# OIE Guideline

## INTERNATIONAL REFERENCE STANDARDS FOR ANTIGEN DETECTION ASSAYS

## 1. Introduction

### 1.1. Purpose

This document provides guidelines for the preparation, validation and distribution of antigens as International Reference Standards for antigen detection assays for infectious diseases of animals. In these guidelines, the term "Standards" refers to antigens 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 Test Protocol

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

#### 1.2.2. 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.

#### 1.2.3. Secondary and Working Standards

Secondary standards are prepared by direct comparison with the International Reference Standard, and should so far as is possible 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 and be designated as the national or local standard.

Working standards may be synonymous with secondary standards, or they may be tertiary standards calibrated against the secondary standard. Working standards should be available in sufficient quantities for use by diagnostic laboratories to standardise routine daily testing.

### 1.3. Scope

International Reference Standards are necessary to ensure that a given antigen detection assay is capable of measuring antigen activity to a specified level of diagnostic sensitivity. Diagnostic sensitivity relates to the risk of a false negative reaction occurring in an antigen assay when in fact an animal is, or has been, infected. 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. The secondary or other working standard, and not the international standard, are to be used on a daily basis to standardise testing.

Some antigen detection assays may utilise diagnostic material directly from a suspect case, whereas other assays may be applied following preliminary *in-vivo* or *in-vitro* culture of the agent (see table).

For a limited number of diseases, there has been international agreement on a system of 'International Units' of antigen activity. In such cases the International Reference Standards define the scale of such units. In the vast majority of animal diseases no such system exists, and

assay systems, working standards, and test samples are defined relative to the International Reference Standards.

### 1.4. Approach

For most assays, three primary reference standards should be established: a strong positive, a weak positive and negative standard. These standards should be selected and characterised by a designated Reference Laboratory using an internationally accepted Standard Operating Procedure (SOP) and internationally accepted reagents.

The weak positive standard is critical for providing assurance of the diagnostic sensitivity of the test. For non-quantitative assays (e.g. immunofluorescence assays, agglutination tests and immuno-histochemical tests) the weak positive reference standard may be the only positive standard required.

For quantitative, non-titration assays (e.g. immunoassays including lateral flow devices, ELISpot and antigen-capture enzyme-linked immunosorbent assay [Ag ELISA]) the strong positive standard should define an arbitrary level of 100% positivity. The weak positive and negative standards should then be assigned a proportional percentage positivity corresponding to their reactivity when tested in the standard test protocol.

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.

## 2. Selection of Materials for use as Standards

### 2.1. Types of material

The majority of International Reference Standards will be prepared by purifying the antigen content by infecting permissive cells with virus, by infecting experimental animals -with the appropriate pathogen or from recombinant protein (antigen) preparations. These should be further purified using column chromatography and purity may be checked by mass spectrometry to produce a homogenous antigen preparation that is free from contaminating proteins. Antigens may be further concentrated by a number of methods, including lyophilisation and ultrafiltration. Viruses and recombinant viruses (including virus-like particles) prepared in cell culture, should whenever feasible, be grown in growth media free of animal proteins derived from serum. If bovine sera must be used, should be from a BSE-free source. Antigens, should where possible, be produced in specific pathogen free or gnotobiotic animals of a species appropriate to the assay being standardised.

### 2.2. 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 which has been validated as inactivating the agent whilst retaining its reactivity in the assay. Examples include treatment with BEI (binary ethyleneimine) or irradiated at 25–30 kilogray (2.5–3.0 Mrad) and kept at –78°C. Irradiating freeze-dried samples is not recommended as the recommended dose may not be enough for complete pathogen inactivation. After treatment, samples should be submitted to appropriate innocuity tests as described in Chapter 1.1.9 *Tests of biological materials for sterility and freedom from contamination* of the *Terrestrial Manual* to ensure that they are free from detectable live agents.

### 2.3. Positive reference standards

Positive reference standards should be selected either from cultures or from infected experimental animals. The timing after inoculation for collection of the material should be determined by the replication of the pathogen in culture or in the animal as measured in the standard test protocol. This may vary according to the pathogenesis of the disease and the assay. Full details of the inoculation schedule and the nature of the inoculum must be provided so that secondary standards can be prepared by equivalent methods. The standards should be free from antigens and organisms that might cross-react in the standard assay or information on this cross-reaction should be provided. The standard may be derived from a single animal or a pool of specimens from a number of animals. Exceptionally, naturally infected animals may be used as the source of the standard where controlled inoculation or infection is not feasible.

### 2.4. Negative reference standards

Negative reference standards should comprise uninfected cultures or be selected from animals that have never been exposed to, or vaccinated against, the agent in question. They should be free from antigens of other pathogens, which might cross-react in the standard assay. The negative standard may be derived from a single specimen or a pool of specimens.

## 3. Characteristics of International Reference Standards

### 3.1. Strong positive reference standard

For immunoassays that demonstrate typical sigmoidal dose/response curves, the strong positive reference standard should exhibit an antigen activity which lies on the linear portion of the curve just below the plateau phase. In other tests, the strong positive reference standard should contain sufficient antigen to produce consistently the maximum reaction within the selected limits of the test, e.g. a clear cut line of identity in an immunodiffusion test or 100% inhibition in a competitive or inhibition Ag ELISA.

### 3.2. Weak positive reference standard

The weak positive reference standard should exhibit antigen activity which again lies on the linear portion of the curve just above the positive/negative threshold. The reaction produced should never be equivocal. In other tests, the weak positive reference standard should contain sufficient antigen to produce consistently the minimum detectable reaction, e.g. a weak but unequivocal line of identity in an immunodiffusion test. For competitive/inhibition assays, which frequently show a sharp transition from positive to negative, the selection of the weak positive standard can be particularly difficult. The same principles apply, in that the standard should give a consistent positive response, just above the positive/negative threshold, in the Standard Test Protocol.

### 3.3. Negative reference standard

This standard should always give a reaction below the positive/negative threshold in the Standard Test Protocol. The reaction produced should never be equivocal.

## 4. Preparation of Reference Standards

### 4.1. Constitution of the standards

Where possible, the positive reference standards should be prepared from materials showing the desired level of reactivity without further dilution. However in many cases it may be necessary for the Reference Laboratory to make a dilution, just the once, of a positive antigen preparation in an appropriate buffer, in order to achieve the desired level of reactivity as specified in Section 3

above. In such cases the weak positive reference standard may be derived from the same positive antigen stock as the strong positive reference standard.

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. The standard should be tested as would any field sample or culture, under routine diagnostic conditions (including any dilution steps which are a normal part of the assay procedure). This prevents the introduction of error or bias related to special handling or preparation. Therefore, the amount of antigen activity in a positive reference standard should be within the accurate detection limits of the diagnostic test.

### 4.2. Stability and storage

All materials should be stored frozen or refrigerated pending evaluation. Repeated freeze–thaw cycles should be avoided. To ensure stability it is recommended that the final standard, after sample inactivation treatment, be freeze-dried, and it would be advantageous to provide the sterile diluent for reconstitution of the material, along with the freeze-dried standard. Sealed glass ampoules, rather than rubber caps, are preferred for long-term storage. Freeze-dried stocks should be stored at 4°C, although short periods at ambient temperature (e.g. during shipment) should not be deleterious. The freeze-drying process may alter the biological quality of antigen; storing the standards in cryotubes at –78°C is the recommended alternative solution.

After freeze-drying, several bottles of the standard should be reconstituted and re-evaluated. There should be no evidence of cross-reacting antigens or other non-specific factors which interfere with the interpretation of assay results. If there is a possibility of cross-reaction with closely related agents, this information should be indicated.

### 4.3. Batch control

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) and a batch can be freeze-dried for a minimum 2-year supply (about 500 tests). For each batch, whether frozen or freeze-dried, batch references must be allocated and full quality control data maintained for each batch.

Each freeze-dried batch must be recalibrated. Each bottle or ampoule should contain 0.5–1 ml.

### 4.4. Labelling

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. 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.

### 4.5. Data sheets

OIE Reference Laboratories issuing international reference standards should ensure that all aliquots are 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.

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.

#### 4.5.1. Data required

The datasheet should repeat all the information specified for the label (see 4.4). The following information must also be supplied in order to facilitate the selection and/or

preparation of secondary reference standards which, as closely as possible, duplicate the primary reference standard.

i) Description of the culture or donor animal for the preparation of the agent, including species, age, reproductive status and origin (i.e. natural production, specific pathogen free, gnotobiotic, etc.);

ii) Details of agent used, i.e. source, strain, serotype, etc.;

iii) Details of experimental infection protocol, i.e. route, dose, immunisation schedules, method and time of sample collection etc.;

iv) Description of growth media, incubation times and temperatures for preparing cultures of the agent;

v) Reference tests used to select positive and negative reference antigen candidates, e.g. Western (immune) blot, agar gel immunodiffusion;

vi) Sample of titration profiles of antigen preparation(s) and criteria for selection of appropriate dilutions of defined activity;

vii) Presence of heterologous antigens, if known, and tests used in detection;

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

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

x) 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.);

xi) Batch number and date of production;

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

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

## 5. 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).

## 6. References

BALAMURUGAN V., VENKATESAN G., SEN A., ANNAMALAI L., BHANUPRAKASH V. & SINGH R.K. (2010). Recombinant protein-based viral disease diagnostics in veterinary medicine. *Expert Rev. Mol. Diagn.,* **10**, 731–753. doi: 10.1586/erm.10.61. (Review).

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## Table: Principles of Antigen Detection Assays

| Method | Specimens | Observations | Characteristics |
|---|---|---|---|
| Enzyme immunoassays, e.g. antigen-capture enzyme immunoassay | Tissues, cells | Agent antigen identified by reaction with antibody of known specificity | Rapid, sensitive and specific, e.g. antigen capture enzyme-linked immunosorbent assay |
| Immunochromatography, immunogold-binding assays | Blood, secretions and excretions | Agent antigen identified by reaction with antibody of known specificity | Rapid, sensitive and specific e.g. lateral flow devices |
| Immunofluorescence | Tissues, cells | Agent antigen identified *in situ* by reaction with antibody of known specificity | Rapid, sensitive and specific, e.g. fluorescent antibody test |
| Immunohistochemistry | Tissues, cells | Agent antigen identified *in situ* by reaction with antibody of known specificity | Slow but sensitive and specific; technically demanding for use in specialised histopathology laboratories |
| Immunoelectron microscopy | Tissues, cells | Aggregation of agent by specific antibody of known specificity | Rapid, sensitive and specific, e.g. extension of diagnostic electron microscopy; technically demanding for use in specialised microscopy laboratories |
| Radioimmunoassay | Tissues, cells | Agent antigen identified by reaction with antibody of known specificity | Complex equipment and reagents required |
| Latex particle agglutination | Extracts from tissues, cells | Agent antigen identified by reaction with antibody of known specificity | Insensitive and subject to nonspecific reactions, e.g. slide agglutination test |
| Immunodiffusion | Extracts from tissues, cells | Agent antigen identified by reaction with antibody of known specificity | Insensitive and subject to nonspecific reactions; simple method |

Table modified from: *Veterinary Virology*, Third Edition, Murphy F.A., Gibbs E.P.J., Horzinek M.C. & Studdert M.J., eds, published 1999, Academic Press, San Diego, California, USA.