyze amplicons using an appropriate percentage agarose gel. Determine the highest dilution factor that produced amplicons which are still visible on the agarose gel. Use a dilution which is 10-100 times more concentrated than the weakest signal in order to test each well in a thermocycler. Perform the PCR, and analyze amplicons on agarose gel. When all wells of the plate have produced a band of about equal intensity, the instrument can be considered fully functional.

II.6. Ice buckets
There are multiple options; one or more can be selected as necessary and applicable for each laboratory.

- II.6.1. Disposable Styrofoam containers (soup cup size)
- II.6.2. Designated ice beads containers/ice buckets for each work station
- II.6.3. Clean Styrofoam shipping boxes that may be discarded weekly

II.7. Automated Extraction Instruments
Perform maintenance, background checks, and calibrations as instructed by the manufacturer and following AAVLD guidelines. If an internal control is used with each extraction, monitor the performance of this control using the accepted ranges for the internal control for verification of system performance. Also, if equipment is moved to another location, or if it is otherwise deemed necessary then perform system performance verification as follows:

- Extract a complete plate of known positive samples in a matrix of choice to determine if all the magnetic pins are performing properly. (The pins may become bent or misaligned causing a failed extraction/purification)

III. Disinfection and cleaning
A stringent cleaning routine needs to be in place to keep all work areas, all bench top work surfaces, and all hoods clean and disinfected. One or more of the following recommendations may be selected as necessary.

- III.1. Spray and wipe surfaces with 10% bleach followed by 70% alcohol to avoid corrosion of metal
- III.2. Spray and wipe surfaces using Virkon® S according to label instructions (except California)
- III.3. Wipe surfaces with bleach wipes (commercial or freshly made) or ‘bleach-in-a-bottle’
III.4. Use the interior UV lights of hoods for their decontamination (timers turn automatically off after 1-2 hours) whenever not in use and/or in between assay setups. Available PRRSV PCR data indicate, however, that UV light alone is NOT a reliable way to control contamination.

IV. Personnel:
Performing diagnostic PCR assays requires good laboratory techniques and highly trained personnel to ensure validity of results. Recommended requirements for new personnel are B.S. degree or equivalent training in Biology, Microbiology, Virology, Biotechnology or Bioengineering, Biochemistry/Chemistry, and equivalent. Preferred skills include analytical, mathematical, organizational, and communication skills. Preferred attributes include dedicated, self-motivated; shows attention to detail, good work ethics, team-oriented person. Preferred experience includes repetitive tasks, and handling and processing diverse biological and environmental samples.
Personnel of a PCR laboratory should be cross trained on all methods and processes to ensure uninterrupted workflow. Cross training and rotations may also provide better job satisfaction. The laboratory should have a proficiency test plan in place that all relevant personnel should participate in.

V. Reagents Quality Control:
Core reagents and supplies should be purchased from reputable vendors with good quality control practices to ensure best results and cost efficiencies. In-house prepared reagents are acceptable with sufficient quality control testing. If laboratories use bulk mastermixes (all components except target), laboratories must have data to demonstrate that there is no significant decrease in assay sensitivity (e.g. positive and negative PCR control, trend tracking or refer to Method Comparison document).
Perform Quality Control testing on each batch of in-house reagents. Quality Control testing should include comparison of “test” and “control” lots of reagents; “test” denotes newly prepared lot and “control” denotes previously tested lots. Passing criteria should be established for specific reagents. Re-quantification of primers and probe upon receipt from the manufacturer is considered a best practice in some laboratories. A method for primer/probe QC is detailed at the Life Technologies website. Include appropriate extraction and amplification controls for each PCR test to monitor consistent and acceptable assay performance (refer to table in Section VII).

VI. Sample Acquisition and Handling

VI.1. Sample Acquisition
Proper collection of samples by technicians and veterinarians is important in order to obtain PCR results that are interpretable. Laboratories should post collection instructions on their web site. The instructions should begin with sample acquisition on farm and in the clinic. Volume of sample and tube size (automation) should be specified. Recent vaccine history including type of vaccine and route should be recorded because modified live vaccine agents can be detected for weeks post vaccination. Vaccination at the same time of sample collection should be discouraged due to contamination concerns. Practitioners must also be cognizant of potential contamination from other environmental sources such as clothing.
and gloves. Blood samples should be collected using a new syringe and needle for each animal. Equipment should be disinfected with hypochlorite or peroxide based solutions because these degrade nucleic acid. However, thorough rinsing is required to prevent false PCR negative results due to residual bleach or peroxide. The timing of sample collection is critical. Viral infections are usually best detected when sampling occurs within 48 hours after onset of clinical signs. Consult laboratory if collection is outside of that time window. Alternatively, shedding of virus or bacteria may be intermittent and require collection on consecutive days. Instructions for pooling should be clearly stated as to whether this can occur on farm or will be done in the laboratory. Validation data should support pooling of 2 or more samples. Often, pooling potential is predicted from the CT level of individual samples and is not empirically derived. Clients should be informed whether it is a predicted number or derived from actual testing. Some veterinary practices require use of substances that may contain PCR inhibitors. Among these are: tattoo ink, milk containers for earnotch samples, EDTA and heparin anticoagulants, bleach, and bacterial transport media. Proper controls to test for the presence of inhibitors are recommended (section VII).

VI.2. Sample transport

Optimally, samples suspected of containing viral, mycoplasma or chlamydial agents are best obtained with dacron or polyester tip, plastic applicator shaft, and sent chilled in viral transport media. Alternatively, a sealed, sterile vial with several drops of saline can be a satisfactory alternative for viral and bacterial detection. Even dry swabs can be suitable if validation data exists to support their use. The following have been found to be unsuitable: viral transport media brands which are stored at room temperature, calcium alginate swabs and bacterial transport media. Samples should be sent using cold packs and next day delivery recommended. Some sample types require special conditions, e.g. semen should be shipped in a liquid nitrogen vapor tank.

VI.3. Processing at the Diagnostic lab

In the laboratory, decontamination of instruments used for preparation of samples should be described in laboratory protocols or disposable tools should be used (e.g. scalpel, biopsy punches or microtome blades). Provide instruction regarding decontamination of surfaces and instruments used in necropsy. Samples potentially containing viral agents should be refrigerated and tested within 24 hr or stored at -70/-80C. Samples potentially containing certain bacteria or parasites (e.g. Cryptococcus) should not be frozen because pre-enrichment may be necessary prior to PCR amplification. Same quality and suitability should be carefully assessed prior to processing. Unsuitable swabs, transport media, or sample type as well as degradation may require rejection or a qualifying comment. In some cases an inappropriate sample is the only sample available. The client should be informed of the unsuitable sample and if the sample is still tested, results should be reported with a cautionary statement.
## VII Appropriate controls - The recommended controls are described in this chart

<table>
<thead>
<tr>
<th>Controls</th>
<th>Definition What is it?</th>
<th>Purpose</th>
<th>Recommended</th>
<th>Best practice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Extraction/purification controls</strong></td>
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<tr>
<td>Internal control</td>
<td>1. Amplifiable synthetic nucleic acid (e.g. ultramer, xeno RNA) added to every sample or endogenous housekeeping gene (e.g. 18s). 2. Present in all samples tested *</td>
<td>Assess efficacy of the extraction and purification method for each sample. Also assess presence of inhibitors which may vary from sample to sample</td>
<td>No, with the following qualifier: After a date to be determined it is strongly recommended that new or replacement assays incorporate an internal control.</td>
<td>Yes</td>
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<tr>
<td>Positive for the extraction/purification step(s) control (one per run/plate)</td>
<td>1. A well characterized positive field sample in a relevant matrix; or synthetic nucleic acid containing the target sequence spiked into the lysis solution 2. Present in one well of each assay</td>
<td>Assess efficacy of reagent batch and technical performance. Also assess reagent lots and technical performance reproducibility</td>
<td>A positive extraction control for each matrix type is recommended. PEC not necessary if IC is used.</td>
<td>Yes</td>
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<tr>
<td>Negative extraction / purification control (one per run/plate)</td>
<td>1. Buffer, PBS, DMEM, VTM, BHI (if the kit contains sufficient carrier) or well characterized negative field sample. 2. Present in one well of each assay</td>
<td>Assess target contamination in extraction reagents</td>
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<td>Yes</td>
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<td><strong>Amplification controls</strong></td>
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<tr>
<td>Positive amplification Control</td>
<td>1. Synthetic nucleic acid or purified target from a well characterized field sample or reference material. 2. Present in one well of each assay/run</td>
<td>Assess PCR mastermix functionality</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>No template control (NTC)</td>
<td>All the components of the PCR master mix with sterile nuclease free water in place of template 2. Present in one well of each assay</td>
<td>Assess target contamination in PCR reagents</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td><strong>qPCR only</strong></td>
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<tr>
<td>Copy number control</td>
<td>1. Transcribed RNA or synthetic DNA that can be quantified without associated cellular nucleic acid 2. Must generate a standard curve</td>
<td>Control to monitor the LOD or copy number detection</td>
<td>Yes if performing qPCR No if performing relative rPCR as is done in most Vet-D labs</td>
<td>Yes if performing qPCR</td>
</tr>
</tbody>
</table>
VII. References

Sample Transport and Handling


Internal Control


1. **Accuracy** - nearness of a measurement to a known/actual/true value. A reference standard of a known value can be used to assess the accuracy of a test method.

2. **Analyte / Target** - analyte and target are used interchangeably in polymerase chain reaction (PCR) methods. A specific component of a test sample that is detected or measured by the test method.

3. **Control sample** - Sample(s) or reagents incorporated in every run of the assay to determine whether the assay is performing within predetermined statistical parameters.
   - Types of controls include:
     - Extraction control (positive and negative) – to assess the efficiency of extraction process
     - Internal control (could be synthetic nonsense sequence) test for inhibitors and efficiency of extraction
     - Amplification control (positive and negative) – to assess the PCR reaction efficiency
     - No template control (NTC) – to evaluate for reagent contamination

4. **Cut-off** - the value used to distinguish between negative and positive results; may include indeterminate or suspicious zone.

5. **Cycle threshold (Ct)** – the cycle at which a value rises above the threshold.

   - **Threshold** – A level of delta Rn used for the Ct determination in real-time assays. The level is set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve. The threshold is the line whose intersection with the amplification plot defines the Ct (threshold cycle).

6. **False-negative reaction** - a known positive sample testing negative.

7. **False-positive reaction** - a known negative sample testing positive.

8. **Fitness for purpose** - characteristics of a test method and related procedures that relate to their suitability for one or more specific diagnostic application(s).

9. **Linearity** - defines the capacity of the method to obtain test results proportional to the concentration of the target analyte.

10. **Linear range** - interval of analyte concentrations over which the method provides suitable precision and accuracy.

11. **Matrix** - all the constituents of a test sample with the exception of the analyte.

12. **Optimisation** - process by which all of the physical, chemical and biological parameters of an assay are evaluated and adjusted in order to ensure that the performance characteristics of the test method are best suited to the intended application.

13. **Performance characteristic** - an attribute of a test method that may include analytical sensitivity and specificity, accuracy and precision, diagnostic sensitivity (DSe) and specificity (DSP) and/or repeatability and reproducibility.
14. **Precision** - the degree of dispersion of results for a repeatedly tested sample tested under similar conditions.

15. **Predictive Value (Negative)** - the probability that an animal is free from exposure or infection given that it tests negative; predictive values are a function of the Diagnostic sensitivity (DSe) and Diagnostic Specificity (DSp) of the assay and the prevalence of infection.

16. **Predictive Value (Positive)** - the probability that an animal has been exposed or infected given that it tests positive; predictive values are a function of the DSe and DSp of the assay and the prevalence of infection.

17. **Prevalence** - the proportion of infected animals in a population at one given point in time.

18. **Repeatability** - level of agreement between replicates of a sample both within (intra-assay) and between (inter-assay) runs of the same test method in a given laboratory.

19. **Reproducibility** - ability of a test method to provide consistent results when applied to aliquots of the same sample tested by the same method in different laboratories.

20. **Robustness** - measure of an assay’s capacity to remain unaffected by small changes or variations introduced in test conditions to mimic anticipated routine laboratory operation, part of optimization studies and reflected in repeatability assessments (e.g. incubation times, reaction temperatures, buffer pH/ionic strength, reagent dilutions, sample condition and/or preparation, etc.).

21. **Ruggedness** - a measure of an assay’s capacity to remain unaffected by substantial changes or substitutions in test conditions anticipated in multi-laboratory utilization, part of fitness studies and reproducibility assessments (e.g. shipping conditions, technology transfer, reagent batches, equipment, testing platforms and/or environments).

22. **Sensitivity**
   a. **Analytical Sensitivity** - synonymous with ‘Limit of Detection’, smallest detectable amount of analyte that can be measured with a defined certainty.

b. **Diagnostic Sensitivity** (DSe) - proportion of known infected reference animals that test positive in the assay; infected animals that test negative are considered to have false-negative results.

c. **Relative Sensitivity** - proportion of reference samples, defined as positive by one or a combination of test methods that also test positive in the assay being compared.

23. **Specificity**
   a. **Analytical Specificity** - degree to which the assay distinguishes between the target analyte and other components in the sample matrix.

b. **Diagnostic Specificity** (DSp) - proportion of known uninfected reference animals that test negative in the assay; uninfected reference animals that test positive are considered to have false-positive results.

c. **Relative Specificity** - proportion of reference samples, defined as negative by one or a combination of test methods, that also test negative in the assay being compared.
24. **Standardization** - process by which a test method is calibrated to a uniform level of analytical sensitivity and adjusted to consistent level of diagnostic agreement when compared to a recognized standard test method, usually achieved by use of calibration reagents and test panels that are typically provided by a reference laboratory.

25. **Validation** - process through which a test method is confirmed to be fit for its intended purpose, against specified requirements for a particular diagnostic application; may also include continuous monitoring.

   a. **Bench/Analytical Validation** – process through which the analytical performance characteristics of a test method are optimized and standardized, may include analytical sensitivity and specificity, accuracy and precision, repeatability, and robustness criteria, as well as, analytical comparisons to other test methods. Note - this process necessarily precedes Field Validation, and an assay cannot be considered fit for most diagnostic purposes without the latter.

   b. **Field/Diagnostic Validation** - process through which the performance characteristics (may include diagnostic sensitivity and specificity) of a test method are established using diagnostic samples, field samples or samples from experimentally infected animals. Note - recommended only for assays that have been thoroughly bench validated.

   c. **Verification** - process by which a laboratory demonstrates a previously validated assay will perform according to its established parameters.

**Types of PCR:**

**Conventional PCR** – real time PCR (rPCR)– quantitative real time PCR
The PCR method originally invented by Kari Mullis is still common to the PCR methods used today. The basic principle is still the exponential amplification of sometimes minute amounts of target nucleic acids in a sample. The difference between the methods lies in the data analysis.

**Conventional PCR**
Conventional PCR requires only a standard thermocycler with heating and cooling capability and is also called end-point PCR: once DNA amplification has been terminated (typically after 40 to 45 cycles) an aliquot of the resulting DNA has to be run on an agarose gel to visualize whether the PCR has been successful, i.e. if there is an amplicon present. Determinations of DNA concentration can be accomplished by running a molecular weight and concentration ladder on the gel with the sample but quantitation in general is not very accurate and does not allow inferences to be made about the concentration of target in the original sample. The limit of detection of DNA in an agarose gel is about 5-10ng so it is possible that a weak positive result goes unnoticed.

**Real time PCR** (TaqMan assay, 5’ nuclease assay)
This method employs, in addition to the primers used in conventional PCR, a third oligonucleotide, commonly called probe. Real time PCR takes advantage of the 5’ to 3’ exonuclease activity of the Taq polymerase. The probe must be complementary to the target DNA located between the two primers and the melting temperature must be several degrees higher than the primers’ melting temperature so that it binds to the target before the primers do. The probe is labeled at the 5’ end with a fluorescent dye (reporter dye) and at the 3’ end with a quencher. The immediate proximity of the reporter and the quencher prevents fluorescence but as soon as the primers have annealed, Taq polymerase has bound and replication commences, the probe is degraded through the Taq’s exonuclease activity. Once removed from the quencher’s immediate proximity, the reporter dye starts fluorescing when externally excited and the increase of fluorescence over time indicates the PCR reaction is successfully carried out.
Real time PCR instruments incorporate visualization of the PCR results as the software measures any changes in fluorescence over the time of the cycling and computes them in relation to a reference dye that is added to the reaction and does not change. The resulting graph in the case of a positive reaction shows the lag time (amplification below the threshold), the exponential phase (amount of fluorescence is proportional to the amount of target in the sample), and the plateau phase (reagents start to exhaust, no more amplification is taking place). Thresholds and baseline values need to be set to enable data analysis. While real time instruments are much more expensive than conventional cyclers and the TaqMan probes are very expensive, too, the fact that no agarose gel is required for visualization of results and data analysis represents a significant advantage. The relationship between visible fluorescence and amount of target DNA in the sample makes the standard real time PCR semiquantitative.

MGB (minor groove binder) and LNA (locked nucleic acid) probes represent modifications of the standard TaqMan probe that allow for a shorter probe with no tolerance for mismatches between probe and target. DNA intercalating dyes like SYBR Green can be added to a real time PCR reaction in place of a probe as it only fluoresces in the presence of double-stranded DNA, i.e. when PCR has been successful and an amplicon has been created.

Semiquantitative real time PCR [relative quantification by real time (rPCR) or reverse transcriptase real time PCR (rRT-PCR)]

The absolute quantitation of nucleic acid targets requires extensive and very costly extra effort (standard curve, running in triplicates) and, therefore, those assays are rarely performed in veterinary diagnostic settings. Alternatively, a relative quantification can be performed. Here, the $C_T$ of a sample is used by itself - in relation to a thoroughly characterized positive control for the run and an equally well characterized internal control in every well – for relative or rough estimation of amount of target nucleic acid in the sample. Highly consistent performance of the positive control and the internal control are required for those estimates to be meaningful.

Quantitative real time PCR

Due to the linear range of amplification when the amount of fluorescence is directly proportional to the amount of target, the real time PCR offers the possibility to actually quantitate the amount of DNA or RNA in a sample. While the actual method is essentially the same, quantitation requires the addition of standard samples of known quantity to be run with the unknown samples. A typical standard curve consists of a serial 10-fold dilutions of a sample where DNA concentration has been determined and is expressed as DNA copies/ml or /ul. The more points the standard curve has the better it can perform. Ideally, the linear range encompasses up to 7 points, i.e. from $10^7$ to $10^1$ copies/ml. Standard curve points and the samples should be run in triplicate to be statistically meaningful and to enable calculation of uncertainty of measurement. The known quantities need to be entered into the software and once the run has finished, the software compares the $C_T$ of the standard curve points with the $C_T$ of the samples and estimates the quantity. Strict quality parameters should be employed, (i.e. slope and $r^2$), to make the quantitation useful.

Acronyms

1. Real-Time Polymerase Chain Reaction (DNA template) rPCR
2. Real-Time Reverse Transcriptase Polymerase Chain Reaction (RNA template) RT-rPCR

* OIE is the source document for definitions #1-25 in this document
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