CHAPTER 1.1.5.

VALIDATION AND QUALITY CONTROL OF POLYMERASE CHAIN REACTION METHODS USED FOR THE DIAGNOSIS OF INFECTIOUS DISEASES

INTRODUCTION

The diagnosis of infectious diseases is performed by direct and/or indirect detection of infectious agents. By direct methods, the particles of the agents and/or their components, such as nucleic acids, structural or non-structural proteins, enzymes, etc., are detected. The indirect methods demonstrate the antibodies induced by the infections.

The most common direct detection methods are isolation or in-vitro *cultivation, electron microscopy, immunofluorescence, immunohistochemistry, antigen enzyme-linked immunosorbent assay* (*antigen-ELISA*), *nucleic-acid hybridisation* (*NAH*), *macro- and microarrays and the various techniques of nucleic acid amplification, such as the polymerase chain reaction* (*PCR*) or the *isothermal amplification methods, such as nucleic acid sequence based amplification* (*NASBA*), *Invader or loop-mediated isothermal amplification* (*LAMP*). *As NAH, macro- and microarrays and the various amplification assays have nucleic acid molecules as targets, they are also termed methods of molecular diagnosis.*

The most common indirect methods of infectious agent detection are serological assays, such as virus neutralisation, antibody-ELISA, haemagglutination inhibition tests, followed by the recently appearing novel methods, such as biosensors, bioluminometry, fluorescence polarisation, chemoluminescence, etc. In general, diagnostic laboratories simultaneously apply both the direct and the indirect methods, in order to provide more certainty in a diagnosis.

The experiences of the last two decades indicate that the PCR techniques will eventually supersede many of the classical direct methods of infectious agent detection. It is clear that the PCR is replacing virus isolation or bacteria cultivation for the detection of agents that are difficult or impossible to culture. There are several reasons for this trend, including that virus isolation requires: i) the presence of replicating organisms (viruses or bacteria); ii) expensive cell culture and maintenance facilities; iii) as long as several weeks to complete the diagnosis in some instances; and iv) special expertise, which is missing or diminishing today in many laboratories. Although PCR assays were initially expensive and cumbersome to use, they have now become relatively inexpensive, safe and user-friendly tools in diagnostic laboratories (2–4, 6, 7, 11–13). The sensitivity and specificity of PCR is generally greater than isolation or antigen capture ELISA procedures. The introduction of various real-time PCR methods, nucleic acid extraction robots, and automated work stations has resulted today in a large arsenal of high throughput, robust, very rapid and reliable assays for molecular diagnosis. In this chapter the diagnostic applicability of the various PCR methods is summarised with special regard to international harmonisation and validation.

A. PCR METHODS USED IN ROUTINE MOLECULAR DIAGNOSTICS

1. The principles of the PCR

Polymerase chain reaction (PCR) implies that there is an enzyme-based amplification reaction in the assay. The term ‘chain reaction’ refers to several cycles of copying a specified stretch of DNA, in this case from the genome of an infectious agent. The region to be amplified is defined by two (or more) short nucleotide sequences, termed primer sites that flank the target sequence. Primers, short oligonucleotides that are complementary to the primer sites, bind to the DNA strand to be copied. Using a polymerase, which is not denatured during heat cycling, it is possible to copy the target sequence by joining free nucleotides to the primers. By repeating the heat-cycling

regime 20–40 times, the amount of copied target DNA increases exponentially, producing enough for further operations, such as detection, cloning or sequencing. The diagnostic sensitivity of the PCR is very high because several million copies of the selected target are produced. The specificity may also be very high, as determined by the specific nucleotide sequences of the selected target, as well as primer design. The primers can be designed to detect very specific nucleotide sequences in the genomes of the selected target infectious agents, or can be designed to be complementary to more conserved regions, thus enabling detection of members within a family or genus of infectious agent. A more detailed overview of molecular techniques has been published (17).

a) DNA amplification

If the genome of the infectious agent is DNA, the amplification is performed directly, with or without previous purification of the target DNA. In many cases, use of DNA extracted and purified from the material to be tested (e.g. blood) will result in increased analytical and diagnostic sensitivity.

b) RNA amplification (reverse-transcription PCR)

The genomes of many infectious agents contain ribonucleic acid (RNA) that cannot be amplified directly by the PCR. For PCR amplification, a single-stranded DNA target is necessary, and this is not available in the case of RNA viruses. This problem can be solved by the addition of a step before the PCR is begun. Using reverse transcriptase, it is possible to transcribe the RNA into complementary DNA (cDNA), which can be used in a PCR assay (the procedure is termed reverse transcriptase PCR). Traditionally, the reverse transcription reaction is performed in a separate reaction vessel and the cDNA produced is then transferred to a new tube for the PCR. However, heat-stable DNA polymerases with reverse transcriptase activity and specific buffers in which RT and DNA polymerases are active are now readily available. Both allow a reverse-transcription PCR amplification to take place in the same tube in direct sequence without any further handling and with less chance of carry-over contamination. In most cases, it will be necessary to extract and purify RNA prior to reverse transcription.

c) PCR amplicon detection

The PCR product, or amplicon, can be detected using a variety of procedures. The most common include nonspecific detection of the PCR product based on amplicon size using electrophoresis in agarose gel and staining of the DNA with a nonspecific, intercalating dye, such as ethidium bromide (it is now possible to replace the latter with non-carcinogenic dyes, e.g. GelRed), or specific recognition of the amplified target sequence using Southern blot transfer of the DNA followed by hybridisation with oligonucleotide probes complementary to the target sequence. Hybridisation probes can be enzyme, chemiluminescent, or radionucleotide-labelled to allow visual detection of the specific target sequence.

Some examples of PCR methods currently used are given below.

2. Conventional PCR

'Conventional PCR' (or simply PCR) uses one pair of oligonucleotide primers to amplify a small part of the genome of the infectious agent. Analytical sensitivity is typically high with a minimum number of 100 to 1000 copies of the target DNA detectable. Analytical specificity can be high, dependent on target selection, primer design, and assay optimisation. Both analytical sensitivity and specificity can be further improved by applying nested PCR (see point 3 below). Detection methods, such as Southern blotting followed by hybridisation probes, can further improve sensitivity and specificity, but are time-consuming, require laboratory handling of amplified DNA, and the interpretation of results can be technically subjective. Based on complexity and expense, these detection methods are not generally considered suitable procedures for common practice in diagnostic laboratories today.

3. Nested PCR

Nested PCR assays use two sets of amplification cycles with four primers, termed external and internal primers. In general, nested PCR assays provide higher analytical sensitivity and specificity compared with conventional PCR assays. However, there is a substantial increased risk of cross contamination as products from the first round of amplification are often used as the starting template in the second round, resulting in the transfer of material between different PCR tubes. The nested PCR has been largely replaced by real-time PCR protocols, which are equally sensitive but have much less risk of contamination. The limit of detection with the nested PCR is typically <10 genomic copies of the target DNA, and analytical specificity is also enhanced because in the nested PCR, four oligonucleotide primers have to bind specifically to the selected targets in order to yield a positive reaction (4).

4. Real-time PCR

Real-time PCR differs from standard PCR; here the amplified PCR products are detected directly during the amplification cycles, using hybridisation probes, which enhance assay specificity. Various real-time methods, such as TaqMan, Scorpion primers, fluorescence resonance energy transfer (FRET), Primer-Probe Energy Transfer

(PriProET), SybrGreen, Light-Upon-eXtension (LUX) and the Molecular Beacon assays have become popular tools for detection of infectious agents. Real-time PCR has been used for the detection of bacteria, viruses or parasites from a range of animal species (2–4, 14, 17). These assays have several advantages over the 'classical' conventional or nested PCR methods. In general, only one primer pair is used, providing sensitivity often close or equal to traditional nested PCR but with a much lower risk of contamination. Fluorescence, indicating the presence of the amplified product, is measured through the lid or side of the reaction vessel, thus there is no need for post-PCR handling of the amplified DNA. These procedures are considerably less time-consuming compared with traditional post-amplification PCR product detection in agarose gels followed by ethidium bromide or equivalent DNA detection stain and again, the risk of contamination is reduced. The use of a 96-well microtitre plate format, without the need for nested PCR, allows the procedure to be automated and suitable for large-scale testing (10, 17). Diagnosis can be further automated by using robots for DNA/RNA extractions and pipetting. Compared with classical amplification methods, a further advantage of the real-time PCR is that it is possible to perform quantitative assays (6, 7). Using real time PCR, the diagnostic time can be shortened from hours to minutes. Real-time PCR can also be used for reverse-transcription PCR using one-step protocols, thus enabling the RT-step and PCR to take place in the same tube during the same PCR protocol (17).

5. Multiplex PCR

PCR using multiple primers directed at different targets in a single assay are referred to as multiplex PCR assays. In multiplex PCR, various infectious agents can be detected and differentiated in a single reaction vessel at the same time. The different PCR targets amplified in a standard PCR assay are identified based on PCR product size. The use of 'classical' nested PCR methods for the construction of a multiplex assay is complicated by the need for targets of different sizes, as well as primers that may 'compete' with each other in the same reaction mix, both of which can negatively impact PCR efficiency. In contrast, the concept of real-time PCR (single primer pairs) provides excellent possibilities for the construction of highly sensitive multiplex systems (4, 9) based on more uniform target size, uniform amplification conditions, and differential detection of targets using specific hybridisation probes labelled with different fluorophores. It should also be noted that common primers can be used to amplify specific regions of the genome of a group of pathogens and fluorogenic (TaqMan) probes can then be employed to discriminate between members of the group. This is not strictly multiplex PCR although it may mistakenly be described as such.

6. Further methods of molecular diagnosis

Although this chapter focuses on the PCR-based molecular diagnosis, it should be briefly mentioned that besides PCR, there are many other novel methods being introduced for the molecular detection of pathogens, for example the various isothermal amplification methods (such as nucleic acid sequence based amplification [NASBA], Invader or loop-mediated isothermal amplification [LAMP] technologies), various macro- and microarrays using padlock probes, rolling cycle amplification and other molecular approaches. There are also other approaches being assessed to detect and analyse PCR products, such as MALDI and Luminex. The advantage of such approaches, in combination with the multiplex PCR, is that typing of different strains or types by of an organism becomes possible. It is evident that the arsenal of molecular diagnosis is further strengthened by these methods.

B. PRINCIPLES OF ASSAY VALIDATION FOR NUCLEIC ACID DETECTION TESTS

When performing analyses of clinical material it is important to produce data of good quality. For this, some key criteria have to be fulfilled. The establishment of quality assurance (QA) and quality control (QC) systems is required, i.e. a set of quality protocols, including the use of control samples that ensure that the system is working properly and confirms data reproducibility and quality. QA and QC systems, together with trained and competent personnel, have already been established in many laboratories world-wide. Assay validation is another essential factor for assuring that test results reflect the true status of the samples (8).

To predict the diagnostic performance of a diagnostic assay, it is necessary to use a validation methodology to document the expected performance of the assay in question. Validation is the evaluation of a diagnostic assay for the purpose of determining how fit the assay is for a particular use. The general principles of assay validation can be found in Chapter 1.1.4 Principles of validation of diagnostic assays for infectious disease. This chapter extends these validation principles to molecular diagnostic assays. For explanations of terms and definitions please consult Chapter 1.1.4.

C. ASSAY VALIDATION – INTRODUCTION

1. Selection of an assay fit for its intended purpose

The fitness of PCR assays for various purposes is broad. Wherever there is a need for direct detection of an infectious agent, it is generally possible to use PCR. During the first years of PCR diagnostic development, many

laboratories had problems with contamination and performance; thus PCR had a poor reputation as a technique suitable for diagnostic use. Achievements in recent years have reversed that view. New technology (i.e. real-time PCR) has made the technique less prone to producing false positive results caused by contamination and is easier to use. Furthermore, automating the extraction and pipetting procedures using robots has substantially lowered the costs, enhanced repeatability and reduced the required work-load. During the 'early years' many in-house assays were developed, and harmonisation and validation were poor or non-existent. The OIE, National Laboratories and the European Community Reference Laboratories (ECRLs) have an important role to play in driving the validation and harmonisation work forward. It is fair to say that PCR, as it is performed today, is safe (substantially lower risk of false-positive results), usually validated in some form and fit for its intended purposes. Some specific examples of the importance of PCR are given below, definitions of intended purpose(s) can be found in Chapter 1.1.4.

- To diagnose infection when antibody levels are so low that previous exposure cannot be confirmed by an antibody test (e.g. enzyme-linked immunosorbent assay [ELISA] repeatedly in the 'gray zone' during the bovine leukaemia eradication programmes).

- To discriminate between infection and maternal immunity in young animals (e.g. young calves in eradication programmes).

- To detect viral or bacterial nucleic acid when the diagnostic specimen is not suitable for virus isolation due to toxicity (e.g. semen, exam of mummified fetus).

- In the final stage of eradication programmes, when thorough investigation of single cases is necessary (e.g. herpesvirus latency and single reactor animals during the Aujeszky's disease eradication programmes).

- To discriminate vaccine strains from field viruses (DIVA [differentiating infected from vaccinated animals] approaches).

- To determine phylogenetic relationship of viruses and use this information for molecular epizootiology.

- To enable fast and safe first diagnosis in outbreak situations (e.g. the 2006 outbreaks of highly pathogenic avian influenza).

- To determine the viral load (e.g. in porcine circovirus type 2 infections).

- Rapid monitoring of vaccinated animals that appear to have clinical signs.

- Detection of drug resistant mutants of pathogens, etc.

- To demonstrate freedom of infection in live animals or animal products. However, it has to be noted that some infected animals may have no detectable nucleic acid in the tissues being examined.

2. Initial assay development considerations

a) Precautions and controls

Considering the uncertainty about the safety and reliability of the PCR in routine diagnosis, special precautions should be applied in any laboratory using PCR for detecting infectious agents so as to avoid false-positive or false-negative results. These, together with internal controls (e.g. mimics) assure the safe evaluation of the results, free from false-positive results caused by contamination. True internal controls using house-keeping genes also complete with the target PCR but only for reagents that are in vast excess such as the polymerase and nucleotides. Minimal competition implies that the level of the true target is known, which cannot be the case for field samples. Armoured RNAs allow the mimic to be added in the extraction process, which is a step towards knowing that the extraction has worked. A true internal control provides more confidence that the extraction has been performed correctly. The down side of using housekeeping genes as internal controls is that they can be present in greater amounts than target pathogens.

b) Precautions taken to avoid false-positive results

False-positive results (negative samples showing a positive reaction), may arise from either laboratory-related issues, such as cross-contamination, or assay-related factors, such as inefficient optimisation or assay performance. Product carry-over from positive samples or, more commonly, from cross-contamination by PCR products from earlier experiments is a possible source of error, and various practices and tools have been applied to prevent false-positive PCR results. Samples and reagents should be handled in separate

laminar air-flow hoods, which are regularly decontaminated using UV light (the use of UV-light demands very careful maintenance to be effective) and bleach. Constructing and using special tube-holders and openers can also help to prevent false-positives (2). In addition, good laboratory practices should be applied, i.e. to perform the basic steps (DNA extraction, mix and primer preparation, sample preparation, agarose gel electrophoresis of amplification products, etc.) in separated laboratory areas or rooms (Figure 1; refs 1, 4, 17). Different sets of pipettes should be used for each of the steps. The use of positive displacement and filtered tips is advisable. It is also, if possible, advisable to have different persons perform the different steps, who are restricted to the respective laboratory areas. Precautions should be taken to prevent the introduction of amplified material from potentially contaminated laboratories into 'clean' laboratory areas by movement restrictions on samples, papers, equipment, persons or any other potential method of contamination. Movement in the opposite direction should only occur after surface decontamination of equipment and tubes etc. and changing of laboratory coats and gloves. If the sample is expected to have a high amount of agent or target nucleic acid, it is preferable to dilute it prior to introducing it into 'clean' laboratory areas.

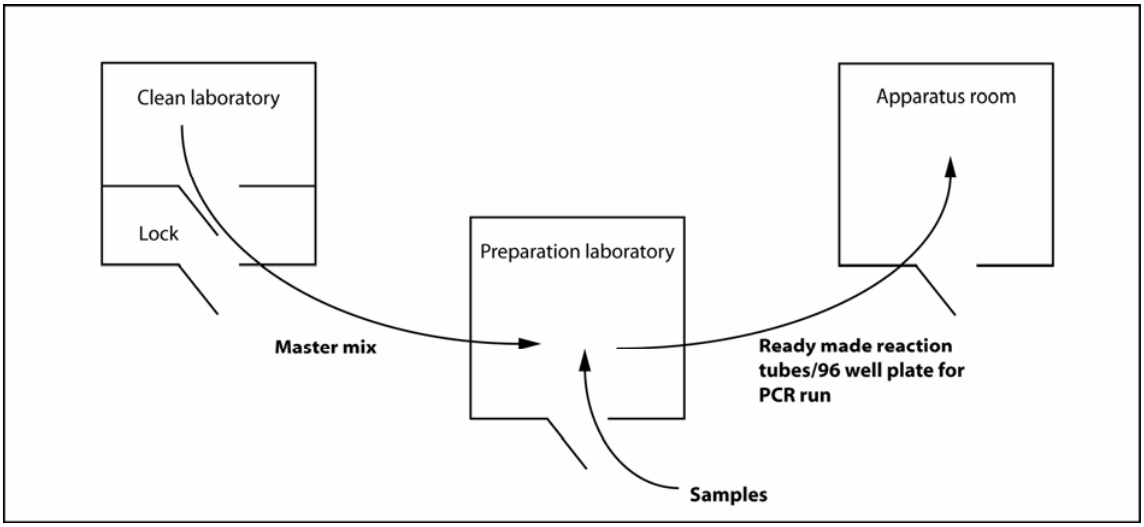

Figure 1. *Recommended laboratory set-up for diagnostic real-time PCR. Samples to be analysed are transferred into the 'Preparation laboratory' for extraction of nucleic acid. PCR master mix is prepared in the 'Clean laboratory' and transferred to the 'Preparation laboratory' for dispensing into PCR plates and adding template. Ready-made reaction tubes/plates are subsequently transferred into the 'Apparatus room' for the PCR run. The 'Clean laboratory' is used only for preparing PCR master mix; no DNA or PCR products are allowed in the room. It is advisable to have an air lock entryway into the 'Clean laboratory' for changing into a lab coat and shoes that are only used herein. The 'Preparation laboratory' is used for processing samples and setting up PCR reactions (with master mix prepared in the 'Clean laboratory'). No PCR products are allowed in this room and nothing from the 'Preparation laboratory' goes back to the 'Clean laboratory'. The 'Apparatus room' contains the PCR machines and nothing from this room goes back to the 'Clean laboratory' or the 'Preparation laboratory'. If the laboratory has a controllable air system, the clean lab should be positive and the other two negative. If nested PCR is going to be performed, two more rooms are recommended. A 'Second PCR laboratory' for setting up the second PCR reaction (which will involve handling PCR products, thus making it impossible to do this in the "Preparation laboratory") and an 'Electrophoreses laboratory' for analysing PCR products in agarose gels.*

It is also very important to include negative controls, i.e. samples that are as similar to the test samples as possible but without having the target. In laboratories experiencing problems with cross-contamination, at least one negative control per five diagnostic samples has been recommended. Both positive and negative control samples should routinely be interspersed with diagnostic samples to assess PCR assay performance.

c) Precautions taken to avoid false-negative results

PCR has proven to be a very effective method of detecting nucleic acids, such as viral genomes in clinical specimens. However, an infected animal in the later phases of infection may no longer have viral nucleic acid in the tissues being examined. Consequently, in such cases the negative PCR results should be considered as one part of a complex diagnostic examination.

False-negative results (samples containing the agent of interest but tested as negative) occur mostly due to inhibitory effects and/or pipetting errors; however, issues attributable to sample handling can also yield false-

negative results. Therefore, internal controls can be used as indicators of PCR assay efficiency. PCR internal controls may include foreign DNA added to the sample or ubiquitous DNA naturally occurring in the sample. Foreign DNA added to the sample, may include DNA or RNA mimics. DNA mimics, manufactured oligonucleotides, have the same primer-binding sequences as the PCR target, but flank a heterologous DNA fragment of a different size. The identical primer-binding nucleotide sequences allow co-amplification of the target and the mimic in the same tube with minimal competition. The size differences provide easy discrimination by Southern blot analysis. Armored RNA®, an identical concept to DNA mimics, uses a control RNA fragment packaged in bacteriophage coat proteins to protect or stabilise the RNA for control or standardisation of RT-PCR assays (further details on internal controls, see above).

With real-time PCR assays, it is also possible to use internal controls, a naturally occurring housekeeping gene, a selected fragment of the host animal's genome such as beta-actin, GAPDH, or ribosomal RNA. By multiplexing such an intrinsic control with a specifically coloured reporter fluorophore, it is possible to check the sample quality and confirm PCR efficiency, as the target agent and intrinsic DNA are simultaneously detected (14).

Internal controls (for example 'mimics') increase the reliability of diagnostic PCR (1, 4). Caution must be used when designing and validating internal controls. Extensive testing is necessary to ensure that PCR amplification of the added internal control does not compete with the diagnostic PCR and thus lower the analytical sensitivity. Internal controls are used in concentrations slightly higher than the detection limit of the diagnostic PCR to ensure the test's performance. It should also be remembered that internal controls have a disadvantage, similar to spiked samples, in not being representative of target nucleic acid and can lead to false-negative results.

d) Preparation of standards

Reference laboratories should provide standard samples representative of a given infectious agent. Such samples can be cultivated infectious agents or clinical specimens, etc., which are distributed in such a manner that the infectious agent is well preserved. Thus the samples are distributed frozen, in organic solvents (e.g. Trizol) or other suitable ways. The samples can also be sent as nucleic acids (frozen, freeze-dried or in ethanol). For specific details, see the individual disease chapters. Reference laboratories should also provide the appropriate mimics.

The availability of standard samples is crucial for a successful assay validation. Unfortunately this is often the most problematic issue to solve when planning a validation project. It is not sufficient to use only cultivated agents or spiked samples; true samples from the field may have very different characteristics than these laboratory-generated samples. It might prove very difficult to obtain samples from the field that are truly negative or positive, especially as PCR generally has a higher analytical sensitivity than most 'Gold Standard' methods, thus making it difficult to determine the status of the intended validation samples using already established assays. As mentioned previously, reference laboratories might be one potential source for such standard samples. Alternatively, Bayesian methods offer probabilistic approaches to validate diagnostic tests in the absence of a gold standard (5), but are not further discussed in this chapter.

D. ASSAY VALIDATION – PART 1

1. Optimisation and standardisation of reagents and determination of critical control parameters

Sample collection, preparation and transport (see Chapter 1.1.1 Collection and shipment of diagnostic specimens) and nucleic acid extraction methods (see Chapter 1.1.7 Biotechnology in the diagnosis of infectious diseases and vaccine development) are all critical parameters in test performance and should be optimised for disease diagnosis. Suitable methods vary depending on sample and organism type. In general, blood serum, body tissues and swab samples are suitable samples for easy extraction of target nucleic acids, while faeces, autolysed material and semen samples are more difficult to handle. Extraction of RNA targets differs from extraction of DNA targets, and RNA is more prone to degradation. Both commercial (robotic, spin columns, magnet-based extractions, etc.) and standard chemistry-based methods are used for DNA or RNA extraction. It is crucial to determine the most reproducible and efficient extraction method before further validation of the assay is performed. If the method of extraction is changed, equivalency data should be generated or the entire validation procedure should be repeated.

All equipment used during the process must be properly maintained. Apparatus (heating blocks, refrigerators, freezers, thermocyclers, pipettes, etc.) that require calibration must be calibrated according to the laboratory's quality assurance protocols. It is also important to properly validate the equipment and protocols used. One good example is the recent implementation of robotic extraction methods for routine diagnostic processing. It is not

sufficient to compare the characteristics of this technique with that of the previously used extraction method. The robot and the protocol must be validated to confirm that there is no danger of cross-contamination, e.g. by running a set of mixed positive and negative samples.

When developing 'classical' or real-time PCR assays, all parameters, protocols and reagents need to be optimised. A standardised assay is a method that consistently gives the same result for a given sample when repeated several times and when performed by different analysts in different laboratories.

During the optimisation of the PCR assay, it is also possible to estimate the capacity of the method to remain unaffected by small changes in the main parameters. To achieve an optimised PCR assay, it is essential to evaluate critical parameters in the assay. Examples of such parameters include: incubation times and temperatures, concentrations of buffers, primers, $MgCl_2$, etc., pH, amounts of other components added (e.g. dNTP, bovine serum albumin, etc.). The characterisation of critical control parameters is crucial for identifying critical points that must be properly controlled in the assay. Intentional variations of parameters can lead to a preliminary expression of assay robustness.

2. Repeatability

Agreement between replicates within and between runs of the assay should be accessed at this stage. This gives important information about the assay before further validation is carried out. If excessive variability is encountered, it should be corrected before continuing the validation process.

Repeatability of a PCR assay requires that each replicate be treated as an independent sample. According to assess variation of a replicate (e.g. a triplicate), three individual aliquots of starting analyte are extracted and amplified, and the variation from the mean value detected is determined as an indication of repeatability. Thus, it is not acceptable to assess triplicate amplifications from one extraction. Likewise replicates from multiple runs must be treated as individual samples. This process will result in estimates of intra- and inter-assay variability. In a real-time PCR assay, the Ct-values produced from the replicated samples can be used to determine the inter-run coefficient of variation (CV; see Chapter 1.1.3 Quality management in veterinary testing laboratories, Section 6.d Uncertainty).

It is important that the analyte to be detected in PCR be in the same matrix as test samples destined for use in the assay. For example, if the assay is to be used for demonstrating freedom of an agent in a matrix known for PCR-inhibitory activity (such as semen with extenders), it is particularly important to thoroughly evaluate repeatability.

When new batches from new manufacturers of oligonucleotides or other reagents are introduced into the assay, the repeatability of the assay needs to be re-established on each occasion.

3. Determination of analytical specificity and sensitivity

Analytical specificity is defined as the ability of an assay to distinguish the target agent from other infectious agents. This ability is determined by analysing genetically related pathogens and clinical material obtained from animals with diseases that may mimic that for which the assay is being designed. It is desirable to obtain field samples from infected animals, but this may prove difficult or even impossible. In such cases viruses grown in cell culture can be used. Acceptable cross-reactivity is largely dependent on the intended purpose of the test and must be determined for each case. It is useful to perform '*in silico'* studies as an adjunct to laboratory assessment.

Analytical sensitivity (or limit of detection) is defined as the smallest amount of an agent detected by the assay, and may be represented as number of genome copies, infectious dose, colony-forming units, plaque forming units, etc. of the agent that can be detected and distinguished from a zero result. To determine analytical sensitivity, an end-point dilution is used until the assay can no longer detect the target in question in more than 5% of the replicates. Cloned fragments of the PCR products in question can be used as standard samples, either as DNA or for RNA targets, the RNA being transcribed *in vitro* into DNA. Estimates of analytical sensitivity can vary substantially for the same assay when different sample matrices are used. When setting up a dilution series, it is important to use a diluent that has qualities that are similar to the sample matrix, i.e. dilute positive semen in negative semen and not in buffer.

E. ASSAY VALIDATION – PART 2

Performance characteristics (or assay parameters) give information about how a method functions under specified conditions. Some typical performance characteristics are given in Chapter 1.1.4.

1. Determining assay performance characteristics

a) Reference animal populations

i) *Negative reference animals*

True negative samples, i.e. samples from animals that have had no possible exposure to the agent, can sometimes be difficult to obtain. Often it is possible to collect samples from countries that have eradicated the disease in question. It is important that the negative samples obtained be representative of the samples that will be analysed, i.e. species, age, sex, breed, etc.

ii) *Positive reference animals*

It is generally problematic to find positive reference animals in sufficient numbers. Naturally infected or experimentally infected animals are needed and their positive status is best demonstrated by isolation of the agent. Before using experimentally infected animals, please see Chapter 1.1.4.

iii) *Reference animal status determined by other assays*

The term 'gold standard' is commonly used to describe any standard of comparison and should be limited to methods that unequivocally classify animals as infected or uninfected. New PCR assays are generally expected to outperform any already existing 'gold standard' method and thus the established 'gold standard' may not suitable to use as a comparison. This is especially true when demonstrating that a negative reference animal is truly negative. Validation of molecular tests by comparing them to a 'gold standard' test may be complicated by the PCR being more sensitive, resulting in apparent reduced specificity. To an extent this may be resolved by assessing sample derivation, clinical history and sequencing any PCR products to confirm identity.

2. Threshold determination

Diagnostic sensitivity (DSe; proportion of known infected reference animals that test positive in the assay) and specificity (DSp; proportion of known uninfected reference animals that test negative in the assay). The number of reference samples required to determine estimates and allowable error of both DSe and DSp can be calculated. To do this, a reasonable prediction of both DSe and DSp must be used. Generally, confidence in the estimate is set at 95%. However, no formula can account for the numerous host/organism factors that can affect the outcome of the test. The number of samples to determine estimates of DSe and DSp is outlined in Chapter 1.1.4. For a disease that is not endemic or widespread, it may be difficult initially to obtain the number of samples to achieve a satisfactory confidence interval; but over time, accrual of additional data will enhance confidence in the threshold. The use of spiked samples in PCR is a last resort because such samples might not be representative of naturally infected samples. If samples from naturally infected animals are unavailable, infections induced by means that mimic natural infections may provide samples that are useful. An example is tick-borne infection induced by exposure to infected ticks.

It is not always possible to conform to suggested guidelines (e.g. OIE recommendations on test validation). Faced with low numbers of samples for test evaluation, or for tests with no gold standard, one approach is to introduce molecular tests as 'partially validated' and then add validation data if significant numbers of clinical samples are tested. In this system, positives are confirmed by other means, such as isolation of the pathogen in question or sequencing, and a sample of negatives is also confirmed as suitable (non-inhibitory) for PCR testing using control genes. This principle of 'on-going' validation allows rapid introduction of new tests and reduces the cost of validation. This process must be used under defined conditions. It is only applicable when there is sound evidence from testing an appropriate range of known cultures, spiked samples (to provide analytical data) and some clinical samples (to show that the target is available in particular tissues) that a test may be released as partially validated (15).

F. ASSAY VALIDATION – PART 3

1. Establishing reproducibility of the assay

Reproducibility is an important parameter in assay precision. Reproducibility is determined in several laboratories using the identical assay (protocol, reagents and controls). Each of at least three laboratories test the same panel of samples (minimum of 20 samples), with identical aliquots going to each laboratory. This effort will yield estimates of ruggedness of the assay. Reproducibility estimates for the assay are essential before it can be considered valid for deployment to other laboratories.

Currently, reproducibility is rarely completely evaluated in veterinary diagnostic laboratories carrying out PCR assays. Traditionally, many laboratories have used tests developed in-house, probably for practical reasons.

When possible, published standardised and validated methods, especially by OIE reference laboratories, ECRLs or National Laboratories, should be followed. In addition inter-laboratory validation processes should be carried out. This work will help to standardise assays, allowing harmonised diagnostic activity in various countries.

G. ASSAY VALIDATION – PART 4

1. Programme implementation

Reference laboratories play a major role in the implementation of new or validating existing molecular assays. OIE Reference Laboratories ECRLs and National Laboratories are urged to assist in the implementation of promising new assays for their disease of interest. An example is the assistance provided by the OIE and CDRL to implement avian influenza molecular diagnostics in Europe.

2. Monitoring validity of assay performance

a) Interpretation of test results – factors affecting assay validity

A primary factor affecting interpretation of test results is the prevalence of the analyte in the target population. A PCR assay that is highly precise and accurate, with estimates of DSe and DSp approaching 99%, may still provide false inferences (see Chapter 1.1.4). For nucleic acid assays, false-positive results are of particular concern in low prevalence populations. In this instance, it may be necessary to confirm PCR-positive results by sequence analysis of the amplified product to assist in correcting errors due to nonspecific target or primer binding.

b) Maintenance of validation criteria

When the assay is used as a routine test, it is important to maintain the internal QC. The assay needs to be consistently monitored for repeatability and accuracy. Reproducibility between laboratories (proficiency testing) is recommended by the OIE to be completed at least twice a year (16). This testing is usually administered by a reference laboratory that distributes panels of samples, receives the results from the laboratories, analyses the data, and reports the results back to the laboratories. If the assay is to be applied in another geographical region and/or population, it might be necessary to revalidate or document equivalency under the new conditions. Revalidation or equivalency should be determined, if the test is applied to a different sample matrix, e.g. validated on blood and used on another tissue, or validated for cattle tissue and used on another species. Different extraction protocols may be needed if a different species or tissues are tested; which possibly contain different inhibitory factors. This is especially true for PCR assays as it is very common for point mutations to occur in many infectious agents (i.e. RNA viruses). Mutations, which may occur within the primer or probe sites, can affect the efficiency of the assay and, by doing so; the established performance criteria are no longer valid. It is also advisable to regularly confirm the target sequence at the selected genomic regions for national or regional isolates of the infectious agents. This is especially true for the primer sites, to ensure that they remain stable so that the validation of the assay cannot be questioned. Validation and estimation of robustness may need to be repeated when the test is transferred from the developing laboratory to the field as the conditions may be less than optimal and the staff less experienced.

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NB: There is an OIE Collaborating Centre for the Biotechnology-based Diagnosis of Infectious Diseases in Veterinary Medicine (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).