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Textiles — Determination of reduction activity of specific proteins derived from pollen, mite and other sources on textile products

Textiles — Détermination de l'activité de réduction des protéines spécifiques provenant du pollen, des acariens et d'autres sources sur les produits textiles

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

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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 38, *Textiles*.

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

Specialty textile products which can have the positive effect on human comfortable and hygienic life, such as antibacterial, antifungal, antiviral treated textiles, have been introduced in the market and are expanding year by year in various applications.

Now, contamination on textile products with specific proteins which show antigen-antibody reaction also can have the negative effect on human comfortable and hygienic life. There are high performance textile products which can reduce the amount of those specific proteins on textile products.

Because those products are relatively new and include the technical aspects of textile and biological technology, the testing methods have been developed by the individual procedures to evaluate the product performance. That has resulted in inexistence of a unified test method, hindering for both consumers and producers a true explanation or understanding of those functional products.

The demand to establish an international standard has been growing in the consumers, retailers, producers, etc. as stakeholders in the market.

This document provides a quantitative test method by using enzyme-linked immunosorbent assay to assess the reduction activity of the specific proteins on textile products by taking proteins derived from pollen and mite-faeces or carcass as an example.

Textiles — Determination of reduction activity of specific proteins derived from pollen, mite and other sources on textile products

WARNING — This document calls for use of the antigen-antibody reactive derived-protein or substances/procedures that can be injurious to the health/environment if appropriate conditions are not observed. It refers only to technical suitability and does not absolve the user from legal obligations relating to health and safety/environment at any stage.

1 Scope

This document specifies a test method for the determination of reduction activity of textile products against specific proteins which shows antigen-antibody reaction. This document only specifies the reduction activity against those proteins on the surface of textile products. It does not specify a testing method to evaluate the allergenic reaction against human beings.

Specific proteins which show antigen-antibody reaction are proteins derived from pollen, mite and other sources. Other specific proteins can be used after appropriate validation described in this document.

Enzyme-linked immunosorbent assay is used to quantify the amount of those proteins in this document.

This document is applicable to textile products include woven, knitted and nonwoven fabrics, fibres, yarns, braids, etc.

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 3696, *Water for analytical laboratory use — Specification and test methods*

3 Terms and definitions

For the purposes of this document, the following terms and definitions 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 https://www.electropedia.org/

3.1
antigen
substance that is recognized as foreign by the immune system and elicits an immune response through stimulating antibody production

[SOURCE: ISO 16577:2016, 3.12]

3.2
antibody
protein (immunoglobulin) produced and secreted by B lymphocytes in response to a molecule recognised as foreign (antigen) and which is capable of binding to that specific antigen

Note 1 to entry: Immunoglobulin is the common synonym for antibody.

[SOURCE: ISO 16577:2016, 3.10]

3.3
specific protein
proteins which act as antigen and are derived from pollen, mite and other sources

3.4
reduction activity of specific protein
reduction rate of *specific protein* (3.3) concentration

3.5
specific protein reduction agents
inorganic or organic chemicals able to reduce the specific protein concentration

3.6
untreated fabric
fabric of the testing sample without treatment by antigen reduction agent

3.7
negative control
blank test to confirm the effect of an empty plastic bag

3.8
control test
test to confirm that the extract chemicals from test sample do not affect to the sensitivity of ELISA measurement

3.9
enzyme-linked immunosorbent assay
ELISA
method that used *antibodies* (3.2) or *antigens* (3.1) covalently bound to enzyme

4 Principle

The suspension of specific protein from pollen, mite and others are deposited onto a test specimen. After specified contact time, the remaining antigen-antibody reactive specific proteins from pollen, mite and others is measured by using the ELISA method, and the reduction activity is calculated by the comparison between the concentration of the test specimen and the negative control.

5 Specific proteins

Examples of specific proteins derived from pollen and mite are shown in Annex A. Other specific proteins from other species can be used after appropriate validations. If the other species are used, the name of the species and the specific reason for their use shall be described in the test report.

6 Apparatus

6.1 Measuring flask, with capacity of 1 l.

6.2 Balance, with the available range of 0,001 g to 100 g with accuracy of 1,0 %.

6.3 Micropipette, having the most suitable volume for each use, with a tip made of glass or plastic, and with an accuracy of 0,5 % or less.

6.4 Freezer, capable of operating at a temperature of (-20 ± 2) °C.

6.5 Refrigerator, capable of operating at a temperature between 2 °C and 8 °C.

6.6 pH meter, with a glass electrode, with a resolution of at least ±0,01 pH unit

NOTE The pH meter are described in ISO 3071.

6.7 Biological safety cabinet, class II.

6.8 Incubator, capable of maintaining at a temperature of (25 ± 1) °C and (37 ± 1) °C.

6.9 Microplate reader, capable of measuring at a 450 nm to 620 nm in wavelength.

6.10 Polyethylene bag with zipper, with (60 ± 2) mm × (85 ± 2) mm.

6.11 Culture container, made of glass bottle

6.12 Specific protein antibody coated plate, having 96 wells coated with specific protein antibody on bottom of wells.

6.13 Gel filtration columns.

7 Reagents and media

All reagents shall have the quality suitable for biological needs. Some of the media are available in the market.

7.1 Water, which shall be analytical-grade water for microbiological media preparation, which is ion-exchanged and/or freshly distilled and/or ultra–filtered and/or filtered with reverse osmosis (RO) or ISO 3696 grade 3.

7.2 Phosphate buffered saline PBS (-)

7.2.1 Prepare a measuring flask of 1 l, and put the following chemicals into a flask (6.1):

— sodium chloride (NaCl), 8 g;

— potassium chloride (KCl), 0,2 g;

— disodium hydrogen phosphate $12H_2O$ (Na_2HPO_4, $12H_2O$), 2,9 g;

— potassium dihydrogen phosphate (KH_2PO_4), 0,2 g.

7.2.2 Add water (7.1) and make up a whole amount to 1 000 ml. Dissolve well.

7.2.3 Transfer the solution (7.2.2) to a culture container (6.11).

7.3 Suspension solution of specific protein

7.3.1 Put the polysorbate 20 (7.7), 0,5 g, in the phosphate buffered saline PBS (-) prepared at 7.2, approximately 700 ml to 800 ml and dissolve well.

7.3.2 Add the solution (7.2) by making up whole amount to 1 000 ml and mixed well.

7.3.3 Then, transfer the solution (7.3.2) to a culture container (6.11).

7.4 ELISA assay reagents, reagents and buffer solutions

ELISA assay reagents, reagents and buffer solutions can be obtained from commercial suppliers which shall be prepared for use in accordance with the manufacturer's instructions.

Examples of ELISA assay reagents, reagents and buffer solutions are shown in Annex B.

7.5 Sodium hydroxide solution.

7.6 Hydrochloric acid solution.

7.7 Polysorbate 20.

8 Preparation

8.1 Preparation of test specific protein suspension

Adjust the concentration of specific protein extract to 10 ng/ml to 20 ng/ml by using suspension solution of specific protein (7.3).

The default concentration of specific protein extract shall be to 10 ng/ml to 20 ng/ml, however, the concentration of specific protein extract can be allowed to increase up to 100 ng/ml according to the experience of the laboratories.

Specific protein extracts are available in the market. The storage of the specific protein extracts shall be in the freezer (6.4) with the temperature below -20 °C and all operation to handle specific protein extracts shall be done in the biological safety cabinet (6.7).

8.2 Preparation of test specimens

8.2.1 Prepare the test specimens of specific protein reduction test sample as specified in Table 1.

Table 1 — Dimension or mass of specimen

Kind of sample	Mass[a]	Specimen
Fabrics (woven, knitted, nonwoven)	0,40 g or more	(50 ± 2) mm × (50 ± 2) mm
	less than 0,40 g	0,40 g ± 0,05 g
Yarns, braid, fibres, wadding and feather		0,40 g ± 0,05 g
[a] Mass of the fabric specimen with a dimension of (50 ± 2) mm × (50 ± 2) mm.		

8.2.2 Prepare six (6) test specimens of the specific protein reduction test sample.

Three (3) specific protein reduction test specimens are used for the control test.

The remaining 3 specific protein reduction test specimens are used for the main test of this document.

If untreated fabrics are used for the main test instead of negative control, prepare 6 test specimens of the untreated fabric. Three untreated fabric test specimens are used for the control test and the remaining 3 untreated fabric test specimens used for the main test.

8.3 Control test

8.3.1 General

The purpose of the control test is to confirm that any chemicals from the test specimen does not reduce the sensitivity of ELISA. In case of using a commercial kit, refer to the technical sheet.

8.3.2 Procedure of verification of the sensitivity of ELISA

8.3.2.1 Put 3 specific protein reduction test specimens in each bag with zipper (6.10) and add 1 ml of suspension solution of specific protein (7.3).

8.3.2.2 Fold the test specimen in four plies or more in the bag and immediately squeeze it as it is in the bag.

8.3.2.3 Collect the solution in the bag and take 0,1 ml of the solution by micropipette (6.3). Put the solution into the 2 wells of specific protein antibody coating plate (6.12).

Additionally, take 0,1 ml of suspension solution for specific protein (7.3) by micropipette (6.3) as negative control and put it into the 2 wells of the plate (6.12).

8.3.2.4 Keep them at 25 °C in the incubator (6.8) for (30 ± 5) min. Then, remove the solution from the well and wash the well three times with 300 µl of suspension solution of specific protein (7.3).

8.3.2.5 Determine the concentration of the test specific protein suspension by ELISA test method using the plate (see 8.3.2.4) described in Annex C.

8.3.3 Requirement for verification of the sensitivity of ELISA

Calculate the specific protein concentrations for the negative control and the test specimen. Obtain the values by using Formula (1). The requirements for verification of the sensitivity of ELISA shall satisfy Formula (1).

$$| (S_n - S_t) / S_n \times 100 | < 20\ \% \quad (1)$$

where

S_n is the average of the specific protein concentration (ng/ml) from 3 negative controls;

S_t is the average of the specific protein concentration (ng/ml) recovered from 3 specific protein reduction test specimens.

If untreated fabrics are used for the main test instead of negative control, the requirements for verification of the sensitivity of ELISA shall also satisfy Formula (2).

$$| (S_n - S_u)/S_n \times 100 | < 20\ \% \quad (2)$$

where S_u is the average of the specific protein concentration (ng/ml) recovered from 3 untreated fabric test specimens.

If the above value is ≥20 %, the negative factor against the sensitivity of ELISA in the solution (see 8.3.2.2) shall be carefully removed by selecting the following method appropriately.

a) Measure pH of the solution by pH meter (6.6). The pH of the solution (see 8.3.2.2) shall be adjusted to pH (7,0 ± 0,2) by adding the sodium hydroxide solution or the hydrochloric acid solution.

b) The solution (see 8.3.2.2) shall be filtrated using the gel filtration columns (6.13).

If the solution (see 8.3.2.2) is modified, the same condition about the solution in 10.4 shall be applied.

9 Preparation of the specific protein calibration curve

The procedure of preparation of the specific protein calibration curve is as follows. The calibration curve shall be obtained before the test in every testing time.

Prepare the specific protein calibration curve using 7 points. The range of the concentration shall be near the detection limit concentration to 20 ng/ml.

a) Dilute the specific protein extract with suspension solution (7.3) to prepare 2-fold dilutions having an accurate concentration.

b) Measure the absorbance with microplate reader (6.9) at 450 nm of each specific protein dilution series by ELISA.

Example of the specific protein calibration curve is shown in Annex D.

Other specific protein calibration curve can be used after appropriate validations. In case of using a commercial kit, refer to the technical sheet.

10 Test procedure

10.1 Preparation of test specimen

All test specimens are prepared in bags with zipper (6.10).

10.2 Deposit of specific protein to the test specimens

Deposit exactly 1,0 ml of the specific protein suspension prepared in 8.1 onto the test specimen at one point of the centre of the test specimen in bags with zipper and into 3 empty bags with zipper as for negative control by micropipette (6.3). Then, zip up all bags.

The default depositing volume to test specimen shall be 1 ml, however, in case, there is difficulty to recover the suspension after depositing the solution from the test specimen, the depositing volume may be increased up to 5 ml.

10.3 Contacting time

Put the bags of 10.2 in the incubator (6.8) and keep for 2 h as a specified contact time at a temperature of 25 °C.

The contact time can be varied and can be determined by the concerned party, but not longer than 24 h.

10.4 Recovery of specific protein from the test specimens

After contacting for 2 h in 10.3, fold the test specimens in four plies or more in the bag and squeeze the test specimens out of the bag to recover the specific protein.

11 Determination of the specific protein concentration

Determine the specific protein concentration of recovered specific protein suspension in 10.4 by ELISA according to Annex C.

In case of using a commercial kit, refer to the technical sheet.

12 Calculation of the specific protein concentration and reduction rate

12.1 Calculate the specific protein concentration, expressed in ng/ml from the calibration curve equation.

12.2 Calculate the reduction rate of specific protein by the comparison between the specific protein reduction test specimen and negative control following to Formula (3):

$$P = (R_n\text{-}R_t)/R_n \times 100 \quad (3)$$

where

P is the reduction rate of specific protein concentration in %;

R_n is the average of the specific protein concentration (ng/ml) recovered from three bags for negative control after 2 h;

R_t is the average of the specific protein concentration (ng/ml) recovered from three specific protein reduction test specimens after 2 h.

If untreated fabrics are available and the concerned party requests, the effect of the reduction agents of specific protein is calculated by Formula (4):

$$P = (R_u - R_t) / R_u \times 100 \quad (4)$$

where

R_u is the average of the specific protein concentration (ng/ml) recovered from three specific protein reduction untreated test specimens after 2 h.

13 Repeatability and reproducibility

The interlaboratory test has been performed by participation of 5 laboratories and results are shown in Annex F and the repeatability and reproducibility were calculated based on Annex F and the results are shown in Annex G.

14 Test report

The test report shall contain the following information:

a) a reference to this document, i.e. ISO 4333:2022;

b) the identification of sample;

c) the details of specific-protein deposited;

d) the method to determine the deposit protein concentration;

e) test result according to Clause 12;

f) any deviation from the specified procedures;

g) date of test;

h) if a commercial ELISA-kit was used, describe the details; manufacture and product name product number or lot number.

Annex A
(informative)

Specific protein derived from pollen and mite

Examples of specific protein derived from pollen and mite used in this document are shown in Table A.1. Other specific proteins can be used after appropriate validation.

Table A.1 — Specific protein derived from pollen and mite used for this document

	Species	
	Pollen	**Mite**
Specific protein	*Cry j 1 (Cryptomeria japonica 1);* derived from pollen surface *Cry j 2 (Cryptomeria japonica 2);* derived from pollen inside	*Der f 1 (Dermatophagoides farinae 1);* derived from mite-feces *Der f 2 (Dermatophagoides farinae 2);* derived from mite-carcass *Der p 1 (Dermatophagoides pteronyssinus 1);* derived from mite-feces *Der p 2 (Dermatophagoides pteronyssinus 2);* derived from mite-carcass

Annex B
(informative)

ELISA assay reagents, reagents and buffer solutions

B.1 Enzymatic reaction-stop reagent

An example of composition of enzymatic reaction-stop reagent is described in Table B.1.

This is used for pH adjustment and stopping the enzymatic reaction. Once fully dissolved, keep at room temperature before use. Use within 4 h of preparation.

Table B.1 — Composition of enzymatic reaction-stop reagent

Reagent	Composition
Sulfuric acid (H_2SO_4)	10 ml
Water	90 ml

B.2 Specific protein antibody coated plate

B.2.1 Specific protein antibody coated plate (6.12) is coated with specific protein antibody on 96 wells plate surface.

B.2.2 An example of composition of specific protein antibody solution is described in Table B.2.

Table B.2 — Composition of specific protein antibody solution

Reagent	Composition
Phosphate buffered saline [PBS (-)] (7.2)	50 ml
Specific protein Antibody	50 µg to 200 µg

For specific protein antibody that will not be used immediately after preparation, it is recommended that it be stored in refrigerator (6.5) at 4 °C and can be used for 1 week and if passed 1 week. discard it.

B.2.3 Add 0,1 ml of specific protein antibody solution (see Table B.2) per 1 well of 96 wells plate. Incubate at 37 °C for 18 h.

B.3 Enzyme-labelled anti specific protein antibody solution

An example of composition of enzyme-labelled anti specific protein antibody solution is described in Table B.3.

Table B.3 — Composition of enzyme-labelled anti specific protein antibody solution

Reagent	Composition
Suspension solution of specific protein (7.3)	50 ml
Bovine serum albumin	0,5 g
Enzyme-labelled anti specific protein antibody	1 appropriate unit

Once fully dissolve, keep in refrigerator (6.5) at 4 °C before use. Use within 4 h after preparation.

B.4 Colorimetric substrates

An example of composition of colorimetric substrates solution is described in Table B.4.

Table B.4 — Composition of colorimetric substrates solution

Reagent	Composition
Phosphate buffered saline [PBS (-)] (7.2)	10 ml
colorimetric substrates	1 unit

Refer to the technical date sheet for the preparation. Once fully dissolve, keep in refrigerator (6.5) at 4 °C before use. Use within 1 h of preparation. Immediately before use, add appropriate amount of hydrogen peroxide (H_2O_2).

Annex C
(informative)

Enzyme-linked immunosorbent assay — Sandwich ELISA test procedure

C.1 Add 100 µl of specific protein suspension to the well of specific protein antibody coated plate (6.12), and incubate at (25 ± 1) °C for 1 h to allow the specific protein to be captured to antibody.

C.2 Wash the well three times with 300 µl of suspension solution of specific protein (7.3). Invert the plate and blot on absorbent paper to remove the residual solution.

C.3 Add 100 µl of enzyme-labelled anti specific protein antibody (B.3) to each well, and incubate at 25 °C for 1 h to allow the specific protein to bind be to enzyme-labelled anti specific protein antibody.

C.4 Wash the well three times with 300 µl of suspension solution of specific protein (7.3). Invert the plate and blot on absorbent paper to remove the residual solution.

C.5 Add 100 µl of colorimetric substrates (B.4), and keep in the shade at room temperature for 15 min.

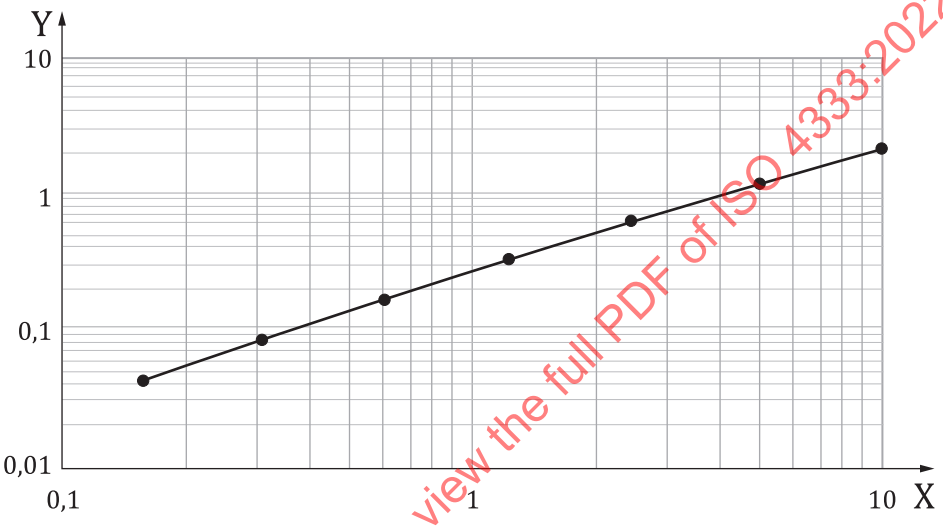
C.6 Add 100 µl of enzymatic reaction-stop reagent (B.1) to each well to stop the enzyme-substrate reaction.

C.7 Measure the absorbance at 450 nm of the plate wells by microplate leader (6.9) to determine the presence and quantity of specific protein.

Annex D
(informative)

Specific protein calibration curve

An example of the specific protein calibration curve is shown in Figure D.1 by using 4-parameter logistic. The regression equation for the calibration curve is usually prepared by the manufacturer of ELISA by the calculation software, if not, calculated by the statistical software available in the market.



Key

X specific protein concentration (ng/ml)

Y absorbance at 450 nm

Figure D.1 — Specific protein calibration curve

Annex E
(informative)

Recovery rate

E.1 General

The recovery rate of specific protein suspension from the test specimen after depositing is one of the verification information, if the appropriate reference fabrics exists. Untreated fabrics are considered as a reference fabric, if available.

E.2 Calculation of recovery rate

E.2.1 Prepare an untreated fabric the specimen according to 8.2.

E.2.2 The test procedure shall follow Clause 10.

E.2.3 The recovery rate (%) is calculated by Formula (E.1):

$$R = R_u/R_n \times 100 \quad \text{(E.1)}$$

where

R is the recovery rate (%) of the untreated fabric test specimens;

R_n is the average of the specific protein concentration (ng/ml) recovered from 3 bags of negative control after 2 h;

R_u is the average of the specific protein concentration (ng/ml) recovered from 3 untreated fabric test specimens after 2 h.

E.3 Recommendation of the recovery rate

Recovery rate (%) is recommended 70 % or higher.

The examples of the recovery rate are shown in Table F.4 and Table F.5.

Annex F
(informative)

Interlaboratory test result

F.1 Participants

Five laboratories in Japan participated in the interlaboratory test.

F.2 Test condition

Materials used in the interlaboratory test and the test conditions applied are summarized in Table F.1.

Table F.1 — Materials and test conditions

Items	Details
Untreated fabric	Cotton 100 % woven fabric without specific protein reduction agents
Sample A	Cotton 100 % woven fabric with specific protein reduction agents A
Sample B	Cotton 100 % woven fabric with specific protein reduction agents B
Negative control	Suspension solution of specific protein (7.3)
Specific protein	*Cryj1 (Cryptomeria japonica 1)*
Specific protein suspension deposited amount	1,0 ml
Contact condition	25 °C, 2 h

F.3 Test result

F.3.1 Control test

The control test result is shown in Table F.2. The value of the verification of the sensitivity is shown in Table F.3. All laboratories satisfied the requirement.

Table F.2 — Interlaboratory test result for control test

		Negative control	Untreated	Sample A	Sample B
Average of the specific protein concentration (ng/ml)	Lab 1	12,29	12,65	11,48	12,86
	Lab 2	17,39	16,66	16,57	16,27
	Lab 3	15,28	14,84	15,13	16,14
	Lab 4	15,05	14,81	15,03	14,05
	Lab 5	13,17	13,65	14,60	13,73
Designation		S_n	S_u	S_t	S_t

Table F.3 — Verification of the sensitivity of ELISA in control test

		Negative control	Untreated	Sample A	Sample B
$\lvert (S_n-S_u)/S_n\times100 \rvert$ or $\lvert (S_n-S_t)/S_n\times100 \rvert$ (%)	Lab 1	—	2,93	6,59	4,64
	Lab 2	—	4,20	4,72	6,44
	Lab 3	—	2,88	0,98	5,63
	Lab 4	—	1,59	0,13	6,64
	Lab 5	—	3,64	10,85	4,25
Requirement for verification		< 20 %			

F.3.2 Reduction activity test

The result of the reduction activity of the specific protein of sample A is shown in Table F.4 and sample B is shown in Table F.5.

Table F.4 — Reduction activity test result for sample A

			Negative control	Untreated	Sample A	Reduction rate (%) of specific protein	Recovery rate (%) of control fabric test specimens	Initial validation
						$P=(R_n-R_t)/R_n\times100$	$R=R_u/R_n\times100$	(ng/ml)
Sampling time (h)			2	2	2			
Average of the specific protein concentration (ng/ml)	Lab 1	1st block	13,20	11,48	0,43	96,7	87,0	13,39
		2nd block	12,55	10,83	0,45	96,5	86,3	12,52
	Lab 2	1st block	13,93	11,93	1,16	91,7	85,6	14,85
		2nd block	15,50	13,30	0,84	94,6	85,8	15,50
	Lab 3	1st block	13,76	14,15	0,84	93,9	102,8	15,01
		2nd block	12,11	10,53	0,90	92,6	87,0	14,26
	Lab 4	1st block	14,19	13,54	1,55	89,1	95,4	15,36
		2nd block	14,83	13,21	0,87	94,1	89,1	15,36
	Lab 5	1st block	9,74	8,35	0,35	96,4	85,7	9,96
		2nd block	10,98	9,22	0,63	94,2	84,0	11,11
Mean			13,08	11,65	0,80	94,0	88,9	13,73
Designation			R_n	R_u	R_t			