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Foodstuffs — Molecular biomarker analysis — Immunochemical methods for the detection and quantification of proteins

Produits alimentaire — Analyse des biomarqueurs moléculaires — Méthodes immunochimiques pour la détection et la quantification des protéines

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

This third edition cancels and replaces the second edition (ISO 21572:2013), which has been technically revised. The main changes compared with the previous edition are as follows:

— the title has been changed to specify that the document is focused on immunochemical protein detection methods;

— an introduction has been added;

— terms, definitions and references have been updated;

— the text has been modified to improve the document's applicability to general protein analysis applications.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Analytical techniques based on highly specific immunochemical-binding interactions have become key tools for analysing many different chemical and macromolecular analytes, including proteins. Methods utilizing these techniques are widely accepted in the scientific and regulatory communities. Immunochemical assay methods are most commonly used to detect (presence or absence) and/or quantify specific protein analytes such as allergenic proteins, disease marker proteins or newly expressed proteins in biotech crops.

Prior to analysis, samples generally need to be ground or processed in a manner that facilitates extraction of the analyte from the sample matrix. An important step in analytical method development is therefore the selection of a suitable extraction buffer that does not interfere with the analytical method performance and that ensures an appropriate level of analyte stability during the analytical process.

The immunochemical assay process generally incorporates at least two steps:

— binding or capturing the analyte of interest present in samples with an antibody targeted specifically to the analyte;

— detection of the antibody-analyte complex using a technique that signals the specific interaction.

Once an analytical method has been developed and optimized, it should be validated to demonstrate that its performance is reliable and suitable for the intended use and to characterize the method limitations. This involves performing several experiments with real samples to evaluate parameters such as accuracy, precision, sensitivity, selectivity and the detection or quantification limits. Validation also allows for the establishment of method performance criteria, against which routine analytical performance can be compared to ensure that acceptable analytical results are consistently reported.

This document provides a set of general procedures and analytical considerations for using immunochemical techniques to analyse target proteins. It discusses aspects of sample processing, extraction, assay set-up, interpretation and reporting of results, and relevant assay performance parameters. Two annexes are included containing example procedures that can be followed when analysing a protein of interest (POI) in a variety of background matrices using methods based on enzyme-linked immunosorbent assays (ELISAs) and lateral flow devices (LFDs).

Foodstuffs — Molecular biomarker analysis — Immunochemical methods for the detection and quantification of proteins

1 Scope

This document specifies performance criteria for immunochemical methods for the detection and/or quantification of a specific protein or protein(s) of interest [POI(s)] in a specified matrix.

The methods discussed are applicable to the analysis of proteins from a variety of sample types. Some uses for these methods include, but are not limited to, analysing proteins involved in crop and food production, food processing, food marketing, food safety, biotechnology or disease indexing.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 16577, *Molecular biomarker analysis — Terms and definitions*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 16577 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
conjugate
material produced by attaching two or more substances together by covalent bond via chemical groups

Note 1 to entry: Conjugates of antibodies with fluorochromes (e.g. chemical entity, such as a molecule or group, that emits light in response to excitation by absorbed incident light), radiolabelled substances, gold or enzymes are often used in immunoassays.

4 Principle

The target protein is extracted according to the procedure described for that specific matrix, and a specific antibody is used to detect or measure the concentration of the POI in the sample. For the detection of specific proteins in ingredients, the basic principle of a protein-based method is to:

— take a representative sample of the matrix;

— extract the proteins;

— detect and/or quantify the specific protein derived from the matrix under study.

5 Reagents

During the analysis, use only reagents of recognized analytical grade and only de-ionized or distilled water or water that has been purified, or equivalent unless indicated otherwise by the manufacturer of the reagents or the kit.

Other reagents, such as antibodies, conjugates, substrates, stop solutions and buffer components are method specific. Refer to the method for specifics regarding reagents such as protein standards or reference materials, antibodies or pre-coated solid surfaces, controls, and samples.

Reagents are specified in A.4.2, A.4.3, B.4.2 and B.4.3.

6 Laboratory equipment

Laboratory equipment is specified in A.5 and B.5.

7 Sampling

Sampling is not part of the method specified in this document, though Annex A and Annex B do provide sampling instructions as per the relevant methods. It is recommended that the parties concerned come to an agreement on this subject.

8 Procedure

8.1 General

Storage conditions and the shelf-life of LFDs, antibodies, conjugate, substrate, etc. shall be clearly specified by the provider.

Use appropriate laboratory equipment with low protein binding capacity (e.g. polypropylene tubes) to prevent protein adsorption during the whole procedure.

For the use of this document, general requirements of quality assurance for laboratories shall be observed (e.g. concerning calibration of apparatus, double determination, blanks, use of reference materials, preparation of calibration curves) Carefully clean all equipment coming into direct contact with the sample to prevent contamination. See ISO/IEC 17025 for more information.

8.2 Preparation of sample solution

Once a representative sample is obtained, prepare the sample solution. Sample preparation procedures are described in Annexes A and B.

Grind samples as specified in the method before test portions are taken, if necessary. Powders/flour can have swelling properties and could require more extraction solution if a manufacturer's method does not specify this information. If the sample is not immediately used, follow the laboratory's procedure for storage (e.g. –20 °C or below).

Weigh an appropriate amount of a representative test sample (as specified in A.6.6.1 and B.6.2.1) for analysis to create a test portion for extraction. Add extraction solution and homogenize or mix.

Laboratory samples containing high amounts of fat can be non-homogeneous and a larger test portion should be extracted to ensure that it is representative. If applicable, instructions can be found in the sample preparation sections of Annexes A and B.

8.3 Extraction

Use an extraction procedure suitable for the matrix. Details of appropriate conditions for the extraction/dilution of the test portions, controls and reference materials are provided in Annex A for ELISA and Annex B for LFDs. Care should be taken to use extraction procedures validated for the matrix. Extracted samples should be immediately used or treated as specified in the procedure for storage.

8.4 Preparation of calibration curves, positive controls, and reference materials

For the preparation of calibration curves, positive controls and reference materials for Annex A, it is recommended to use matrix-matched reference materials or reference materials that have been validated for the matrix. Calibration curves are not required for qualitative application such as LFDs. However, positive and negative controls can be prepared at the discretion of the analyst.

8.5 Assay procedure

For a quantitative test, select the required number of wells, (e.g. in ELISA) for the test portion(s) to be analysed, including blanks, positive controls and negative controls, and add each of them, at minimum in duplicate and properly diluted so as to be within the range of the assay.

For a qualitative test or semi-quantitative test, select the required number of tests (e.g. ELISA or LFDs) needed for the test portions to be analysed, including blanks, positive controls and negative controls. The stability of the final signal can vary. Read the results in a timely manner as specified in Annexes A and B.

According to the method chosen, follow the instructions of each method for sample analyses, including blanks, reference materials and/or measurement standards (if necessary). Allow the reaction to occur at a specified temperature range and time. If necessary, terminate the reaction according to the method described in A.6.6.2.7 and B.6.4.2. For example, if the ELISA method requires acquiring data on a spectrophotometer, perform this step. In the case of qualitative tests, follow the kit instructions. Generally, these are interpreted visually.

9 Interpretation and expression of results

9.1 General

The parameters to interpret vary depending on whether the assay is qualitative, semi-quantitative or quantitative.

For quantitative methods, the coefficient of variation (C_V) of optical density values resulting from replicate measurements of a sample test solution, in general, should not exceed 15 %. The coefficient of variation of calculated concentrations resulting from replicate measurements of a sample test solution, in general, should not exceed 20 %.

If the coefficient of variation limit is exceeded, the analyses should be repeated on freshly prepared sample test solution. To establish a coefficient of variation, in this case, at least three determinations shall be carried out (e.g. values from three micro-titre wells).

Negative results shall be reported as “negative at the limit of detection” and the limit of detection (LOD) shall be reported.

Positive results below the limit of quantification shall be reported as “positive above the limit of detection, but below the limit of quantification”. The limits of quantification and detection shall be reported.

9.2 Quantitative and semi-quantitative analysis

For quantitative and semi-quantitative analysis, the following parameters shall be evaluated:

— raw data of the sample test solution;

— blanks;

— reference materials or measurement standards;

— negative controls;

— % C_V between replicates;

— % C_V of standards;

— % C_V of control samples.

In accordance with ISO/IEC 17025, measurement uncertainty should be reported, where applicable.

Quantitative results shall not be reported by extrapolating above the highest or below the lowest calibration point.

9.3 Qualitative analysis

For qualitative tests, including all applications thereof, the corresponding parameters are described in Annexes A and B. The LOD shall always be reported. Negative results shall be reported as “negative at the limit of detection”.

Positive results shall also report the LOD.

10 Specific parameters that can influence results

10.1 General

The performance criteria listed in the method of Annex A are a set of performance specifications established for each method during the development, validation and routine use of the method. These parameters shall be estimated and evaluated for each method to ensure they are reliable and of consistently high quality. Each time a method is implemented, the data generated shall be evaluated and compared with the established method performance criteria.

When a value (e.g. coefficient of variation of replicate determinations) does not agree with the assay specifications, it signals that the result is atypical and warrants closer evaluation of the data. The list of specifications shall be taken as whole. In certain instances, individual parameters may not meet the specifications but the data are still perfectly acceptable. If any of the criteria are not met, this should, however, be acknowledged in writing and the data evaluated to determine if the analysis of results should be adjusted, or if a particular sample or a set of samples should be repeated. These decisions should be based on the judgement of the technical expert interpreting the entire set of criteria.

In contrast to the method described in Annex A, the performance criteria of LFD assays as described in Annex B are evaluated during the development of the method by the manufacturer of the kit. The method should be evaluated for repeatability in the laboratory prior to use on test samples. Non-performing kits shall not be used.

10.2 Special considerations

10.2.1 Selectivity

Adequate selectivity of the assay for a particular analyte shall be demonstrated for each POI or analyte (protein) to be measured in each matrix to be tested. Where appropriate, cross-reactivity should be evaluated for analogues (proteins with a similar sequence or structure). To test for the absence of the POI in non-POI sample, assay the non-POI containing sample and POI-containing sample at the appropriate dilutions and compare.

This is generally done during the development and validation of the method and is not necessary during routine analysis of samples for which the method has previously been validated. Selectivity of the test kits, either ELISA or LFD-based methods, should be addressed by the manufacturer of the kit (e.g. listed in the manufacturer's product inserts).

10.2.2 Extraction efficiency

Special care shall be taken to assess the influence of process parameters applied for the production of a given laboratory sample.

In order to provide for the greatest sensitivity of the immunoassay, extraction efficiency should be as high as possible, especially for quantitative methods. The assay performance is matrix dependent. Extraction efficiency should be determined and documented for each matrix.

The extraction procedure shall be demonstrated to be reproducible and the method of calibration (if applicable) should account for incomplete extraction.

10.2.3 Matrix effects

The scope of application clearly and exactly defines the matrices for which the given immunoassay is applicable. The use of matrix matched reference materials allows for direct comparison between reference materials and samples. However, if samples are to be analysed against reference materials that are not the same matrix, then the matrix effects will have to be evaluated.

For example, prepare a negative extract for each sample (matrix) to be analysed by the method and an extract of a positive control of known concentration. Prepare a series of dilutions of the positive control in the negative extract and compare the resulting dose response curve with the calibration curve from the method. If the two curves are different, then there is a matrix effect. Use a matrix that most closely represents the true samples that will be tested. A dilution curve with a positive control of known concentration should also be included as a reference. The shape of the calibration curve should not change due to a matrix effect.

10.2.4 Assay applicability

Food processing will generally lead to degradation or denaturation of the POI, which could result in a substantial change in immunoreactivity. Immunoassays should be evaluated for applicability to the POI in processed products.

10.2.5 Hook effect

In an antibody-based LFD and plate format assay, a hook (saturation) effect could lead to a false-negative result. A thorough demonstration that the working concentration range comfortably covers the practical need of POI test samples is necessary.

10.2.6 Parallelism/linearity

For quantitative analyses, the expected dynamic range of the immunoassay should be explicitly stated in the scope of applications for all matrices covered by it. The relationship of the instrument response to known POI concentration may not be linear and shall be established for each quantitative immunoassay

method by the manufacturer. This relationship is typically hyperbolic if the POI concentration is plotted on a linear scale or sigmoidal with a logarithmic concentration scale. Either a 4-parameter logistic or 5-parameter logistic regression model provides the best fit for an ELISA calibration curve, with the linear portion in the centre of the sigmoidal curve representing the optimal region for quantitative analysis. Other regression models (e.g. linear, quadratic, logit-log, spline-fit, third order polynomial) may also be suitable to fit limited portions of the calibration curve.

A minimum of four calibration points, reflecting the usable portion of the curve, shall be evaluated for quantification purposes, although a 4-parameter logistic fit requires at least five points and a 5-parameter logistic fit requires at least six points.

10.2.7 Limits of detection

Results should not be interpreted below the LOD. In this case, reporting of results shall be stated according to applicable method as described in 9.1 to 9.3.

10.2.8 Limits of quantification

The limits of quantification for each set of calibrants (or dilutions) shall be stated explicitly.

The estimated concentration of unknown sample test solutions shall be interpolated and not extrapolated.

Results shall not be extrapolated below the limit of quantification or above the highest or below the lowest calibration points.

11 Confirming method

To establish the credibility of assays, another method such as western blot, high-performance liquid chromatography (HPLC), mass spectrometry (MS) or a functional assay can be used to measure split analytical samples of known concentration. The results of both methods are then qualitatively/quantitatively compared. This is especially important for immunoassays, since antibodies could cross react with other analytes present in a matrix.

12 Test report

The test report shall contain at least the following information:

a) all information needed to identify the laboratory;

b) all information needed to identify the laboratory sample;

c) reference to this document, i.e. ISO 21572, and to the method used, and an indication of whether it was a qualitative, quantitative or semi-quantitative method;

d) LOD;

e) lower and/or upper limits of quantification;

f) date and type of sampling procedure used (if known);

g) date of sample receipt;

h) analysis start date or other appropriate documentation;

i) amount of the test portion;

j) amount of the test sample;

k) results and the units used to report them;

l) any special points observed during testing;

m) if known, limitations should be also listed such as possible cross-reactants or selectivity of each method;

n) any operation not specified in this method or considered to be optional, but that can have an effect on the results.

Annex A
(informative)

Detection of a protein by ELISA

A.1 General

ELISAs have been successfully and routinely used for qualitative or quantitative detection of protein analytes.

Most of the commercial ELISA methods are applicable to samples where little or no treatment and processing have been performed, and thus the POIs have not been denatured. Heating, steaming and drying are just a few examples of conditions to which food ingredients are subjected during processing, which will denature proteins, and thus impact or greatly diminish the ability of the immunological reagents used in the ELISAs to bind to the specific proteins in their non-native states. Selectivity of the method should be reported since, in certain cases, ELISAs will also react with similar proteins.

ELISA methods are routinely utilized in a qualitative manner for presence or absence testing and for quantification purposes. Applicability to different matrices (e.g. grain, leaf) and crops are clearly identified by the kit manufacturer in the kit documentation and shall be supported by the manufacturer's method validation. The limit of detection (LOD) is defined under these parameters. The LOD for an ELISA kit is usually established by the kit manufacturer to be consistent with the expression in the lowest expressing variety. Cross-reactivity with other proteins is examined. ELISA kits are commercially available worldwide.

The alternate use of kits, on other crops and matrices (e.g. foods), shall be supported by independent validation and LOD determination by the laboratory performing the off-label testing.

A.2 Scope of the method

This annex outlines a generic example of a procedure for using ELISA to determine if the POI is present, and to quantitate the amount of the POI present in the sample. The method is applicable to samples where little or no treatment or processing has been carried out, and, thus, the POI is not denatured. For example, high temperatures at which food ingredients are processed can impact the ability to detect the POI. Each manufacturer shall supply the ELISA procedure with the kit and specify what matrix can be tested using the ELISA along with acceptance and rejection criteria that have been established through development and validation of the method.

A.3 Principle

A direct sandwich ELISA is used for detection of a POI as follows and as shown in Figures A.1 to A.4.

— Step 1: The surface of a micro titre plate is coated with a specific capture antibody.

— Step 2: When the sample of interest is added, the capture antibody binds the antigen. Unbound components of the sample are removed by washing.

— Step 3: After washing, a polyclonal antibody, covalently linked (for example) to horseradish peroxidase (HRP) is added, which is specific for a second antigenic site on the bound POI.

— Step 4: After washing, a tetramethylbenzidine (TMB) chromogenic substrate for horseradish peroxidase is added. The horseradish peroxidase generates a colour signal that is proportional to the concentration of the antigen in a linear range. To stop the colour development, a stop solution is added. The degree of colour produced is measured at a wavelength of 450 nm.

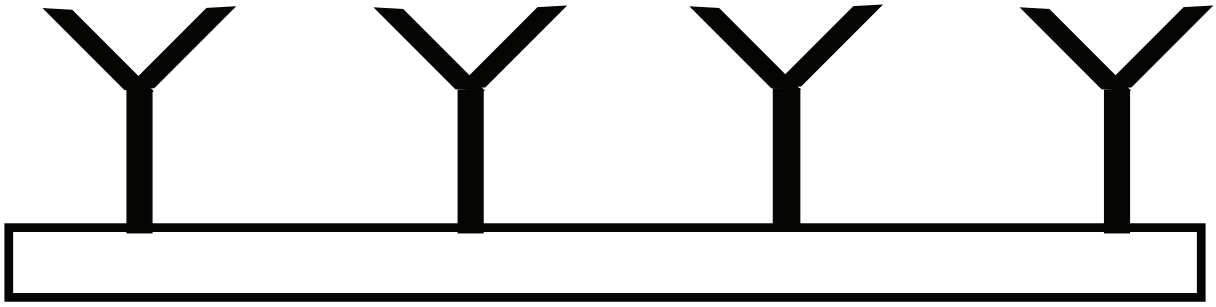


Figure A.1 — Step 1

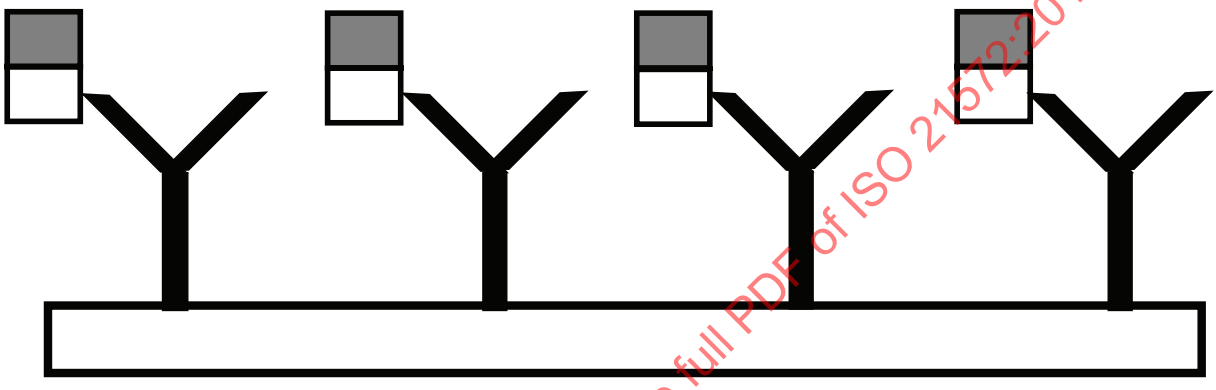


Figure A.2 — Step 2

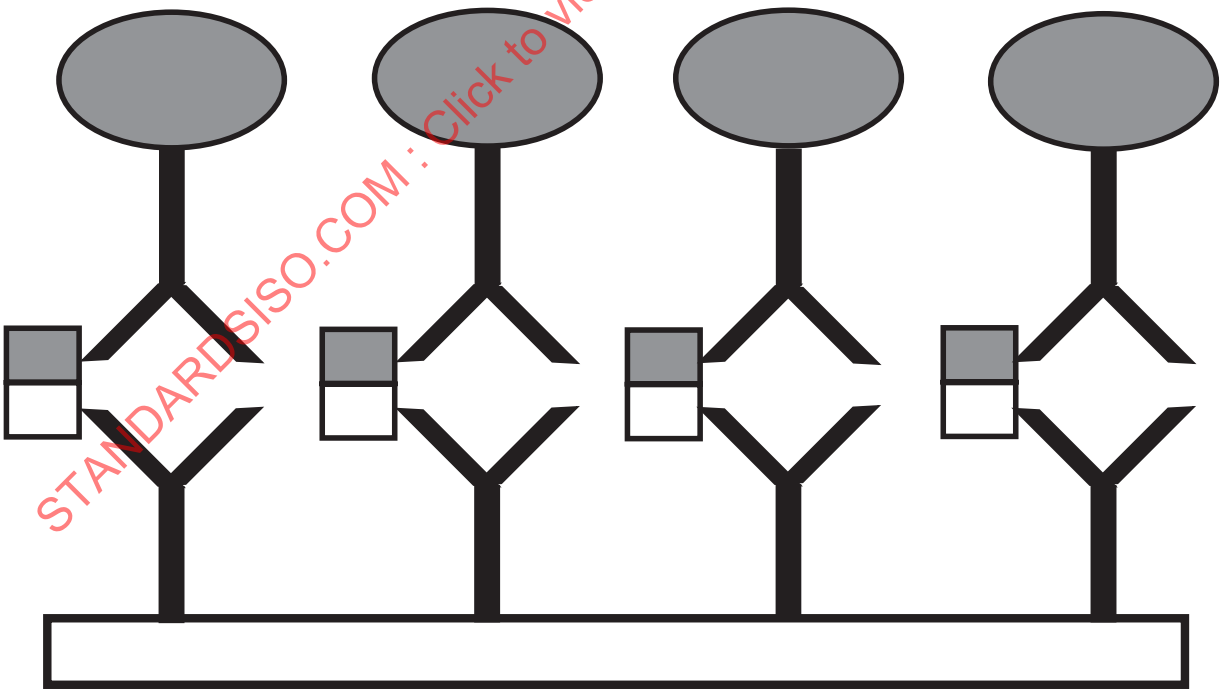


Figure A.3 — Step 3

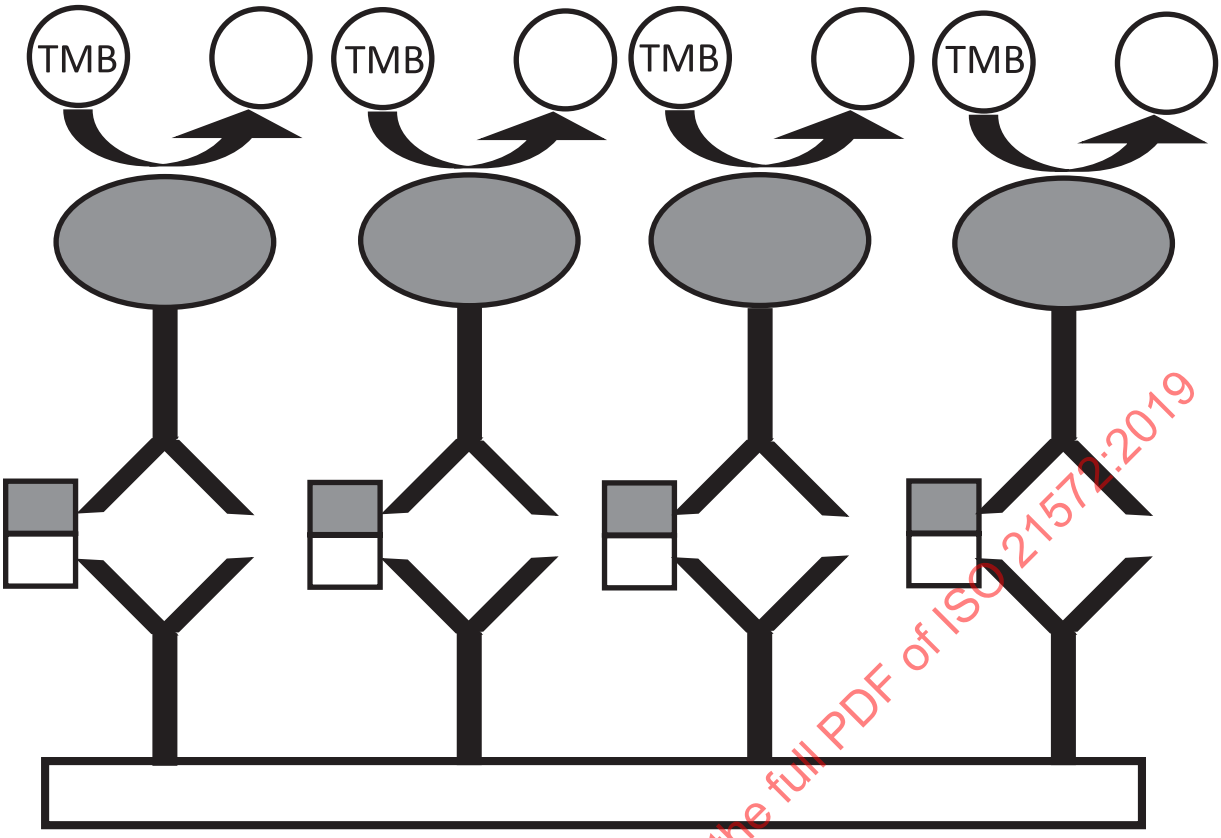


Figure A.4 — Step 4

A.4 Reagents

A.4.1 General

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and deionized or distilled water.

Any deviation from the defined performance criteria can indicate a lack of reagent stability. For example, turbid buffer or turbid conjugate solutions should not be used.

All kit components should be stored at approximately 2 °C to 8 °C. The shelf-life of the kit components is indicated by an expiration date.

The antibody conjugate stock solution (A.6.3.1) and the antibody conjugate working solution (A.6.3.2) can be stored at 2 °C to 8 °C according to the kit instructions for dilution and storage. The diluted wash buffer should also be stored according to the manufacturer's instructions or according to the laboratory's established standard procedures.

A.4.2 Reagents and materials usually provided with the test kit

A.4.2.1 Extraction buffer.

A.4.2.2 Assay buffer.

A.4.2.3 Coated strip wells or plates.

Not all kits contain coated strip wells or plates. Coating the strip wells or plates with antibody can be done by the user as an additional step. Users shall follow the manufacturer's instructions for the preparation of coating buffer and strip well or plate coating steps.

A.4.2.4 A conjugated detection antibody.

A.4.2.5 Conjugate diluent buffer.

A.4.2.6 Chromogenic substrate.

A.4.2.7 Stop solution.

A.4.2.8 Wash buffer concentrate.

A.4.2.9 Matrix matched negative and positive reference materials.

A.4.3 Chemicals not supplied with the test kit

A.4.3.1 Alcohol, such as methanol, volume concentration of 70 %, or ethanol, volume concentration of 95 %.

A.4.3.2 Detergent for ultrasonic bath (optional).

A.5 Laboratory equipment

Usual laboratory equipment and, in particular, the following.

A.5.1 Refrigerator, set to an operating range of approximately 2 °C to 8 °C.

A.5.2 Polypropylene conical centrifuge tubes, sealable, e.g. 15 ml.

A.5.3 Plastic wrap or aluminium foil (optional).

A.5.4 Plastic tape to prevent strip movement during plate washing **(optional)**.

A.5.5 Wash bottle, e.g. of volume of 500 ml.

A.5.6 Precision pipettes, capable of delivering e.g. 20 µl to 500 µl.

A.5.7 Small sample mixer.

A.5.8 Balance, capable of weighing to the nearest 0,01 g.

A.5.9 Centrifuge, capable for producing a relative centrifugal force (RCF) of at least 5 000*g*.

A.5.10 Microtitre plate reader, capable of reading absorbance at 450 nm or the wavelength specified in the kit.

A.5.11 Incubator oven or water bath, capable of maintaining 37 °C (if required).

A.5.12 Sieve of aperture size of 450 µm, or equivalent (optional).

A.5.13 Sieve of aperture size of 150 µm (100 mesh), or equivalent (optional).

A.5.14 Multi-channel pipette, e.g. of 50 µl to 300 µl, **(optional)**.

A.5.15 Reagent reservoirs for multi-channel dispensing (optional).

A.5.16 Automated plate washer (optional).

A.5.17 Test tube rack for 15 ml centrifuge tubes (optional).

A.5.18 Ultrasonic bath (optional).

A.6 Procedure

WARNING — Precautions should be taken when using acidic stop solutions or chromogenic substrate solutions containing tetramethylbenzidine (TMB). Standard laboratory ventilation is sufficient.

A.6.1 Limitations of the procedure

For thermally processed food samples and composite food samples, the method may not be fit for purpose, unless specifically designed for the matrix.

The ELISA test kit is designed to give optimum performance at ambient temperatures of between 15 °C and 30 °C. The absorbance of the highest reference material should be greater than 0,8 optical density (OD) and should not fall outside the linear range of the reader. At temperatures greater than 30 °C, OD values will rise more rapidly and a reduced substrate incubation time could be necessary. At low temperatures (less than 15 °C), the substrate incubation time should be increased.

A.6.2 Sampling

A.6.2.1 Sample preparation

Take a homogeneous test sample from the laboratory sample in duplicate.

For whole grains, the amount of test portion taken will influence the LOD and limit of quantification of the method. For raw grain, it is typical that 2 000 g is blended and ground until fine enough to be sieved. For quantitative analysis, it is typical that a particle size of less than 150 µm should be obtained and less than 450 µm for qualitative analysis. To avoid contamination, care should be taken during the sieving step. Furthermore, care should be taken to avoid excessive heating. The action of the blender will both mix and grind the sample. From the ground material, approximately 100 g should be taken and passed through a sieve of 450 µm pore size (A.5.12). At least 90 % of this sample should pass through the 450 µm sieve. For a qualitative assay, this material can be used directly. For a quantitative assay, the sieved material should be further sieved using a sieve of 150 µm pore size (A.5.13). The material passing through the 450 µm sieve has been shown to be homogeneous. Therefore, it is necessary to sieve only enough material to provide an analytical sample through the 150 µm sieve.

This procedure is typical for a large grain such as maize or soybean. Certain samples can consist of material that should be handled frozen or are already a homogenate.

A.6.2.2 Measures to avoid contamination during sample preparation

A.6.2.2.1 General

The ELISA test system is a sensitive technique capable of detecting very small quantities of POIs. For this reason, it is imperative that all equipment used to process samples be thoroughly cleaned between sample batches. The procedures given in A.6.2.2.2 and A.6.2.2.3 involve a first step of the physical removal of as much particulate material as possible. The second step, a wash with alcohol, is to denature and render non-reactive any protein that remains on the equipment.

This procedure is typical for blenders that cannot be washed in a commercial washing operation.

A.6.2.2.2 Grinder or blender cleaning

Brush clean with a soft bristle brush.

Rinse with alcohol (this may be stored and dispensed from a spray or squirt bottle). Two rinses or sprays are recommended. Then rinse thoroughly with water.

Air dry or, if rapid reuse is required, use a flow of air such as from a commercial hair dryer.

Periodically wash the brush and soak it in an alcohol solution (A.4.3.1). Dry the brush before subsequent use. Wipe with a soft cloth or laboratory towel.

A.6.2.2.3 Sieve cleaning

Sieves tend to become caked with powder. Sharply tap sieves on hard surfaces to dislodge caked material.

Brush the sieves with clean soft bristle brushes. Soak the sieves in alcohol for at least 5 min and rinse thoroughly with water. Air dry or, if rapid reuse is required, use a flow of air such as from a commercial hair dryer.

An alternative method is to use an ultrasonic bath followed by air drying, or to wash the sieve with hot water and detergent in a dishwasher.

Periodically wash the brush and soak it in an alcohol solution (A.4.3.1). Dry the brush before subsequent use. Wipe with a soft cloth or laboratory towel.

A.6.2.2.4 Cleanliness of work area

Avoid dust contamination in the work area. Do not allow dust from one processing to contaminate equipment to be used in a subsequent processing. For optimum performance, run assay in a room separated from the facility where sample grinding and preparation is conducted to avoid potential dust contamination.

A.6.3 Preparation of antibody conjugate

A.6.3.1 Antibody conjugate stock solution

Prepare the antibody conjugate (A.4.2.4) as per the kit instructions.

Store the antibody conjugate stock solution at 2 °C to 8 °C for no longer than the expiration date of the kit, as per the manufacturer's instructions.

A.6.3.2 Antibody conjugate working solution

Add the conjugate stock solution (A.6.3.1) to the conjugate diluent buffer (A.4.2.5) according to the method used.

Store the antibody conjugate working solution at 2 °C to 8 °C for no longer than the expiration date of the kit.

A.6.4 Preparation of wash buffer

Prepare the wash buffer as per the manufacturers' instructions or to a laboratory standard operating procedure (SOP).

A.6.5 Assay procedure

Allow all reagents to reach room temperature.

Remove the coated strips (A.4.2.3) and strip holder or the ELISA plate from the foil bag. Always seal the foil bag each time after removing the appropriate number of strips. Typically, 10 to 12 wells are required for reference materials and assay blanks. Each plate shall have its own standards and controls. A typical procedure is summarized in A.7.

A.6.6 Test performance

A.6.6.1 Extraction of test portion and reference standard

The test portions and negative and positive reference materials are extracted under the same conditions in duplicate.

A typical process is illustrated as follows.

Weigh out 0,5 g ± 0,01 g of each reference material and the test portion into individual 15 ml polypropylene centrifuge tubes. When weighing, weigh out each reference material in increasing order of concentration. Subsequently, weigh out the test portions. To avoid contamination, clean the spatula by wiping it with an alcohol-soaked tissue (A.4.3.1) followed by drying, or use a disposable spatula between each reference material and test portion.

Add extraction buffer (A.4.2.1) into each centrifuge tube.

Mix the test portion or reference material with extraction buffer by shaking vigorously and agitating (vortex) until they become a homogeneous mixture.

Defatted flour and protein isolate need a prolonged time of mixing, sometimes more than 15 min. Full fat flour easily becomes a homogenous mixture (in less than 5 min).

Centrifuge the mixtures at approximately 5 000*g* for 15 min, preferably at 4 °C.

Carefully remove approximately 1 ml supernatant of each sample solution and standard reference extract and place each into an individual clean polypropylene centrifuge tube.

Stability of the sample solution should be established. It is recommended that sample solutions are stored at 2 °C to 8 °C, but no longer than one working day.

Prior to starting the assay, dilute the sample solutions and the reference standard solutions with the assay buffer (A.4.2.2).

A summary of the extraction steps can be found in Table A.2.

A.6.6.2 ELISA Immunoassay procedure

A.6.6.2.1 General

The ELISA assay kit can be run in different formats using any number of the 8-well strips, or a whole 96-well ELISA plate. It is recommended to follow a randomized loading scheme when validating the assay,

i.e. test samples and controls not always added to the same wells of each assay run to avoid position effects in the plate, if any.

Each extract should be assayed in at least duplicate and the mean absorbance value calculated. Each run includes an assay blank, the sample blank and the positive reference standard solutions. An assay blank or sample blank should be described in the instructions. If the manufacturer does not recommend a reagent, use the diluent buffer as the assay blank. For the sample blank, use the diluted control containing no POI.

When an assay has been started, all steps should be completed without interruption. A summary of the ELISA procedure can be found in Table A.3.

To establish a coefficient of variation in this case, at least three determinations shall be carried out (e.g. values from three wells). These three determinations can include analysis of multiple extracts of the same sample.

A.6.6.2.2 Incubation

Using a pipette, add diluted sample and reference material solutions and the assay blank to the appropriate wells. Use separate disposable tips for each pipetting step to avoid carry-over contamination. Cover the plate with plastic wrap or aluminium foil (A.5.3) to prevent contamination and evaporation.

Before starting incubation, the microtitre plate may be mixed by moving the plate from side to side. Alternatively, ELISAs may be continually shaken during the incubation, if validated as such.

Incubate the microtitre plate at 37 °C for 1 h or as the manufacturer recommends.

A.6.6.2.3 Washing

Wash with wash buffer as per the instructions or validated SOP.

Manual washing: Empty the wells by inverting over a sink or suitable waste container. Using a wash bottle containing a working wash solution, fill each well to the top, allow to stand for 60 s, then empty the plate as described above. Repeat the washing step three times. Remove residual liquid and bubbles by tapping upside down on several layers of paper towels.

If using a multi-strip plate it is recommended to prevent the strips from falling out of the frame by securing with adhesive tape.

Automatic washing: At the end of the incubation period, aspirate the contents of all wells using a plate washer, then fill the wells with a working wash buffer. Repeat the aspiration/fill step three times. Finally, use the washer to aspirate all wells then tap the inverted plate into a stack of paper towels to remove residual droplets of wash buffer and bubbles. Plate washers may automate one or more of these steps.

Do not let wells dry out, as this it can affect assay performance.

NOTE Inadequate washing will cause erroneous results. Whether using a manual or automated washer, it is important to ascertain that each assay well is washed with the same volume as all other wells.

A.6.6.2.4 Addition of antibody conjugate

Add the antibody conjugate working solution (A.6.3.2) to each well using a pipette. Cover the plate to prevent contamination and evaporation.

The plate is mixed (if appropriate) and incubated (with or without shaking as per the manufacturer's instructions or validated SOP).

A.6.6.2.5 Washing

At the end of the incubation period, repeat the washing step as described above (A.6.6.2.3).

A.6.6.2.6 Substrate addition

Add the chromogenic substrate (A.4.2.6) to each well using a pipette. Gently mix the plate and incubate (typically) for 10 min at room temperature.

The addition of chromogenic substrate should be completed without interruption. Maintain the same sequence and time interval during the pipetting.

A.6.6.2.7 Stop solution addition

At the end of the incubation period, add the stop solution (A.4.2.7) to each well, pipette the stop solution in the same sequence as the colour reagent was added. Gently agitate the plate to ensure mixing of the well contents and the stop solution.

The addition of stop solution should be completed without interruption. Protect the microtitre plate from prolonged exposure to light, otherwise the colour intensity can change over time.

A.6.7 Absorbance reading

Measure the absorbance or chromogen intensity of each well using a microtitre plate reader fitted with a filter or monochromator appropriate to the wavelength needed for the ELISA chromogenic system (see Table A.1). All readings should be completed within 30 min of adding the stop solution.

Absorbance for the HRP enzyme and TMB substrate example provided in this annex should be measured with a plate reader fitted with a 450 nm filter.

Record the results obtained and calculate the mean absorbance values or use a software application.

Table A.1 — Typical enzyme chromogenic systems

Enzyme	Substrates	Wavelength (nm)/Colour
Horseradish peroxidase (HRP)	3,3′-diaminobenzidine (DAB)	405/yellow
	3,3′,5,5′-tetramethylbenzidine (TMB)	450 (652)/yellow (blue)
	2,2′-Azinobis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS)	410 (650)/green (blue)
	o-phenylenediamine dihydrochloride (OPD)	490 (450)/green (orange)
Alkaline phosphatase (AP)	Combination of nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP)	Blue
	p-Nitrophenyl Phosphate (PNPP)	405/yellow
Glucose oxidase (GO)	Nitro blue tetrazolium chloride (NBT)	Black/brown
β-galactosidase	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (BCIG or X-Gal)	Blue

A.7 Flowcharts

Table A.2 — Extraction flowchart

Procedure	Description
Weigh out 0,5 g	Weigh out analytical samples, blank, reference standards.
Addition of 4,5 ml	Addition of extraction buffer (A.4.2.1).
Mixing	Mix the test portion with extraction buffer until it becomes homogeneous, full fat flour less than 5 min, defatted flour, protein isolate more than 15 min.
Centrifugation at 5 000*g*	Centrifuge the sample at 5 000*g* for 15 min, preferably at 4 °C. Remove supernatant and place it into a clean tube.

Table A.2 *(continued)*

Procedure	Description
Dilution: 1 → 300 or 1 → 10 according to the material investigated	Dilute the resulting test solution, assay blank and reference standards.
Protein quantification	Immediately after dilution, proceed to ELISA as described in A.6.6.2 or store the test samples and dilutions thereof, assay blank and reference standards according to the recommended storage condition.

Table A.3 — ELISA procedure flowchart

Procedure	Volume	Description
Addition	100 µl	Pipette diluted sample test solutions, blank and reference standards into appropriate assay well and mix.
Incubation		Incubate 1 h at 37 °C.[a]
Washing		Wash three times with wash buffer (A.6.4).
Addition	100 µl	Dispense antibody conjugate (A.4.2.4) into each assay well and mix.
Incubation		Incubate 1 h at 37 °C.[a]
Washing		Wash three times with wash buffer (A.6.4).
Addition	100 µl	Dispense chromogenic substrate (A.4.2.6) into each well and mix.
Incubation		Incubate for 10 min at ambient temperature.[a]
Addition	100 µl	Dispense stop solution (A.4.2.7) into each assay well and mix.
Absorbance measurement		Measure the absorbance or chromogen intensity of each well using a microtitre plate reader fitted with a filter or monochromator appropriate to the wavelength needed for the ELISA chromogenic system.

[a] Incubation times and temperatures will be based on the manufacturer's recommendations and conditions established during validation.

A.8 Evaluation

Data should be recorded.

Standard values should be used to develop a standard curve. The value from the assay blank should be subtracted for all values. The average corrected values from each duplicate reference point should be used to create a standard curve. The average data from each duplicate sample test solution should then be used to interpolate a concentration from this curve.

The curve should provide a linear function upon transformation. ELISA assays often show a nonlinear line without transformation, and thus curve fitting programs give the best result.

A.9 Accept/reject criteria

Each assay procedure shall have a set of criteria for accepting and rejecting results. The specific criteria developed and validated for each procedure may vary. Table A.4 provides a set of example criteria. To be considered valid, each assay run shall meet the procedure-specific criteria. The run consists of an assay blank, positive reference materials, negative reference materials (sample blank) and unknown samples. All sample test solutions, reference material solutions and assay blanks will be run in duplicate. If an assay run does not meet the assay acceptance criteria, the entire run shall be repeated. Individual sample test solutions that do not pass the acceptance criteria in any run shall be re-run a second time.

Table A.4 — Example criteria for accepting or rejecting results[a]

Assay buffer blank	Absorbance at appropriate wavelength of < 0,30
0 % POI standard (sample blank)	Absorbance at appropriate wavelength of < 0,30
2,5 % reference standard	Absorbance at appropriate wavelength of ≥ 0,8
All positive reference standards, OD	C_V (OD) of replicates ≤ 15 %
Unknown samples, solution	C_V (OD) of replicates ≤ 20 %

[a] As applied to horseradish peroxidase (HRP) detection in the sandwich ELISA example, the wavelength is 450 nm.

Annex B
(informative)

Detection of a protein or proteins by lateral flow devices

B.1 General

LFDs (also known as lateral flow strips or dipsticks) have been successfully and routinely used for the qualitative or semi-quantitative detection of antigens including, for example, novel proteins expressed in crops derived from modern biotechnology (GMO, bioengineered).

Most of the commercial LFD methods are applicable to samples where little or no treatment and processing have been performed, and, thus, the POIs have not been denatured. Heating, steaming and drying are just a few examples of processes to which food ingredients are subjected during processing, which will denature proteins, and thus impact or greatly diminish the ability of the immunological reagents used in the LFDs to bind to the specific proteins in their non-native states. Typically, specific separate applications should be developed, if required, for detecting POIs in processed fractions such as meals. Selectivity of the method should be reported since, in certain cases, LFDs will also react with similar proteins.

In cases of a semi-quantitative application for the detection of a specific antigen, the method can only be applied to food samples consisting entirely of products in which protein can be detected. The LFD methods are routinely utilized in a qualitative manner for presence or absence testing, although approaches have been developed to obtain quantitative outputs. The confidence in the results is high, and these methods usually test against a threshold. These methods can be easily transformed for semi-quantitative applications by combining them with sampling plans and statistics[2] for field applications[3].

The LOD for a LFD kit is usually established by the kit manufacturer to be consistent with the expression in the lowest expressing variety. Cross-reactivity with other proteins is examined. The LFD kits are commercially available world-wide. Applications for other (i.e. non-grain) protein containing matrices, such as leaf, are clearly identified by the kit manufacturer in the documentation that is part of the kit and should be supported by manufacturer's method validation data.

The alternate use of kits, on other crops and matrices (e.g. foods), shall be supported by independent validation and LOD determination by the laboratory performing the off-label testing.

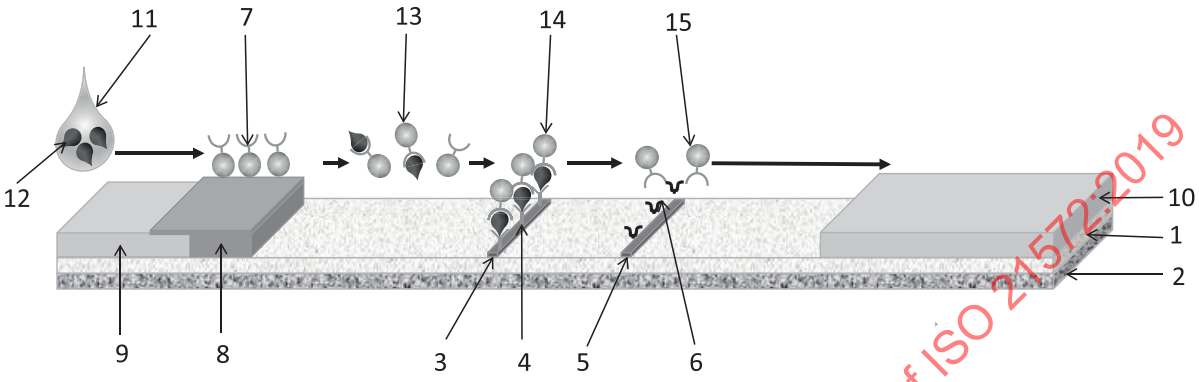
B.2 Scope of the method

This annex specifies LFD for the determination of POIs that are present in herbicide-tolerant or pest-resistant crops or ingredients processed from biotechnology derived crops. This annex provides general guidelines for LFD methods for the detection of POIs in a specified matrix and, in general, addresses the existing procedures for LFD-based methods.

B.3 Principle

B.3.1 General

As shown in Figure B.1, a typical LFD consists of a nitrocellulose membrane (1) on a backing material (2) with two lines: the test line (3) immobilizes the antigen-specific capturing antibody (4), and the control line (5) immobilizes another type of antibody that recognizes the labelled antigen-specific conjugate antibodies (6).



Key

1 nitrocellulose membrane
2 backing material
3 test line
4 antigen-specific capturing antibody
5 control line
6 antibody specific for gold-labelled antibody conjugate
7 gold-labelled antigen-specific antibody conjugate
8 gold-labelled antibody conjugate pad
9 sample and buffer pad
10 wicking pad
11 extract of positive sample
12 target antigen/protein of interest (POI)
13 antigen complex to gold-labelled antibody conjugate
14 sandwich formation with capturing antibody
15 unbound conjugated antibody captured by the anti-conjugate antibody

NOTE Adapted from Reference [4].

Figure B.1 — Diagram and description of a lateral flow device

The antigen-specific detection antibody is conjugated to gold (7) and dried into a fibre pad (antibody conjugate pad or gold pad (8)). Optimized buffers necessary for the test performance are incorporated into the sample pad (9). On the opposite end, the strip also contains a wicking pad (10) made of fibre, which provides the necessary wicking for the fluids to move through the nitrocellulose membrane. When a strip is immersed into an extract of a positive sample (11), the target antigen (12) in the sample first binds to the gold-labelled specific antibody (13) and flows through the membrane. When it passes over the test line, it forms a sandwich with the capturing antibody (14) present in the test line. This results in the formation of a visible pink to reddish line, and the result is interpreted as positive. The excess gold-labelled antibody (15) further moves and when it passes over the control line it is recognized by the immobilized antibodies in this line and allows for the development of the second line. The second line, often termed as the “control line”, serves as an internal control. The test and control lines can be of varying intensities. The device is negative if only a control line is observed. This negative result

should not be construed as proving that there is no analyte present. Refer to 9.1 for further details on reporting results.

B.3.2 Visualized test results by LFD

Examples of a positive, negative and invalid results obtained by LFDs are demonstrated in Figure B.2. Development of the control line within the required reaction time indicates that the LFD has functioned properly. Any LFD that does not develop a control line (in Figure B.2, it is closest to the top of the strip) should be discarded and the test portion shall be analysed again using a new test strip. If the sample extract contained the POI, a second line (in Figure B.2, the test line is closest to the arrows) will develop on the LFD within the required reaction time. The test results should be interpreted according to the manufacturer's instructions for the POI. If no test line is observed after the required reaction time, the results should be interpreted according to the manufacturer's instructions for the POI. If the results are interpreted as negative, i.e. the test portion does not contain the POI, then the POI could be present at levels below the LOD or the POI was modified, rendering it undetectable (e.g. a test portion was highly processed, such as defatted flour).

Figure B.2 — Example lateral flow device results

B.4 Test kit reagents and equipment

B.4.1 General

Any deviation from the defined performance criteria can indicate a lack of reagent stability. Extraction may be performed using a supplied or prepared buffer or, in cases where the buffer is incorporated in the strip, the sample may be extracted in water. Water containing high levels of copper, iron and other divalent cations should not be used to extract samples or for preparation of buffers.

The LFDs should be stored according to the manufacturer's specifications. Usually, they can be stored at room temperature, or in some cases refrigerated for longer shelf life, and will perform according to the principles in this annex for a period equal to one year from the date of shipment of the product or the expiration date marked on the product packaging. Prolonged exposure to deviant conditions, especially to high temperatures or moisture conditions, will adversely affect the test results. In cases