the vaccine is not acceptable if:

$$X_{\text{test}} - X_{\text{ref}} > C_1$$

the vaccine may be retested once if:

$$C_1 < X_{\text{test}} - X_{\text{ref}} < C_2$$

If the vaccine is retested, the means of the lesion scores for the vaccine to be tested and the reference vaccine are recalculated. The vaccine is not acceptable if:

$$\frac{X_{(\text{test 1}+\text{test 2})} - X_{(\text{ref 1}+\text{ref 2})}}{2} > C_3$$

The constants C_1, C_2 and C_3 are calculated from the expressions:

$$C_1 = 2.3\sqrt{\frac{2s^2}{N_1}}$$

$$C_2 = 2.6\sqrt{\frac{2s^2}{N_1}}$$

$$C_3 = 1.6\sqrt{\frac{2s^2}{N_1}}$$

N_1 = number of positive monkeys per vaccine test,

N_2 = number of positive monkeys in the two tests,

2.3 = normal deviate at the 1 per cent level,

2.6 = normal deviate at the 0.5 per cent level,

1.6 = normal deviate at the 5 per cent level.

A neurovirulence test in which the mean lesion score for the reference (X_{ref}) is not compatible with previous experience is not used for assessing a test vaccine. If the test is valid, the mean lesion score for the vaccine to be tested (X_{test}) is calculated and compared with that of the homotypic reference vaccine.

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2.6.20. ANTI-A AND ANTI-B HAEMAGGLUTININS (INDIRECT METHOD)

Prepare in duplicate serial dilutions of the preparation to be examined in a 9 g/l solution of *sodium chloride R*. To each dilution of one series add an equal volume of a 5 per cent *V/V* suspension of group A_1 red blood cells previously washed three times with the sodium chloride solution. To each dilution of the other series add an equal volume of a 5 per cent *V/V* suspension of group B red blood cells previously washed three times with the sodium chloride solution. Incubate the suspensions at 37 °C for 30 min then wash the cells three times with the sodium chloride solution. Leave the cells in contact with a polyvalent anti-human globulin reagent for 30 min. Without centrifuging, examine each suspension for agglutination under a microscope.

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2.6.21. NUCLEIC ACID AMPLIFICATION TECHNIQUES

1. INTRODUCTION

Nucleic acid amplification techniques are based on 2 different approaches:

1. amplification of a target nucleic acid sequence using, for example, polymerase chain reaction (PCR), ligase chain reaction (LCR), or isothermal ribonucleic acid (RNA) amplification,
2. amplification of a hybridisation signal using, for example, for deoxyribonucleic acid (DNA), the branched DNA (bDNA) method. In this case signal amplification is achieved without subjecting the nucleic acid to repetitive cycles of amplification.

In this general chapter, the PCR method is described as the reference technique. Alternative methods may be used, if they comply with the quality requirements described below.

2. SCOPE

This section establishes the requirements for sample preparation, *in vitro* amplification of DNA sequences and detection of the specific PCR product. With the aid of PCR, defined DNA sequences can be detected. RNA sequences can also be detected following reverse transcription of the RNA to complementary DNA (cDNA) and subsequent amplification.

3. PRINCIPLE OF THE METHOD

PCR is a procedure that allows specific *in vitro* amplification of segments of DNA or of RNA after reverse transcription into cDNA.

Following denaturation of double-stranded DNA into single-stranded DNA, 2 synthetic oligonucleotide primers of opposite polarity, anneal to their respective complementary sequences in the DNA to be amplified. The short double-stranded regions which form as a result of specific base pairing between the primers and the complementary DNA sequence, border the DNA segment to be amplified and serve as starting positions for *in vitro* DNA synthesis by means of a heat-stable DNA polymerase.

Amplification of the DNA occurs in cycles consisting of:

- heat denaturation of the nucleic acid (target sequence) into 2 single strands;
- specific annealing of the primers to the target sequence under suitable reaction conditions;
- extension of the primers, which are bound to both single strands, by DNA polymerase at a suitable temperature (DNA synthesis).

Repeated cycles of heat denaturation, primer annealing and DNA synthesis results in an exponential amplification of the DNA segment limited by the primers.

The specific PCR product known as an amplicon can be detected by a variety of methods of appropriate specificity and sensitivity.

Multiplex PCR assays use several primer pairs designed for simultaneous amplification of different targets in one reaction.

4. TEST MATERIAL

Because of the high sensitivity of PCR, the samples must be protected against external contamination with target sequences. Sampling, storage and transport of the test material are performed under conditions that minimise degradation of the target sequence. In the case of RNA target sequences, special precautions are necessary since RNA is

highly sensitive to degradation by ribonucleases. Care must be taken since some added reagents, such as anticoagulants or preservatives, may interfere with the test procedure.

5. TEST METHOD

5.1. Prevention of contamination

The risk of contamination requires a strict segregation of the areas depending on the material handled and the technology used. Points to consider include movement of personnel, gowning, material flow and air supply and decontamination procedures.

The system should be sub-divided into compartments such as:

- master-mix area (area where exclusively template-free material is handled, e.g. primers, buffers, etc.),
- pre-PCR (area where reagents, samples and controls are handled),
- PCR amplification (amplified material is handled in a closed system),
- post-PCR detection (the only area where the amplified material is handled in an open system).

5.2. Sample preparation

When preparing samples, the target sequence to be amplified needs to be efficiently extracted or liberated from the test material in a reproducible manner and in such a way that amplification under the selected reaction conditions is possible. A variety of physico-chemical extraction procedures and/or enrichment procedures may be employed.

Additives present in test material may interfere with PCR. The procedures described under 7.3.2. must be used as a control for the presence of inhibitors originating from the test material.

In the case of RNA-templates, care must be taken to avoid ribonuclease activity.

5.3. Amplification

PCR amplification of the target sequence is conducted under defined cycling conditions (temperature profile for denaturation of double-stranded DNA, annealing and extension of primers; incubation times at selected temperatures; ramp rates). These depend on various parameters such as:

- the length and base composition of primer and target sequences;
- the type of DNA polymerase, buffer composition and reaction volume used for the amplification;
- the type of thermocycler used and the thermal conductivity rate between the apparatus, reaction tube and reaction fluid.

5.4. Detection

The amplicon generated by PCR may be identified by size, sequence, chemical modification or a combination of these parameters. Detection and characterisation by size may be achieved by gel electrophoresis (using agarose or polyacrylamide slab gels or capillary electrophoresis) or column chromatography (for example, liquid chromatography). Detection and characterisation by sequence composition may be achieved by the specific hybridisation of probes having a sequence complementary to the target sequence or by cleavage of the amplified material reflecting target-specific restriction-enzyme sites. Detection and characterisation by chemical modification may be achieved, for example, by incorporation of a fluorophore into the amplicons and subsequent detection of fluorescence following excitation.

Detection of amplicons may also be achieved by using probes labelled to permit a subsequent radioisotopic or immuno-enzyme-coupled detection.

6. EVALUATION AND INTERPRETATION OF RESULTS

A valid result is obtained within a test only if the positive control(s) is unambiguously positive and the negative control(s) is unambiguously negative. Due to the very high sensitivity of the PCR method and the inherent risk of contamination, it is necessary to confirm positive results by repeating the complete test procedure in duplicate, where possible on a new aliquot of the sample. The sample is considered positive if at least one of the repeat tests gives a positive result. As soon as a measurable target threshold is defined, a quantitative test system is required.

7. QUALITY ASSURANCE

7.1. Validation of the PCR assay system

The validation programme must include validation of instrumentation and the PCR method employed. Reference should be made to the *ICH guidelines* (topic Q2B) Validation of Analytical Method: Methodology.

Appropriate official working reference preparations or in-house reference preparations calibrated against International Standards for the target sequences for which the test system will be used are indispensable for validation of a PCR test.

7.1.1. Determination of the positive cut-off point

During validation of qualitative tests, the positive cut-off point must be determined. The positive cut-off point is defined as the minimum number of target sequences per volume sample which can be detected in 95 per cent of test runs. The positive cut-off point depends on interrelated factors such as the volume of the sample extracted and the efficacy of the extraction methodology, the transcription of the target RNA into cDNA, the amplification process and the detection.

To define the detection limit of the assay system, reference must be made to the positive cut-off point for each target sequence and the test performance above and below the positive cut-off point.

7.1.2. Quantitative assay systems

For a quantitative assay, the following parameters are determined during validation: accuracy, precision, specificity, quantitation limit, linearity, range and robustness.

7.2. Quality control of reagents

All reagents crucial for the methodology used have to be controlled prior to use in routine applications. Their acceptance/withdrawal is based on pre-defined quality criteria.

Primers are a crucial component of the PCR assay and as such their design, purity and the validation of their use in a PCR assay require careful attention. Primers may be modified (for example, by conjugation with a fluorophore or antigen) in order to permit a specific method of detection of the amplicon, provided such modifications do not inhibit accurate and efficient amplification of the target sequence.

7.3. Run controls

7.3.1. External controls

In order to minimise the risk of contamination and to ensure adequate sensitivity, the following external controls are included in each PCR assay:

- positive control: this contains a defined number of target-sequence copies, the number being close to the positive cut-off value, and determined individually for

each assay system and indicated as a multiple of the positive cut-off value of the assay system;

– negative control: a sample of a suitable matrix already proven to be free of the target sequences.

7.3.2. Internal control

Internal controls are defined nucleic acid sequences containing, unless otherwise prescribed, the primer binding sites. Internal controls must be amplified with defined efficacy, and the amplicons must be clearly discernible. Internal controls must be of the same type of nucleic acid (DNA/RNA) as the material to be tested. The internal control is preferably added to the test material before isolating the nucleic acid and therefore acts as an overall control (extraction, reverse transcription, amplification, detection).

7.3.3. Threshold control

The threshold control for quantitative assays is a test sample with the analyte at a concentration which is defined as the threshold not to be exceeded. It contains the analyte suitably calibrated in IU and is analysed in parallel in each run of a quantitative assay.

7.4. External quality assessment

Participation in external quality assessment programmes is an important PCR quality assurance procedure for each laboratory and each operator.

The following section is published for information.

Validation of nucleic acid amplification techniques (NAT) for the detection of hepatitis C virus (HCV) RNA in plasma pools: guidelines

1. SCOPE

The majority of nucleic acid amplification analytical procedures are qualitative (quantal) tests for the presence of nucleic acid with some quantitative tests (either in-house or commercial) being available. For the detection of HCV RNA contamination of plasma pools, qualitative tests are adequate and may be considered to be a limit test for the control of impurities as described in the *Pharmeuropa* Technical Guide for the elaboration of monographs, December 1999, Chapter III "Validation of analytical procedures". These guidelines describe methods to validate only qualitative nucleic acid amplification analytical procedures for assessing HCV RNA contamination of plasma pools. Therefore, the 2 characteristics regarded as the most important for validation of the analytical procedure are the specificity and the detection limit. In addition, the robustness of the analytical procedure should be evaluated.

However, this document may also be used as a basis for the validation of nucleic acid amplification in general.

For the purpose of this document, an analytical procedure is defined as the complete procedure from extraction of nucleic acid to detection of the amplified products.

Where commercial kits are used for part of or the complete analytical procedure, documented validation points already covered by the kit manufacturer can substitute for the validation by the user. Nevertheless, the performance of the kit with respect to its intended use has to be demonstrated by the user (e.g. detection limit, robustness, cross contamination).

2. SPECIFICITY

Specificity is the ability to unequivocally assess nucleic acid in the presence of components which may be expected to be present.

The specificity of nucleic acid amplification analytical procedures is dependent on the choice of primers, the choice of probe (for analysis of the final product) and the stringency of the test conditions (for both the amplification and detection steps).

When designing primers and probes, the specificity of the primers and probes to detect only HCV RNA should be investigated by comparing the chosen sequences with sequences in published data banks. For HCV, primers (and probes) will normally be chosen from areas of the 5' non-coding region of the HCV genome which are highly conserved for all genotypes.

The amplified product should be unequivocally identified by using one of a number of methods such as amplification with nested primers, restriction enzyme analysis, sequencing or hybridisation with a specific probe.

In order to validate the specificity of the analytical procedure, at least 100 HCV RNA-negative plasma pools should be tested and shown to be non-reactive. Suitable samples of non-reactive pools are available from the European Directorate for the Quality of Medicines.

The ability of the analytical procedure to detect all HCV genotypes will again depend on the choice of primers, probes and method parameters. This ability should be demonstrated using characterised reference panels. However, in view of the difficulty in obtaining samples of some genotypes (e.g. genotype 6), the most prevalent genotypes (e.g. genotype 1 and 3 in Europe) should be detected at a suitable level.

3. DETECTION LIMIT

The detection limit of an individual analytical procedure is the lowest amount of nucleic acid in a sample which can be detected but not necessarily quantitated as an exact value.

The nucleic acid amplification analytical procedure used for the detection of HCV RNA in plasma pools usually yields qualitative results. The number of possible results is limited to two, either positive or negative. Although the determination of the detection limit is recommended, for practical purposes, a positive cut-off point should be determined for the nucleic acid amplification analytical procedure. The positive cut-off point (as defined in the General Chapter (*2.6.21*)) is the minimum number of target sequences per volume sample which can be detected in 95 per cent of test runs. This positive cut-off point is influenced by the distribution of viral genomes in the individual samples being tested and by factors such as enzyme efficiency and can result in different 95 per cent cut-off values for individual analytical test runs.

In order to determine the positive cut-off point, a dilution series of a working reagent or of the *hepatitis C virus BRP*, which has been calibrated against the WHO HCV International Standard 96/790, should be tested on different days to examine variation between test runs. At least 3 independent dilution series should be tested with a sufficient number of replicates at each dilution to give a total number of 24 test results for each dilution to enable a statistical analysis of the results.

For example, a laboratory could test 3 dilution series on different days with 8 replicates for each dilution, 4 dilution series on different days with 6 replicates for each dilution, or 6 dilution series on different days with 4 replicates for each dilution. In order to keep the number of dilutions at a manageable level, a preliminary test (using, for example, log dilutions of the plasma pool sample) should be done in order to obtain a preliminary value for the positive cut-off point (i.e. the highest dilution giving a positive signal). The range of dilutions can then be chosen around the predetermined preliminary cut-off point (using, for example, a dilution

factor of 0.5 log or less and a negative plasma pool for the dilution matrix). The concentration of HCV RNA which can be detected in 95 per cent of test runs can then be calculated using an appropriate statistical evaluation.

These results may also serve to demonstrate the intra-assay variation and the day-to-day variation of the analytical procedure.

4. ROBUSTNESS

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The evaluation of robustness should be considered during the development phase. It should show the reliability of the analytical procedure with respect to deliberate variations in method parameters. For NAT, small variations in the method parameters can be crucial. However, the robustness of the method can be demonstrated during its development when small variations in the concentrations of reagents (e.g. $MgCl_2$, primers or dNTP) are tested. To demonstrate robustness, at least 20 HCV RNA negative plasma pools (selected at random) spiked with HCV RNA to a final concentration of 3 times the previously determined 95 per cent cut-off value should be tested and found positive.

Problems with robustness may also arise with methods which use an initial ultracentrifugation step prior to extraction of the viral RNA. Therefore, to test the robustness of such methods, at least 20 plasma pools containing varying levels of HCV RNA, but lacking HCV specific antibodies, should be tested and found positive.

Cross contamination prevention should be demonstrated by the accurate detection of a panel of at least 20 samples consisting of alternate samples of negative plasma pools and negative plasma pools spiked with high concentrations of HCV (at least 10^2 times the 95 per cent cut-off value or at least 10^4 IU/ml).

5. QUALITY ASSURANCE

For biological tests such as NAT, specific problems may arise which may influence both the validation and interpretation of results. The test procedures must be described precisely in the form of standard operating procedures (SOPs). These should cover:

- the mode of sampling (type of container, etc.),
- the preparation of mini-pools (where appropriate),
- the conditions of storage before analysis,
- the exact description of the test conditions, including precautions taken to prevent cross contamination or destruction of the viral RNA, reagents and reference preparations used,
- the exact description of the apparatus used,
- the detailed formulae for calculation of results, including statistical evaluation.

The use of a suitable run control (for example, an appropriate dilution of *hepatitis C virus BRP* or plasma spiked with an HCV sample calibrated against the WHO HCV International Standard 96/790) can be considered a satisfactory system suitability check and ensures that the reliability of the analytical procedure is maintained whenever used.

Technical qualification: an appropriate installation and operation qualification programme should be implemented for each critical piece of the equipment used. Confirmation of analytical procedure performance after change of critical equipment (e.g. thermocyclers) should be documented by conducting a parallel test on 8 replicate samples of a plasma pool spiked with HCV RNA to a final concentration of 3 times the previously determined 95 per cent cut-off value. All results should be positive.

Operator qualification: an appropriate qualification programme should be implemented for each operator involved in the testing. To confirm successful training each operator should test at least 8 replicate samples of a plasma pool spiked with HCV RNA to a final concentration of 3 times the previously determined 95 per cent cut-off value. This test (8 replicate samples) should be repeated twice on two separate days, i.e. a total of 24 tests performed on three different days. All results should be positive.

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2.6.22. ACTIVATED COAGULATION FACTORS

Where applicable, determine the amount of heparin present (*2.7.12*) and neutralise the heparin, for example by addition of *protamine sulphate R* (10 µg of protamine sulphate neutralises 1 IU of heparin). Prepare 1 to 10 and 1 to 100 dilutions of the preparation to be examined using *tris(hydroxymethyl)aminomethane buffer solution pH 7.5 R*. Place a series of polystyrene tubes in a water-bath at 37 °C and add to each tube 0.1 ml of *platelet-poor plasma R* and 0.1 ml of a suitable dilution of a phospholipid preparation to act as a platelet substitute. Allow to stand for 60 s. Add to each tube either 0.1 ml of 1 of the dilutions or 0.1 ml of the buffer solution (control tube). To each tube add immediately 0.1 ml of a 3.7 g/l solution of *calcium chloride R* previously heated to 37 °C, and measure, within 30 min of preparing the original dilution, the time that elapses between addition of the calcium chloride solution and the formation of a clot. The test is not valid unless the coagulation time measured for the control tube is 200 s to 350 s.

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2.6.24. AVIAN VIRAL VACCINES: TESTS FOR EXTRANEOUS AGENTS IN SEED LOTS

GENERAL PROVISIONS

a) In the following tests, chickens and/or chicken material such as eggs and cell cultures shall be derived from chicken flocks free from specified pathogens (SPF) (*5.2.2*).

b) Cell cultures for the testing of extraneous agents comply with the requirements for the master cell seed of chapter *5.2.4. Cell cultures for the production of veterinary vaccines*, with the exception of the karyotype test and the tumorigenicity test, which do not have to be carried out.

c) In tests using cell cultures, precise specifications are given for the number of replicates, monolayer surface areas and minimum survival rate of the cultures. Alternative numbers of replicates and cell surface areas are possible as well, provided that a minimum of 2 replicates are used, the total surface area and the total volume of test substance applied are not less than that prescribed here and the survival rate requirements are adapted accordingly.

d) For a freeze-dried preparation, reconstitute using a suitable liquid. Unless otherwise stated or justified, the test substance must contain a quantity of virus equivalent to at least 10 doses of vaccine in 0.1 ml of inoculum.