OIE Guideline

International Reference Standards for Polymerase Chain Reaction assays

1. Introduction

1.1. Purpose

This document provides guidelines for the preparation, validation and distribution of controls of molecular assays as International Reference Standards for polymerase chain reaction (PCR) assays applied for the diagnosis of infectious diseases of animals. Diagnosis by PCR has become the state of art for most of the infectious diseases of animals relevant to OIE. While PCR techniques, and in particular real-time PCR, provide excellent performance characteristics in terms of sensitivity and specificity, proper control is pivotal. In these guidelines, the term "Standards" refers to nucleic acids unless indicated otherwise. Such standard preparations are designated by the OIE as primary reference standards for use in conjunction with tests described in the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (*Terrestrial Manual*).

1.2. Definitions

1.2.1. Standard

A Standard is defined as a substance (nucleic acid in this case) that has been shown by an extensive set of analytical tests to be an authentic material of high purity, from a recognised source, carefully prepared, scientifically confirmed, obtained from clinical materials or artificially synthesised and of known status.

1.2.2. Standard Test Protocol

Standard Test Protocol refers to a validated, internationally accepted test procedure, as referenced in the OIE *Terrestrial Manual.*

1.2.3. International Reference Standard

The term International Reference Standard is synonymous with primary reference standard. It represents the standard against which all others are compared and calibrated.

1.2.4. Secondary Reference Standards

Secondary standards are prepared by direct comparison with the International Reference Standard, and should mimic the characteristics of the primary standard when used in the Standard Test Protocol. A Secondary Standard would typically be prepared by a national reference laboratory to be distributed and used by the designated national or local laboratories. In that case, the Secondary Standard would be designated as the National or Local Standard.

1.2.5. Working or in-house Standards

Working or in-house standards may be synonymous with Secondary Standards, or they may be Tertiary Standards calibrated against the Secondary Standard. Working Standards are defined as an appropriately characterised material prepared from a Primary Reference Standard lot to support routine testing of lots for quality control purposes in biological assays of PCR. It is always calibrated against the Primary reference standard or the official reference standard and should be available in sufficient quantities for use by diagnostic laboratories to standardise routine daily testing.

1.3. Scope

Reference standards play a critical role in calibrating and confirming validity of test as well as quality control of the conclusions obtained from the data analysed, and are key elements to ensure continuity of the test, quality of results, stability of the reagents and comparability between experiments. Establishing and using reference standards are essential in quality controlled testing to ensure the accuracy of results and to monitor assay performance.

International Reference Standards are normally for use by international, national and other reference laboratories in calibrating standard assays and as templates for the production of secondary standards, while the secondary or other working standard, and not the international standard, are to be used on a daily basis to standardise testing. International Reference Standards are necessary to ensure that a given PCR assay is capable of measuring presence of target nucleic acid to a specified level of diagnostic sensitivity. Diagnostic sensitivity relates to the risk of a false negative reaction occurring in a PCR assay when in fact an animal is, or has been, infected.

They are so important that appropriate protocols for manufacture and qualification of reference standards should be in place to ensure that they are well characterised, qualified and stable. The Reference Standards should be selected and characterised by a designated Reference Laboratory using an internationally accepted Standard Operating Procedure (SOP) and internationally accepted reagents. For most assays, three primary Reference Standards should be established:

- A strong positive with a target copy number in at least 10,000 fold excess of the limit of detection (LOD) of the specific PCR assay (see Chapter 3.6.3 *Development and optimisation of nucleic acid detection assays* of the OIE *Terrestrial Manual*).

- A weak positive with a target copy number in excess of at least 50-fold the LOD, which can be generated by defined dilution of the strong positive control. The weak positive standard is critical for providing assurance of the diagnostic sensitivity of the test.

- For semi-quantitative approaches (e.g. real-time reverse transcriptase PCR [RT-PCR]) the strong positive standard could be used to produce a series of dilutions with known copy numbers of the target sequence, which could be used as a calibration curve. For non-quantitative and quantitative assays (e.g. conventional RT-PCR), the weak positive reference standard may be the only positive standard required.

- A negative standard containing a high copy number of an irrelevant target.

1.4. Approach

The approach to creating and using standards are often unique to the type of standard material. Approaches other than the one presented here can also be acceptable, this document is not binding regulatory it is just a guidance to illustrate how to qualify new standards and the challenges to maintain them. OIE Reference Laboratories producing an International Standard should liaise with other OIE Reference Laboratories especially those designated for the same disease with the aim of organising an inter-laboratory system to improve the consistency of results across participant laboratories. Performing a proficiency test would give an added value to the International Standard, would ensure harmonisation across laboratories, and would promote networking and cooperation among the OIE Reference Centres.

Some general guidelines to address the specifications for good standards are:

- Define a well thought qualification programme that includes a correct characterisation strategy;
- Minimise the number of reference standards in circulation;
- Provide assurance that they are permanently available;
- Maximise their implementation making them easily accessible to all groups that need them;
- Collect and compare scientific data to guarantee consistency, conformance, and accuracy;

- Compile and procure historical data for shelf live, preservation, and extinction of the standards;
- Consider instances when a new reference standard must be qualified and assigned.

2. Selection of Material for use as Standards

2.1. Considerations for selecting standard materials

Selecting the type of material to be used as standard is probably the most critical step during their preparation. Some nucleic PCR assays utilise diagnostic material obtained directly from a suspect case, whereas the same or other assays may be applied following preliminary *in-vivo* or *in-vitro* cultivation/multiplication of the infectious agent(s).

However, several aspects should be considered when preparing Primary Reference Standards.

2.1.1. Material suitability

According to the purpose of the test, the original source for preparing the standard requires a rigorous assessment of the "true" value of a material or at least establish and statistically justify its accurate value

2.1.2. Material specificity

The standard should be representative of the possible targets for the test, including representation of all known variations and blend of scenarios expected for the process.

2.1.3. Material constituents

Similarity of the original sample to the sample of choice for the assay. The condition of having the same attributes, including impurities, as the clinical material to be tested is of most importance for the preparation of the Primary Reference Standards

2.1.4. Material abundance

Replacing Primary Reference Standards should be kept to the absolute minimum because it requires extensive testing effort and copious amounts of data to qualify as a replacement for the previous Primary Standard. So it is strongly recommended to consider a source capable to supply standards for an extended period of time to all the possible users.

2.2. Types of material

Depending on the PCR assay either DNA or RNA controls will be required. Due to its fragility distribution of "naked" RNA standards calls for more complex shipment conditions (e.g. dry ice, etc.) than DNA.

Preparation of controls by extraction of nucleic acids directly from an infectious agent provides the highest similarity with most of clinical samples. However, it is discouraged due to risks of residual infectivity, and lack of a standardised copy-based enumeration of targets.

Recombinant plasmid DNA solubilised in a matrix of negative reference standard, or the most appropriate buffer solution, is considered stable for distribution.

Controls for molecular assays involving RT-PCR should be based on RNA as this also enables control of the reverse transcription step. Although not as common as the use of recombinant DNA, it is also possible to obtain RNA for control on RT-PCR through *in-vitro* transcription of DNA plasmids containing specific promoter sequences.

Encapsidating standard control DNA or RNA into a rigid proteinaceous shell (so-called armouring) increases stability significantly, especially in the case of RNA. Armoured nucleic acid controls have

the added advantage over "naked" DNA/RNA controls to enable the analysis of the efficacy of the nucleic acid extraction part of the SOP. However, use of such armoured controls (e.g. recombinant bacterial phages) may be limited due to their potential GMO status.

2.3. Constitution of the Standards

The first step in preparing International Reference Standards is the selection of the negative stock from single source or a pool of samples from animals that have never been exposed or vaccinated against the organism in question. This material should be carefully screened to ensure that there is no evidence of cross-reacting or other nonspecific factors that may interfere with the test, despite demonstrating an admissible range of background activity representative of the majority of negative samples. For International Reference Standards it might be advisable the use of a pool of negative samples in order to minimise particularities of a single specimen.

The second step is the selection of the positive material to prepare both the strong and the weak reference standards. Like in the previous case, because there is a number of factors that may influence the outcome of an infection, or just the response to the phenomenon under study, it is advisable to use a pool representative enough of the diversity of positive cases. If the positive material is obtained from experimental conditions, it should be confirmed that it mimics the natural course of events as close as possible to avoid deviations in calibration of the test and interpretation of the positive results.

2.3.1. Negative Reference Standards

Negative Reference Standard should be derived from a similar sample of pool of samples with typical background activity or, at least comprise nucleic acid prepared in the same way as the positive standard but derived from an irrelevant target sequence. They must be free from potentially inhibitory substances that might mislead the interpretation of the negative result, artificially cross-react or otherwise interfere in the standard assay in a different way than the positive standard material.

2.3.2. Positive Reference Standards

The Positive Reference Standards should be based on a selected reference isolate of the infectious agent to be detected by the molecular assay, either derived from a similar sample or pool of samples, or a recombinant product that typifies the target sequence. The selected isolates must be representative for the group of agents targeted by the specific assay. Preferably the full length nucleotide sequence of the selected isolate should be known but at least the sequence of the target region is required.

Most of the Reference Standards should be prepared from a one-time dilution the negative standard matrix to yield activities that are comparable with the tested field material, the reaction produced should never be equivocal. However, for some specific applications of the PCR, it is advisable that the Positive Reference Standard be free from other nucleic acids that might cross-react or otherwise interfere in the standard assay.

The activity of the Positive Reference Standards should be defined by specific points in the linear portion of the dose–response curve of the target.

2.3.2.1. Strong Positive Reference Standard

The strong positive should represent activity midway between the upper and the central points of the linear portion of the dose–response curve.

2.3.2.2. Weak Positive Reference Standard

The weak positive should represent activity midway between the central and the lower points of the linear portion of the dose–response curve. The weak positive reference standard should produce positive results just above the positive/negative threshold in the standard assay protocol.

2.4. Safety

The reference standards should be prepared so that they are free of infectious material. To facilitate shipment between countries it is recommended that the standards in the wet state be either treated by a method that has been validated as inactivating residual infectivity whilst retaining its reactivity in the assay. Examples include nucleic acid extraction by validated phenol/chloroform methods or by use of methods depending on chaotropic salts and heating. After treatment, samples should be submitted to appropriate innocuity tests as described in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use* of the *Terrestrial Manual* to ensure that they are free from detectable live agents.

3. Use of International Reference Standards

3.1. Preparation

An International Reference Standard should not require any special manipulation (e.g. pre-dilution) by the recipient laboratory prior to its use in the assay in question. Hence, when possible, the positive reference standards should be prepared from materials showing the desired level of reactivity without further dilution.

However, to prepare Secondary and Working reference standards, it will be necessary for the laboratory to make dilutions from the International Reference Standard material in order to achieve the desired level of reactivity as specified in Sections 2.3.1 and 2.3.2, for either Negative or Positive (strong and weak) Reference Standards, respectively. It would be advantageous to provide the sterile diluent for reconstitution of the material, along with the freeze-dried standard.

Before using the standard as actual controls of the routine tests, the standards should be tested as would any field sample or culture, under routine diagnostic conditions (including any extraction and dilution steps that are a normal part of the assay procedure). This will confirm that the amount of target nucleic acid in the reference standards are specific and sensitive within the accurate detection limits of the diagnostic test.

3.2. Quality control of Standards

Ideally, the original reference material must begin as one single stock with enough to last at least 5 years. This can be kept frozen (preferably at –70°C or below) in aliquots so every batch can last for a minimum of about 500 tests supply.

After production, several units of the standard should be reconstituted and re-evaluated over time. Recalculation of copy-based target numbers is required for quality control of standards for quantitative assays. If there is a possibility of decay of activity over time, this information should be indicated.

For each batch, whether frozen or freeze-dried, batch references must be performed to demonstrate adequacy and "true" value

3.2.1. Batch references

- A complete description of the source, date of preparation, manufacture process, and analytics for characterisation (sensitivity/specificity).
- A plot of the dose–response curve to quantify activity strength.

3.2.2. Batch stability

- Periodical testing of single thawed aliquots of the material during the expected lifespan of the batch should be performed.
- A demonstration of the effects that freeze-drying, lyophilisation, or any other process performed for batch conservation may produce in the biological quality of the standard.
- Data from different conditions used for the standard process, or if a new standard process is using these standards should be collected.
- Data of the natural decay of activity and/or factors that may induce degradation of the material should be gathered.

3.2.3. Data sheets

Every batch of reference standards should be accompanied by an information sheet including potency data, performance characteristics and operational aspects such as:

i) The datasheet should repeat all the identification information specified in the label along with batch number and date of production;

ii) Description of the donor infectious agent for the preparation of the standard, including source, strain, origin, and accession number of its genome sequence in a public database;

iii) A warning that the strong positive standard may be causing cross contamination of samples if not handled appropriately;

iv) Details of the standard production protocol, i.e. plasmid designation and origin, bacterial host, purification methods, RNA run-off transcription (if RNA) etc.;

v) Description of the proteineous shell if armoured DNA/RNA;

vi) Reference tests used to select positive and negative reference standard candidates, e.g. conventional or real-time (RT) PCR;

vii) Sample of titration profiles of target nucleic acid on a copy-based scheme and criteria for selection of appropriate dilutions of defined activity for different assay formats (e.g. conventional versus real-time PCR);

viii) Presence of heterologous nucleic acids, if known, and tests used in detection;

ix) Details of any safety testing carried out on the materials;

x) A statement that the standard is for *in vitro* use only;

xi) Description of sterilisation methods, including type of irradiation and dose and condition of sample at time of sterilisation (i.e. liquid, frozen, freeze-dried, etc.);

xii) Batch number and date of production;

xiii) Recommended reconstitution (type of reconstituting fluid, and volume), handling and storage conditions;

xiv) Full contact address, fax, email of the Reference Laboratory as a source of further information.

3.3. Storage

All materials should be stored frozen or refrigerated. Freeze-dried stocks should be stored at 4°C, although short periods at ambient temperature (e.g. during shipment) should not be deleterious. Storing the standards in cryotubes at –78°C is the recommended alternative solution. Sealed glass ampoules, rather than rubber caps, are preferred for long-term storage.

Repeated freeze–thaw cycles should be avoided, this is particularly relevant for "naked" RNA standards, so reference standards should always be aliquoted and stored to preserve a homogenous, stable standard over time. Using a working standard for routine tests to limit usage of the primary

standard is strongly recommended. If possible, single use aliquots to be used immediately and discarded after use are preferable.

3.4. Labelling and Identification

OIE Reference Laboratories issuing international reference standards for PCR assays should ensure that all aliquots are conveniently identified and accompanied by an appropriate data sheet. It should be made clear to requesting laboratories that international reference standards are intended for use in the calibration of their own assay and for promotion of international harmonisation.

The label should contain the following minimum information: OIE logo; OIE international reference standard for (disease) (test); specify if strong positive, weak positive or negative; the name of the Reference Laboratory; reconstitution method; and storage conditions. In case of standards for quantitative assays: target copy numbers/volume. The space available on the label may prevent the inclusion of all these items; abbreviations may be used and some of the items may need to be put on the data sheet instead of on the label.

In order for a diagnostic laboratory to prepare a secondary reference standard for its own use, it will be necessary for the OIE Reference Laboratory to supply specific data on the selection and/or preparation of the primary reference standards.

4. Approval of Reference Standards by OIE

An International Reference Standard may not be issued under the name of OIE unless it has been endorsed by the OIE Biological Standards Commission acting under authority of the OIE World Assembly.

The full technical and statistical data on the evaluation of the candidate reference standards, together with the full data sheet information as specified above, should be submitted to OIE. The OIE Biological Standards Commission will review the information. If the Biological Standards Commission approves, the reference standard will be added to the list of International Reference Standards available. This list will be supplied to all OIE Members Countries on request, and may also be accessed on the OIE Web site (http: www.oie.int).

5. References

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