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**Foodstuffs — Molecular biomarker  
analysis — Protein-based methods**

*Produits alimentaires — Analyse des biomarqueurs moléculaires —  
Méthodes basées sur les 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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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.

ISO 21572 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

This second edition cancels and replaces the first edition (ISO 21572:2004), which has been technically revised. It also incorporates the Technical Corrigendum ISO 21572:2004/Cor. 1:2005.

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# Foodstuffs — Molecular biomarker analysis — Protein-based methods

**WARNING — Follow all instructions provided by the kit/reagent manufacturers and other standard laboratory safety procedures. Read and implement the material safety data sheets (MSDS).**

## 1 Scope

This International Standard provides general guidelines and performance criteria for methods for the detection and/or quantification of specific proteins or protein(s) of interest [POI(s)] in a specified matrix.

These general guidelines address existing antibody based methods. Methods other than those described in [Annex A](#) or [Annex B](#) can also detect the POI. The same criteria as outlined in this International Standard apply generally.

## 2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 24276, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions*

## 3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 24276 and the following apply.

### 3.1 General

#### 3.1.1

##### **sample**

subset of a population made up of one or more sampling units

[SOURCE: ISO 3534-2:2006, 1.2.17]

#### 3.1.2

##### **laboratory sample**

*sample* (3.1.1) as prepared for sending to the laboratory and intended for inspection or testing

[SOURCE: ISO 78-2:1999, 3.1]

#### 3.1.3

##### **test sample**

*sample* (3.1.1) as prepared for testing or analysis, the whole quantity or part of it being used for testing or analysis at one time

[SOURCE: ISO 3534-2:2006, 5.3.11]

#### 3.1.4

##### **test portion**

part of a *test sample* (3.1.3) which is used for testing or analysis at one time

[SOURCE: ISO 3534-2:2006, 5.3.12]

### 3.1.5

#### **matrix**

products submitted for analysis, which might have differences in chemical composition and physical state

[SOURCE: ISO 22174:2005, 3.1.4]

### 3.1.6

#### **denaturation of proteins**

physical and/or (bio)chemical treatment which destroys or modifies the structural, functional, enzymatic, or antigenic properties of the POI or the analyte

## 3.2 Terms relating to antibodies

### 3.2.1

#### **antibody**

protein (immunoglobulin) produced and secreted by B lymphocytes in response to a molecule recognized as foreign (antigen) and capable of binding to that specific *antigen* (3.2.2)

### 3.2.2

#### **antigen**

substance that stimulates the production of *antibodies* (3.2.1) and reacts with them

### 3.2.3

#### **clone**

population of identical cells derived from a single cell

### 3.2.4

#### **cross-reactivity**

binding of the *antibody* (3.2.1) to substances other than the analyte of primary interest

### 3.2.5

#### **monoclonal antibody**

*antibody* (3.2.1) produced from a single hybridoma *clone* (3.2.3) and directed to a single *antigen* (3.2.2) determinant

### 3.2.6

#### **polyclonal antibody**

mixture of immunoglobulin molecules, secreted against a specific immunogenic substance, each recognizing a different epitope

[SOURCE: ISO 19001:2013, 3.11]

### 3.2.7

#### **selectivity of an antibody**

ability of an *antibody* (3.2.1) to specifically bind to an *antigen* (3.2.2) determinant and not to other similar structures or other antigens

## 3.3 Terms relating to techniques

### 3.3.1

#### **conjugate**

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

**EXAMPLE** Conjugates of antibodies with fluorochromes (e.g. chemical entity, such as a molecule or group, which emits light that is in response to being stimulated by absorption of incident light), radiolabelled substances, gold or enzymes are often used in immunoassays.

**3.3.2****western blotting protocol  
protein immunoblot**

transfer of a protein to a binding surface following separation by electrophoresis that may be visualised using a variety of methods

**EXAMPLE** One example of such a method is with a specific radiolabelled or enzyme-conjugated antibody followed by the addition of an enzyme-specific substrate to form a coloured reaction product.

**3.3.3****enzyme linked immunosorbent assay  
ELISA**

*in vitro* assay used for qualitative, semi-quantitative, or quantitative purposes that combines enzyme-linked antibodies and a substrate to form a coloured reaction product

**3.3.4****test kit**

set of chemicals, materials and instructions for use, packaged together and intended for use as specified by the manufacturer of the kit

**3.3.5****lateral flow immunochromatographic assay  
lateral flow device/strip**

qualitative or semiquantitative, simple rapid assay formats intended to detect the presence (or absence) of a POI in a sample where an *antibody* (3.2.1) or an analyte is coated to a solid surface and dipped into a test liquid to provide a measure of the POI in the liquid

**Note 1 to entry:** The test sample flows along a solid substrate via capillary action. After the liquid sample enters the test strip, it encounters a coloured reagent which mixes with the sample and transits the substrate encountering lines or zones which have been pretreated with an antibody or antigen. Depending on the analytes present in the sample the coloured reagent can become bound at the test line or zone. These assays can operate as either competitive or sandwich assays.

**3.4 Terms relating to control****3.4.1****reference material**

material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process or in examination of nominal properties

[SOURCE: ISO/IEC Guide 99]

**3.4.2****measurement standard**

measured material, measuring instrument, *reference material* (3.4.1) or measuring system intended to define, realize, conserve or reproduce one or more values to serve as a reference or preparation of known characteristics used to standardize the analysis

**3.5 Terms relating to validation****3.5.1****accuracy**

closeness of agreement between a test result or measurement result and a reference value

**Note 1 to entry:** The term “accuracy”, when applied to a set of test results or measurement results, involves a combination of random components and a common systematic error or *bias* (3.5.3) component.

**Note 2 to entry:** When applied to a test method, the term accuracy refers to a combination of trueness and *precision* (3.5.2).

[SOURCE: ISO 3534-2:2006, 3.3.1, modified — Notes 1 and 2 differ from the original and there is no Note 3.]

### 3.5.2

#### **precision**

closeness of agreement between independent test/measurement results obtained under stipulated conditions

Note 1 to entry: Precision is normally expressed in terms of standard deviation.

[SOURCE: ISO 3534-2:2006, 3.3.4]

### 3.5.3

#### **bias**

difference between the expectation of a test result or measurement result and a true value

Note 1 to entry: Bias is the total systematic error as contrasted to random error. There may be one or more systematic error components contributing to the bias. A larger systematic difference from the true value is reflected by a larger bias value.

[SOURCE: ISO 3534-2:2006, 3.3.2]

### 3.5.4

#### **sensitivity**

quotient of the change in the indication of a measuring system and the corresponding change in the value of the quantity being measured

Note 1 to entry: The sensitivity can depend on the value of the quantity being measured. Sensitivity usually is meant as the smallest quantity or concentration of the analyte that can be reliably distinguished from background.

[SOURCE: ISO/IEC Guide 99]

### 3.5.5

#### **selectivity**

extent to which a method can determine particular analyte(s) in a mixture(s) or matrice(s) without interferences from other components of similar behaviour

Note 1 to entry: Selectivity is the recommended term in analytical chemistry to express the extent to which a particular method can determine analyte(s) in the presence of other components. Selectivity can be graded.

[SOURCE: Pure Appl. Chem.]

### 3.5.6

#### **detection limit**

#### **limit of detection**

#### **LOD**

lowest concentration or content of the POI per defined amount of matrix that can be consistently detected under the experimental conditions specified in the method

[SOURCE: ISO 22174:2005, 3.1.8, modified — “LOD” has been added and “of the target organism” became “of the POI”.]

### 3.5.7

#### **determination limit**

#### **limit of quantification**

#### **LOQ**

lowest concentration or content of the POI per defined amount of matrix that can be measured with reasonable statistical certainty consistently under the experimental conditions specified in the method

**3.5.8**

**applicability range**  
**range of quantification**  
**range of linearity**  
**dynamic range**

upper and lower limits of quantification as expressed by a set of reference materials (or dilutions) with a suitable level of precision and *accuracy* (3.5.1)

**3.5.9**

**repeatability conditions**

observation conditions where independent test/measurement results are obtained with the same method on identical test/measurement items in the same test or measuring facility by the same operator using the same equipment within short intervals of time

Note 1 to entry: Repeatability conditions include: the same measurement procedure or test procedure; the same operator; the same measuring or test equipment used under the same conditions; the same location and repetition over a short period of time.

[SOURCE: ISO 3534-2:2006, 3.3.6, modified — the Note has been deleted.]

**3.5.10**

**repeatability**

precision under *repeatability conditions* (3.5.9)

[SOURCE: ISO 3534-2:2006, 3.3.5]

**3.5.11**

**repeatability limit**

*r*

value less than or equal to which the absolute difference between two test results obtained under *repeatability conditions* (3.5.9) may be expected to be with a probability of 95 %

[SOURCE: ISO 5725-1:1994, 3.1.6]

**3.5.12**

**repeatability standard deviation**

standard deviation of test results or measurement results obtained under *repeatability conditions* (3.5.9)

Note 1 to entry: It is a measure of the dispersion of the distribution of test or measurement results under repeatability conditions.

Note 2 to entry: Similarly, “repeatability variance” and “repeatability coefficient of variation” can be defined and used as measures of the dispersion of test or measurement results under repeatability conditions.

[SOURCE: ISO 3534-2:2006, 3.3.7]

**3.5.13**

**reproducibility conditions**

observation conditions where independent test/measurement results are obtained with the same method on identical test/measurement items in different test or measurement facilities with different operators using different equipment

[SOURCE: ISO 3534-2:2006, 3.3.11]

**3.5.14**

**reproducibility**

precision under *reproducibility conditions* (3.5.13)

[SOURCE: ISO 3534-2:2006, 3.3.10; ISO 78-2:1999, 3.13]

### 3.5.15

#### **reproducibility limit**

*R*

value less than or equal to which the absolute difference between two test results obtained under *reproducibility conditions* (3.5.13) may be expected to be with a probability of 95 %

[SOURCE: ISO 5725-1:1994, 3.20]

### 3.5.16

#### **reproducibility standard deviation**

standard deviation of test results or measurement results obtained under *reproducibility conditions* (3.5.13)

Note 1 to entry: It is a measure of the dispersion of the distribution of test or measurement results under reproducibility conditions.

Note 2 to entry: Similarly, “reproducibility variance” and “reproducibility coefficient of variation” can be defined and used as measures of the dispersion of test or measurement results under reproducibility conditions.

[SOURCE: ISO 3534-2:2006, 3.3.12]

## 4 Principle

The POI 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 POI derived from the matrix under study.

## 5 Reagents

During the analysis, use only reagents of recognized analytical grade and only deionized 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. Please 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 International Standard, although [Annex A](#) and [Annex B](#) do provide sampling instructions according to 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 shelf-life of lateral flow strips, antibodies, conjugates, substrates, 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 International Standard, 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, specific sample preparation procedures may be found in the annexes.

Grind samples as specified in the method before test portions are taken, if necessary. Powders/flour might have swelling properties and may require more extraction solution if a manufacturer's method does not specify this information. If the sample is not immediately used, follow your laboratory's procedure for storage (e.g.  $-20^{\circ}\text{C}$  or below).

Laboratory samples containing high amounts of fat may be nonhomogeneous and a larger test sample should be extracted. If applicable, instructions may be found in the annexes.

Weigh an appropriate amount (as specified in the relevant annex) of a representative test sample for analysis to create a test portion for extraction. Add extraction solution and homogenize or mix.

### 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 lateral flow strips. 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 routinely required for qualitative application such as lateral flow strips, 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, measurement standards, and add each of them at minimum in duplicate, properly diluted to the assay.

For a qualitative test or semiquantitative test, select the required number of test (e.g. lateral flow strips or ELISA) needed for the test portions to be analysed. The stability of the final signal can vary. Read the results in a timely manner as specified in the annexes.

According to the method chosen, follow the instructions of each method for sample analyses, including blanks and 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 the relevant

annex. For example, if ELISA method requires acquiring data on a spectrophotometer, perform this step. In the case of qualitative tests, generally these are interpreted visually, follow the kit instructions.

## 9 Interpretation and expression of results

### 9.1 General

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

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

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

Negative results shall be reported as “negative at the limit of detection” and the limit of detection 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 semiquantitative analysis

The following parameters shall be evaluated: raw data of sample test solution, blanks, reference materials or measurement standards, and negative controls; percentage CV between replicates, percentage CV of standard and percentage CV of control samples.

According to ISO/IEC 17025:2005, 5.10.3.1 c), 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 the annexes. The limit of detection shall always be reported and negative results shall be reported as “negative at the limit of detection”.

Positive results shall also report the limit of detection.

## 10 Specific parameters which may 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 and 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. CV 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, individual parameters may in certain instances not meet the specifications, but the data may still be perfectly acceptable. If any of the criteria are not met, it 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.

## 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 lateral flow device 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 has to 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) shall 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 which are not the same matrix, 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.

## 10.3 Assay applicability

### 10.3.1 General

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

### 10.3.2 Hook effect

In an antibody-based lateral flow device and plate format assay, a hook (saturation) effect can 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.

### 10.3.3 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. It can be linear in a very narrow range of the POI, but in most cases, immunoassays show a more complex relationship which has been previously described as a quadratic or four-parameter function.

The number of calibration points supplied should be at least four and reflect the usable portion of the curve.

### 10.3.4 Limits of detection

Results should not be interpreted below the limit of detection. In this case, reporting of results shall be stated according to applicable method as described in [9.1](#) to [9.3](#).

### 10.3.5 Limits of quantification

The limits of quantification for each set of calibrants (or dilution) 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 Confirmation method

To establish the credibility of assays, another method, such as western blot, HPLC or 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 can cross react with other analytes present in a matrix.

## 12 Test report

The test report shall contain at least the following information:

- a) all the information needed to identify the laboratory;
- b) all the information needed to identify the laboratory sample;
- c) reference to this International Standard, i.e. ISO 21572, and to the method used, and an indication of whether it was a qualitative, quantitative or semiquantitative method;
- d) limit of detection;
- 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, 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.

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## Annex A (informative)

### Detection of a protein by ELISA

#### A.1 ELISA methods

ELISAs, have been successfully and routinely used for qualitative or quantitative detection of antigens, including novel proteins expressed in crops derived from modern biotechnology. The expressed proteins generally confer agronomic or quality characteristics, such as tolerance to specified herbicide (e.g. glyphosate, glufosinate) or resistance to insects (e.g. Cry-1Ab, Cry3Bb1, Cry1F). The proteins produced by the biotechnology derived plants may be detected using ELISA-based methods.

Most of the commercial ELISA methods for modern biotechnology are applicable to samples where little or no treatment and processing have been performed, and, thus, the POIs have not been denatured. Heating, steaming, drying, are just a few examples 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 its non-native states. Selectivity of the method should be reported, in certain cases ELISAs will also react with similar proteins that are produced in various varieties of the biotechnology derived crops, such as Cry1Ab in Bt11 corn.

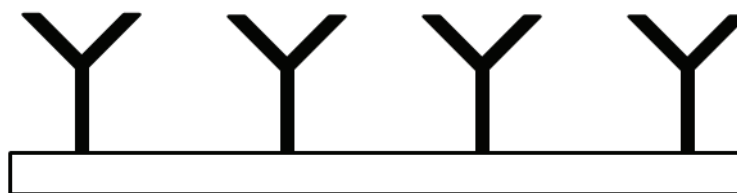
The ELISA methods are routinely utilized in a qualitative manner for presence or absence testing as well as in a quantitative manner. The limit of detection (LOD) for ELISA kit is usually established by the kit manufacturer to be consistent with the expression in the lowest expressing variety. Cross-reactivity with other proteins specific for biotechnology derived crops is examined. The ELISA kits are commercially available worldwide. Applications for other (i.e. non-grain) protein-containing matrices, such as leaf, are clearly identified by the kit manufacturer in the product inserts that are part of the kit and should be supported by manufacturer's method validation data.

#### A.2 Procedure

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 may impact the ability to detect the POI. Each manufacturer shall supply the ELISA procedure with the kit and specify which matrix can be tested using the ELISA along with acceptance and rejection criteria that have been established through development and validation of the method.

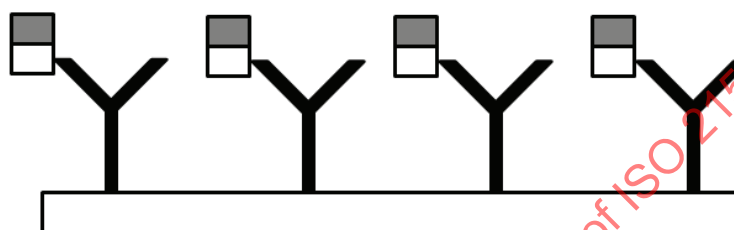
#### A.3 Principle

A direct sandwich enzyme linked immunosorbent assay (ELISA) is used for detection of a POI as shown in [Figures A.1](#) to [A.4](#).



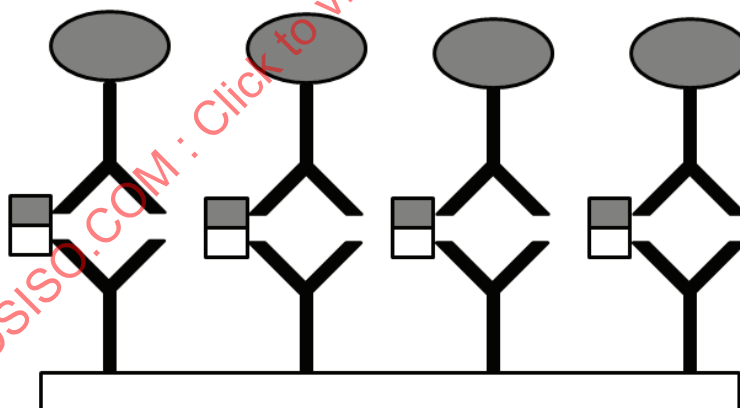
The surface of a microtitre plate is coated with a specific monoclonal capture antibody.

**Figure A.1 — Step 1**



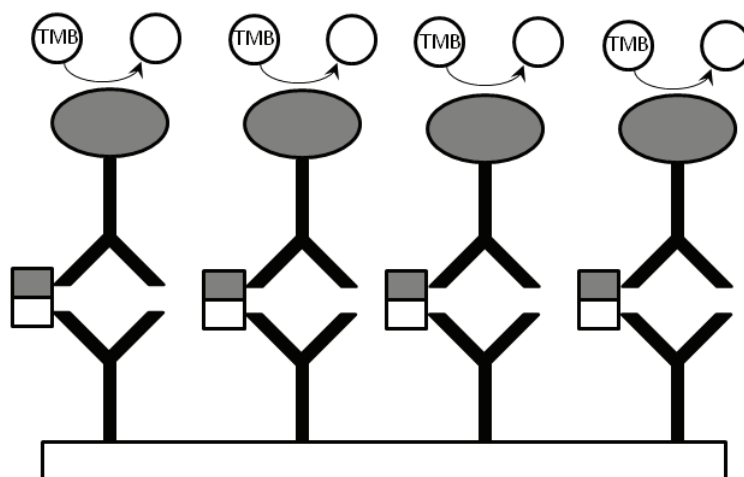
When the sample of interest is added, the capture antibody binds the antigen. Unbound components of the sample are removed by washing.

**Figure A.2 — Step 2**



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.

**Figure A.3 — Step 3**



After washing, a tetramethylbenzidine (TMB) chromogenic substrate for horseradish peroxidase is added. The horseradish peroxidase generates a colour signal which 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.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 between 2 °C and 8 °C, and should not contradict the manufacturer's instructions. The kit solutions should be stored according to the manufacturer's instructions. However, if no storage instructions are indicated, the conjugate stock solution (A.6.4.1) and the antibody conjugate working solution (A.6.4.2) may be stored at 2 °C to 8 °C for dilution and storage.

The antibody conjugate stock solution (A.6.4.1) and the antibody conjugate working solution (A.6.4.2) 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

#### 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 standards**

**A.4.3 Chemicals not supplied with the test kit**

**A.4.3.1 Alcohol**

**A.4.3.2 Detergent, for ultrasonic bath (optional)**

## **A.5 Laboratory equipment**

### **A.5.1 General**

Usual laboratory equipment and, in particular:

**A.5.2 Refrigerator, working at approximately 4 °C**

**A.5.3 Polypropylene conical centrifuge tubes, sealable, e.g. 15 ml**

**A.5.4 Plastic wrap or aluminium foil (optional)**

**A.5.5 Plastic tape to prevent strip movement during plate washing (optional)**

**A.5.6 Wash bottle, e.g. of volume of 500 ml**

**A.5.7 Precision micropipettes capable of delivering e.g. 20 µl to 500 µl**

**A.5.8 Small sample mixer**

**A.5.9 Balance capable of weighing to the nearest 0,01 g**

**A.5.10 Centrifuge capable for producing a relative centrifugal force (RCF) of at least 5 000 × g**

**A.5.11 Microtitre plate reader capable of reading absorbance at 450 nm or the wavelength specified in the kit**

**A.5.12 Incubator oven or water bath capable of maintaining 37 °C (if required)**

**A.5.13 Sieve of aperture size of 450 µm, or equivalent (optional)**

**A.5.14 Sieve of aperture size of 150 µm (100 mesh), or equivalent (optional)**

**A.5.15 Multi-channel pipette, e.g. of 50 µl to 300 µl (optional)**

**A.5.16 Reagent reservoirs for multi-channel dispensing (optional)**

**A.5.17 Automated plate washer (optional)**

#### **A.5.18 Test tube rack for 15 ml centrifuge tubes (optional)**

#### **A.5.19 Ultrasonic bath (optional)**

### **A.6 Procedure**

#### **A.6.1 Warning and/or precautions**

Follow all instructions provided by the kit/reagent manufacturers and other standard laboratory safety procedures. Read and implement the material safety data sheets (MSDS).

#### **A.6.2 Limitation of the procedure**

For thermally processed food samples and composite food samples, the method will 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 of the linear range of the reader. At temperatures greater than 30 °C, OD values will rise more rapidly, a reduced substrate incubation time may be necessary. At low temperatures (less than 15 °C), the substrate incubation time should be increased.

#### **A.6.3 Sampling**

##### **A.6.3.1 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 limit of detection and limit of quantification of the method. For raw grain, it is typical that 2 000 g may be 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.5.13). 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.5.14). 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 may consist of material that is to be handled frozen or are already a homogenate.

##### **A.6.3.2 Measures to avoid contamination during sample preparation**

###### **A.6.3.2.1 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 following suggested procedures involve a first step of 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.6.3.2.2 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.6.3.2.3 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 e.g. commercial hair dryer.

An alternative method would be 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.6.3.2.4 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 the assay in a room separated from the facility where sample grinding and preparation is conducted to avoid potential dust contamination.

**A.6.4 Preparation of antibody conjugate****A.6.4.1 Antibody conjugate stock solution**

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

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

**A.6.4.2 Antibody conjugate working solution**

Add the conjugate stock solution (A.6.4.1) to conjugate diluent buffer (A.4.2.5) according to the method used.

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

**A.6.5 Preparation of wash buffer**

Prepare the wash buffer according to the manufacturers' instructions or a laboratory standard operating procedure.

**A.6.6 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.6.7 Test performance

### A.6.7.1 Extraction of test portion and reference standard

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

**EXAMPLE** A typical process is illustrated as follows.

Weigh out  $0,5\text{ g} \pm 0,01\text{ g}$  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 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 prolonged mixing time, sometimes more than 15 min. Full fat flour easily becomes a homogenous mixture (in under 5 min).

Centrifuge the mixtures at approximately  $5\,000 \times g$  for 15 min preferably at  $4\text{ }^{\circ}\text{C}$ .

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 be stored at  $2\text{ }^{\circ}\text{C}$  to  $8\text{ }^{\circ}\text{C}$ , but no longer than one working day.

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

A summary of the extraction steps can be found in [Figure A.5](#).

### A.6.7.2 ELISA immunoassay procedure

#### A.6.7.2.1 General

The ELISA assay kit can be run in different formats using any number of the eight-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.

All reactions should be run at minimum in 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 then use diluent buffer as the assay blank in place of the diluted test portion. For the sample blank, the diluted test portion should be substituted with a non-POI containing diluted control portion.

When an assay has been started, all steps should be completed without interruption. A summary of the ELISA procedure can be found in [Figure A.6](#).

To establish a CV, in this case, at least three determinations shall be carried out (e.g. values from three wells).

#### A.6.7.2.2 Incubation

Using a micropipette, 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 plate with plastic wrap or aluminium foil (A.5.4) 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 microtitre plate at 37 °C for 1 h or as the manufacturer recommends.

#### **A.6.7.2.3 Washing**

Wash with wash buffer according to instructions or validated SOP.

For manual washing: empty the wells by inverting over a sink or suitable waste container. Using a wash bottle containing 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 for a total of 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 working wash buffer. Repeat the aspiration/fill step for a total of 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.

NOTE 1 Do not let wells dry out, as this can affect assay performance.

NOTE 2 Inadequate washing can cause erroneous results. Whether using manual or automated washer, it is important to ascertain that each assay well is washed with the same volume as all other wells.

#### **A.6.7.2.4 Addition of antibody conjugate**

Add antibody conjugate working solution (A.6.4.2) to each well using a micropipette. Cover the plate to prevent contamination and evaporation.

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

#### **A.6.7.2.5 Washing**

At the end of the incubation period, repeat the washing step as described above (A.6.7.2.3).

#### **A.6.7.2.6 Substrate addition**

Add chromogenic substrate (A.4.2.6) to each well using a micropipette. 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.6.7.2.7 Stop solution addition**

At the end of the incubation period, add stop solution (A.4.2.7) to each well, pipette the stop solution in the same sequence as the colour reagent was added. Gently mix the plate to stop colour development and uniformly distribute 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.6.7.2.8 Absorbance reading

Using microtitre plate reader fitted with a filter appropriate for reading at 450 nm, measure the absorbance of each assay well. All readings should be completed within 30 min of adding the stop solution. Other chromogenic systems may require a different wavelength in the reader.

Record the results obtained and calculate the mean absorbance values or use a computer program.

### A.7 Flowcharts

Procedure	Description
Weigh out 0,5 g	Weigh out analytical samples, blank, reference standards.
Addition of 4,5 ml	Add the 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 x g	Centrifuge the sample at 5 000 x g for 15 min, preferably at 4 °C. Remove supernatant and place it into a clean tube.
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 Figure A.6 or store the test samples and dilutions thereof, assay blank and reference standards according to the recommended storage condition.

Figure A.5 — Extraction 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.
Washing		Wash three times with wash buffer (A.6.7).
Addition	100 µl	Dispense antibody conjugate (A.4.2.4) into each assay well and mix.
Incubation		Incubate 1 h at 37 °C.
Washing		Wash three times with wash buffer (A.6.7).
Addition	100 µl	Dispense chromogenic substrate (A.4.2.6) into each well and mix.
Incubation		Incubate for 10 min at ambient temperature.
Addition	100 µl	Dispense stop solution (A.4.2.7) into each assay well and mix.
Absorbance measurement		Measure absorbance value of each assay well in plate reader at 450 nm.

Figure A.6 — ELISA procedure flowchart

### 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 for diluted test sample solutions and reference material solutions. 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.

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

## A.9 Acceptance/rejection criteria

Each run shall meet the acceptance/rejection criteria in the procedure to be valid as listed in [Figure A.7](#). The run consists of the following: assay blank, positive reference materials, negative reference materials and unknown samples. All sample test solutions, reference material solutions and assay blanks will be run in duplicate. If a run does not meet the assay acceptance criteria, the entire run shall be repeated. Sample test solutions that do not pass the acceptance criteria in any run shall be re-run a second time. Each assay shall have a criteria developed and validated for accepting or rejecting results. [Figure A.7](#) is an example of such criteria.

Assay buffer blank	$A_{450\text{ nm}} < 0,30$
0 % POI standard	$A_{450\text{ nm}} < 0,30$
2,5 % reference standard	$A_{450\text{ nm}} \geq 0,8$
All positive reference standards, OD	CV (OD) of replicates $\leq 15\%$
Unknown samples, solution	CV (OD) of replicates $\leq 20\%$

**Figure A.7 — Example criteria for accepting or rejecting results**

## Annex B (informative)

### Detection of protein(s) by lateral flow devices

#### B.1 General

Lateral flow devices (LFD), also known as lateral flow strips, immunostrips or dipsticks) have been successfully and routinely used for qualitative or semiquantitative detection of antigens, including novel proteins expressed in crops derived from modern biotechnology. The expressed proteins generally confer agronomic or quality characteristics such as tolerance to specified herbicide (e.g. glyphosate, glufosinate) or resistance to insects (e.g. Cry-1Ab, Cry3Bb1, Cry1F). The proteins produced by the biotechnology derived plants may be detected using LFD-based methods.

Most of the commercial LFD methods for modern biotechnology 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 its non-native states. Typically, specific applications should be developed for detecting biotechnology derived POIs in processed fractions such as meals. For example, an LFD method was developed for highly processed product like toasted soy meal to detect the presence of CP4 EPSPS soybeans.<sup>[9],[15]</sup> Selectivity of the method should be reported, in certain cases LFDs will also react with similar proteins that are produced in various varieties of the biotechnology derived crops, such as Cry1Ab in Bt11 corn.

In the case of semiquantitative application for detection of a specific antigen, the method can only be applied to food samples consisting entirely of derived products in which protein can be detected. The LFD methods are routinely utilized in a qualitative manner for presence or absence testing. The confidence in the results is high, but these methods usually test against a threshold. These methods can be easily transformed into a semiquantitative application by combining it with sampling plans and statistics<sup>[14]</sup> for field applications.<sup>[10]</sup>

The limit of detection (LOD) for 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 specific for biotechnology derived crops is examined. The LFD kits are commercially available worldwide. Applications for other (i.e. non-grain) protein-containing matrices, such as leaf, are clearly identified by the kit manufacturer in the product inserts that are part of the kit and should be supported by the manufacturer's method validation data.

#### B.2 Procedure

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

#### B.3 Principle

As shown in [Figure B.1](#),<sup>[13]</sup> a typical LFD consists of a nitrocellulose membrane on a backing material with two lines: the test line immobilizes antigen-specific capturing antibody and the control line immobilizes another type of antibody that recognizes the labelled antigen-specific antibodies.

The antigen-specific detection antibody is conjugated to gold and dried into a fibre pad (antibody conjugate pad or gold pad). Optimized buffers necessary for the test performance are incorporated into the sample pad. On the opposite end, the strip also contains a wicking pad 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, the target antigen in the sample first binds to gold-labelled specific antibody and flows through the membrane. When it passes over the test line it forms a sandwich with the capturing antibody present in the test line. This results in formation of a visible pink to reddish line, and the result is interpreted as positive. The excess gold-labelled antibody 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 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, and this negative result should not be construed as proving that there is no analyte present. For further details on reporting results, see [9.1](#).

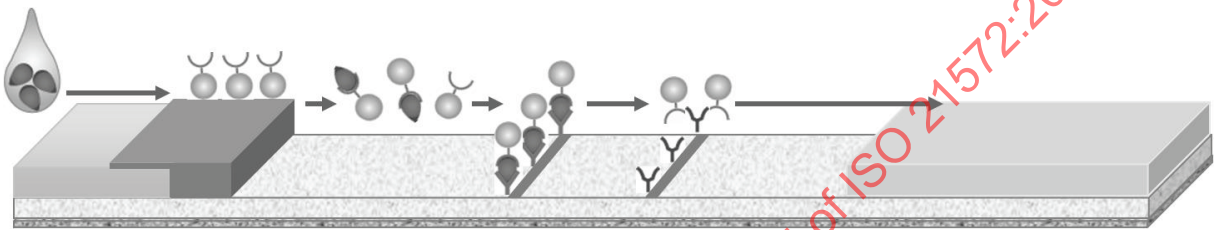


Figure B.1

Examples of a positive, negative, and invalid result, obtained by LFDs are demonstrated in [Figure B.2](#).<sup>[8]</sup>

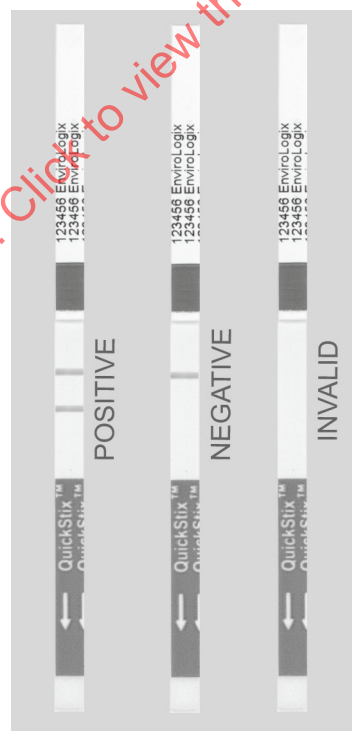


Figure B.2

Visualized test results by LFD. 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 lot number) should be discarded and the test portion should 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