Designation: D6499 − 16

Standard Test Method for The Immunological Measurement of Antigenic Protein in Natural Rubber and its Products[1]

This standard is issued under the fixed designation D6499; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method covers an immunological method to determine the amount of antigenic protein in natural rubber and its products using rabbit antisera specific for natural rubber latex (NRL) proteins. This immunoassay procedure quantitatively measures the level of antigenic latex proteins in solution using an inhibition format. The samples may include glove or other rubber product extracts which have been collected in order to measure the latex protein levels. Although this method detects antigenic proteins, it should not be considered as a measure of allergenic proteins. Correlation of protein/antigen levels with the level of allergenic proteins has not been fully established.

1.2 For the purpose of this test method, the range of protein will be measured in terms of microgram to milligram quantities.

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D4483 Practice for Evaluating Precision for Test Method Standards in the Rubber and Carbon Black Manufacturing Industries

D5712 Test Method for Analysis of Aqueous Extractable Protein in Latex, Natural Rubber, and Elastomeric Products Using the Modified Lowry Method

E177 Practice for Use of the Terms Precision and Bias in ASTM Test Methods

E691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method

3. Terminology

3.1 *Definitions:*

3.1.1 *allergens, n*—protein antigens which induce allergic immune reactions typically mediated through IgE antibodies.

3.1.2 *antibody, n*—an immunoglobulin, a protein that is produced as a part of the immune response which is capable of specifically combining with the antigen.

3.1.3 *antigen, n*—any substance that provokes an immune response when introduced into the body.

3.1.4 *background absorbance, n*—the absorbance reading in the solution resulting from the presence of chemicals, ions etc. other than the substrate being determined.

3.1.5 *blocking solution, n*—a non-reactive protein solution used to prevent nonspecific antibody adsorption.

3.1.6 *calibration, n*—the standardization of an instrument setting or an assay configuration.

3.1.7 *concentration range, n*—the recommended analyte concentration range in µg/mL that produces an absorbance reading of 0.1 to 2.0 units.

3.1.8 *enzyme linked immunosorbent assay (ELISA), n*—an immunological test method to quantify antigen or antibody levels using an enzyme as the detection mechanism.

3.1.9 *primary antibody, n*—the antibody used first in a sequence that is specific for the antigen.

3.1.10 *reference solution, n*—the solution to which the test sample is being compared against.

3.1.11 *repeatability, n*—the variability or test error between independent test results obtained within a single laboratory.

3.1.12 *reproducibility, n*—the variability or error between test results obtained in different laboratories.

3.1.13 *secondary antibody, n*—the enzyme conjugated antibody used second in the sequence that is specific for the heavy chain of the primary antibody.

3.1.14 *standard solution, n*—the preparation of standard analyte used as a reference to which the unknown sample being measured is compared.

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[1] This test method is under the jurisdiction of ASTM Committee D11 on Rubber, and is the direct responsibility of Subcommittee D11.40 on Consumer Rubber Products.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

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3.1.15 *substrate, n*—the material or substance upon which an enzyme reacts.

3.1.16 *titer, n*—the strength of the antibody solution (for example, concentration and affinity of antibody).

4. Summary of Test Method

4.1 The latex device is extracted for 2 h in an aqueous buffer. The extract is recovered and the antigen levels are determined using inhibition Enzyme Linked ImmunoSorbent Assay (ELISA) technology (**1**).[3] The ELISA assay is based on polyclonal antiserum which can detect NRL proteins. ELISA technology takes advantage of the specificity and sensitivity of the antibody-antigen reaction. A variation of the ELISA method (an inhibition ELISA) has been developed for the detection and quantification of latex protein antigens. In the inhibition ELISA, the latex antigen is immobilized by absorption to the wells of a 96-well test plate. The sample extract is mixed with antibody specific for NRL protein in a dilution plate. Following a brief incubation to allow for antibody recognition of the relevant NRL antigens, the mixture is added to the immobilized antigen in the assay plate. Anti-NRL antibody which is not bound to the soluble NRL protein in the sample will bind to the immobilized antigen. The plate is washed to remove the soluble antigen antibody complexes and a secondary antibody (enzyme-labeled anti-immunoglobulin) is added which attaches to the immobilized antigen-bound specific antibody. Next, the enzyme substrate is added and the reaction of the enzyme on the substrate results in a color change. A reduction in the amount of color in comparison to an uninhibited control is an indicator of the amount of antigen present in the sample. Comparison to a standard curve generated using known amounts of NRL protein permits quantification. The assay is highly sensitive and can quantitate NRL proteins in the nanogram per millilitre range.

5. Significance and Use

5.1 Type 1 latex allergy most commonly manifests as localized urticaria after contact of skin with natural rubber but can also include symptoms of allergic rhinoconjunctivitis, asthma and rarely anaphylaxis. This immediate (Type I) allergy is caused by natural proteins inherent to the rubber tree, which remain on the finished natural rubber products. The quantification of protein levels in NRL products using the standard colorimetric protein assays may give spurious results due to chemical additives in the latex formulations that interfere with the assay (**2,3**). Furthermore, the amount of protein found in NRL products are often below the detection limits of the standard colorimetric protein assay (**4,5**).

5.2 This test method describes an immunological method for quantitation of natural rubber latex proteins using rabbit anti-NRL serum. Rabbits immunized with NRL proteins react to the majority of the proteins present, and their sera have the capability to detect most if not all of the proteins in NRL. Therefore, although rabbit antibody reacts with antigenic material, this should not be considered as quantitative measure of total protein levels.

6. Interferences

6.1 Substances such as detergents or surfactants have the potential to prevent antibody binding to antigen and could interfere in an ELISA assay. However, due to the sensitivity of the ELISA assay, these interferences often can be controlled by serially diluting the sample.

7. Apparatus

7.1 *96-Well Microtiter Assay Plate,* (recommended Nunc MaxiSorb, #442-404, round robin testing found this plate to provide more consistent results).

7.2 *Dilution Plate,* a low protein binding 96 well plate for sample dilution and antibody reaction (recommend Corning #25880-96, or equivalent).

7.3 *Multichannel Pipettors.*

7.4 *Analytical Balance.*

7.5 *Centrifuge,* (capable of 1000 × g) and tubes.

7.6 *An Incubator,* capable of regulating the temperature at ~37°C.

7.7 *Microtiter Plate Reader,* and optional computer for data analysis.

7.8 *ELISA Plate Sealing Tape or Plastic Lids.*

7.9 It is expected that all laboratories will adhere to good laboratory practices (GLP) and ensure that all reagents used are within their shelf life and that all equipment used has been calibrated or verified before use.

8. Reagents and Materials

8.1 *Buffers*—Buffers and solutions should be prepared before beginning the protocol. Make sure that all solutions containing protein are made in polypropylene tubes throughout the assay.

8.1.1 *Carbonate Buffer pH 9.6:*

Na_2CO_3	0.795g
$NaHCO_3$	1.465g
NaN_3	0.1 g

Dissolve above in distilled H_2O and dilute to a final volume of 500 mL. Check pH and adjust if necessary.

NOTE 1—Carbonate buffer can be stored for at least one month at 4 ± 3°C. Alternatively, carbonate buffer capsules can be purchased from a commercial source.

8.1.2 *Phosphate-Buffered Saline (PBS),* pH 7.4; 10X stock:

$NaH_2PO_4 . H_2O$	5.125 g
$Na_2HPO_4 . 7H_2O$	45 g

Dissolve above in 1.5 L distilled water and adjust to pH 7.4, if necessary. Add 175.3 g NaCl and distilled water up to a total of 2 L. Prior to use, dilute an appropriate volume of 10X stock 1:10 v/v with distilled water to obtain 1X PBS.

NOTE 2—Alternatively, PBS buffer solution can be purchased from a commercial source.

[3] The boldface numbers given in parentheses refer to a list of references at the end of the text.

8.1.3 *T-PBS Wash Buffer*—To prepare T-PBS washing solution, add 0.5 mL Tween 20 to 1 L of 1X PBS (0.05 %), mix well.

8.2 *Dry Milk Solutions:*

8.2.1 *Blocking Solution*—Prepare 100 mL of 3 % w/v nonfat dry milk in T-PBS (for blocking of assay plate and dilution plate).

8.2.2 Dilution buffer: Prepare 100 mL 0.2 % w/v nonfat dry milk in T-PBS (for dilution of antibodies and blocking in the competitive inhibition step.

8.3 *Reference Reagents*—The lyophilized standard reference antigen (StAg) and the reference anti-NRL serum evaluated during development of this protocol will be supplied to the test users.[4] Details of the preparation procedure for the standard antigen and the protocol for rabbit immunization are described in an ASTM Research Reports for the Industry Reference Material (IRM).[5]

NOTE 3—Do not use frost free freezers which have temperatures that fluctuate and can result in degradation of proteins, enzyme activity, or antibody reactivity. To reduce possible protein loss, all procedures that involve protein containing solutions must be performed in polypropylene tubes or vessels. Polystyrene or glass vessels must be avoided.

8.3.1 *Standard Antigen (StAg) Solutions (IRM # 913)*—The lyophilized preparation of NRL protein is reconstituted with distilled H_2O to a concentration of 1 mg/mL. Aliquot this stock solution into small polypropylene tubes and store at –20 ± 10°C. Aliquots, once thawed for use in the assay, should be stored at 4 ± 3°C.

8.3.1.1 *Coating Antigen*—Prepare a 3 µg/mL solution of the standard antigen in carbonate buffer for coating the assay plate, as described in 12.2.1.

8.3.1.2 *Reference Standard*—Prepare a 2 µg/mL solution of StAg in dilution buffer for the reference standard to be used in the competitive inhibition 12.4.3.

8.3.2 *Antisera (IRM # 914):*

8.3.2.1 *Primary Antisera*—An anti-NRL protein reference antisera was produced in rabbits using the same NRL protein as the antigen. This reference sera must be used for this standard protocol. Analyst should dilute 1:5 in dilution buffer, aliquot into convenient aliquots (for example, 50 µl), and store at –20 ± 10°C until use.

8.3.2.2 *Secondary Antibody*—A horseradish peroxidase (HRP) conjugated anti-rabbit IgG (recommend Sigma #A-0545) is to be used to detect the primary antibody recognition of the NRL protein bound to the solid phase. Analyst should dilute 1:5 in dilution buffer, aliquot into convenient aliquots (for example, 50 µL), and store at –20 ± 10°C until use.

8.4 *Substrate Development Solution*—A yellow colored reaction product is produced using o-phenylenediamine (OPD) and hydrogen peroxide. Dissolve the OPD tablet in dH_2O and add the appropriate volume of H_2O_2 following the manufacturers instructions. For example: A 10 mg tablet of OPD from Sigma is dissolved in 10 mL of distilled H_2O and 30 µL of 30 % H_2O_2 is added just prior to use.

9. Hazards

9.1 Working personnel should adhere to standard Good Laboratory Practices. Care should be taken when working with all chemical reagents including acids and bases.

10. Sample Extraction and Preparation

10.1 Sample extraction is designed to be compatible with Test Method D5712 to allow total protein and antigenic protein to be determined for the same sample extract.

10.2 An aqueous buffer of pH 7.4 and a minimum of 25 mM must be used as the extraction medium. Phosphate buffered saline is recommended.

10.3 The temperature of the extraction medium should be 25 ± 5°C.

10.4 The entire natural rubber product or device should be weighed and the total weight per device recorded. When possible, the surface area of the device should be recorded.

10.5 The length of the extraction period should be 120± 5 min with all surfaces evenly exposed to the extraction medium. If the product is too large for all surfaces of the material to be evenly exposed to extraction medium, it should be cut into pieces of appropriate size to accommodate the extraction vessel. The extraction vessel should be continuously rotated by a mechanical device to ensure even exposure to the extraction medium. Alternatively, the extraction vessel should be shaken three separate times for 15 s intervals at the beginning, middle and end of the extraction period (see Test Method D5712).

10.6 A volume of 5 to 10 mL of extraction medium should be used per gram of natural rubber material. The ratio of extraction medium volume to the weight of natural rubber shall not exceed 10 mL per gram of material. Extraction ratios of less than 5:1 can be used provided that the volume of extract is sufficient to cover all surfaces of the test item. The material must be extracted in polypropylene vessels to reduce the possible loss of proteins by adsorption to the inner surface of the container walls.

10.7 Remove the test specimen from the extraction solution. Transfer the solution containing the extractable protein into a polypropylene tube and centrifuge for 15 min at not less than 500 × g to remove particulate matter. Alternatively, filter the extract through a low protein binding 0.45 µm filter into a polypropylene tube.

10.8 The aqueous extracts of residual proteins should be used immediately but can be stored up to two days at 4 ± 3°C and for greater than two days at or below –15°C.

11. Calibration and Standardization

11.1 *Microtiter Plate Spectrophotometer Warm-Up*—Under normal operation, switch "on" the spectrophotometer and allow to warm up following the manufacturer's recommendations.

[4] The sole source of supply of the reference reagents known to the committee at this time is Akron Rubber Development Lab, 2887 Gilchrist Rd., Akron, OH 44305. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

[5] Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR:D11-1094.

11.2 Zero the instrument as required in the manufacturer's manual.

12. Inhibition ELISA Assay Procedure

DAY 1

12.1 *Blocking the Dilution Plate*—Block a Corning low protein binding plate by adding 300 µl of blocking buffer overnight at 4 ± 3°C.

12.2 *Coating the Assay Plate:*

12.2.1 Prepare the coating antigen (StAg) at 3 µg/mL in carbonate buffer, pH 9.6

12.2.2 To coat the Nunc assay plate, place 100 µL of StAg solution (3 µg/mL) in carbonate buffer, pH 9.6 into all wells of the plate. Cover the plate and incubate for 2 h ± 5 min. at 37 ± 3°C, wash one time with T-PBS, and remove the contents of the plate. If the procedure is to be continued the next day, store the empty plate at –20 ± 10°C overnight. Alternatively, the plate may be incubated with the coating antigen overnight at 4 ± 3°C and washed one time before proceeding to 12.3. The plate must be covered during this step and all subsequent incubation steps.

NOTE 4—*Wash Procedure*—Flick (use a snap of the wrist) the contents of the plate into a sink. Using a squirt bottle, fill each well completely with T-PBS wash buffer and flick the contents into the sink again, invert the plate and forcefully pat the upside down plate on a stack of clean paper towels (to remove any remaining liquid from the wells). Washing may also be accomplished by using a multiple fluid dispenser or automatic plate washer.

DAY 2

12.3 *Blocking the Assay Plate:*

12.3.1 Wash assay plate two times with T-PBS just prior to use.

12.3.2 Place 300 µL of blocking buffer in each well, cover, and incubate the plates for 1 h at 37 ± 3°C.

12.4 *Inhibition Step:*

12.4.1 Wash the low protein binding dilution plate 2 times with T-PBS.

12.4.2 Add 100 µL of dilution buffer per well except in the Row A.

12.4.3 Add 200 µL StAg (2 µg/mL) in wells A1-2 and add 200 µL of sample in duplicate wells A3-12.

12.4.4 Make 7 two-fold serial dilutions by taking 100 µL from the first row and placing it in the second row. Mix by pipetting up and down five times, remove 100 µL and place it in the third row and so on. At the last dilution, (Row G) discard 100 µL after mixing. Wells with no inhibition (Row H) must contain 100 µL of dilution buffer per well (see template).

12.4.5 Prepare the solution of the primary antibody in dilution buffer to a final dilution as recommend for the reference sera. The concentration of primary antibody is given in the Certificate of Analysis supplied along with the reagent. The final working concentration of the primary antibody may be adjusted by each laboratory to obtain a maximum O.D. in the range of 0.8 to 2.0 units in the "no inhibition" wells.

12.4.6 Add 100 µL of diluted primary antibody to each well according to the template. (See 12.9 or Table 1.)

12.4.7 For control wells without primary antibody, add 100 µL of dilution buffer instead of the primary antibody (all wells should have 200 µL total volume).

12.4.8 Cover the plate and incubate 2 h ± 5 min. at 35 to 39°C.

12.5 *Adding the Samples to the Assay Plate*—All succeeding steps take place in the assay plate.

12.5.1 Wash assay plate two times with T-PBS and remove all wash solution. Be sure that all wash solution is removed from the assay plate prior to the addition of the inhibition mixture and that the plate does not dry out.

12.5.2 Into the coated and blocked assay plate, transfer 100 µL/well of each sample from the inhibition plate in 12.4. Use a new pipette tip for each well.

TABLE 1 Sample Template for Inhibition Plates

	1	2	3	4	5	6	7	8	9	10	11	12
A	StAg 2 µg/mL	StAg 2 µg/mL	Extract 1 1:1	Extract 1 1:1	Extract 2 1:1	Extract 2 1:1	Extract 3 1:1	Extract 3 1:1	Extract 4 1:1	Extract 4 1:1	Extract 5 1:1	Extract 5 1:1
B	StAg 1 µg/mL	StAg 1 µg/mL	Ex 1 1:2	Ex 1 1:2	Ex 2 1:2	Ex 2 1:2	Ex 3 1:2	Ex 3 1:2	Ex 4 1:2	Ex 4 1:2	Ex 5 1:2	Ex 5 1:2
C	StAg 0.5 µg/mL	StAg 0.5 µg/mL	Ex 1 1:4	Ex 1 1:4	Ex 2 1:4	Ex 2 1:4	Ex 3 1:4	Ex 3 1:4	Ex 4 1:4	Ex 4 1:4	Ex 5 1:4	Ex 5 1:4
D	StAg 0.25 µg/mL	StAg 0.25 µg/mL	Ex 1 1:8	Ex 1 1:8	Ex 2 1:8	Ex 2 1:8	Ex 3 1:8	Ex 3 1:8	Ex 4 1:8	Ex 4 1:8	Ex 5 1:8	Ex 5 1:8
E	StAg 0.125 µg/mL	StAg 0.125 µg/mL	Ex 1 1:16	Ex 1 1:16	Ex 2 1:16	Ex 2 1:16	Ex 3 1:16	Ex 3 1:16	Ex 4 1:16	Ex 4 1:16	Ex 5 1:16	Ex 5 1:16
F	StAg 0.063 µg/mL	StAg 0.063 µg/mL	Ex 1 1:32	Ex 1 1:32	Ex 2 1:32	Ex 2 1:32	Ex 3 1:32	Ex 3 1:32	Ex 4 1:32	Ex 4 1:32	Ex 5 1:32	Ex 5 1:32
G	StAg 0.031 µg/mL	StAg 0.031 µg/mL	Ex 1 1:64	Ex 1 1:64	Ex 2 1:64	Ex 2 1:64	Ex 3 1:64	Ex 3 1:64	Ex 4 1:64	Ex 4 1:64	Ex 5 1:64	Ex 5 1:64
H	No inhibition	No inhibition	No inhibition	No inhibition	No secondary	No secondary	No secondary	No secondary	No Primary	No Primary	No Primary	No Primary

12.5.3 Cover and incubate the plate for 2 h ± 5 min. at 37 ± 3°C.

12.6 *Incubation with the Secondary Antibody (HRP-Conjugated Anti-Rabbit IgG):*

12.6.1 Prepare a 1/5000 final dilution of secondary antibody in dilution buffer (for example, 10 µL of 1:5 stock antibody in 10 mL). The working concentration of the antisera should be established by each laboratory.

12.6.2 Wash each plate three times with T-PBS.

12.6.3 Add 100 µL/well of the secondary antibody, except to wells designated as "no secondary" according to the template, which receive 100 µL of dilution buffer, cover, and incubate for 1 h ± 5 min. at 37 ± 3°C.

12.7 *Substrate Reaction:*

12.7.1 Dissolve an OPD tablet in the appropriate volume of distilled H_2O.

12.7.2 Add the appropriate volume of H_2O_2 to the OPD substrate solution, just prior to use.

12.7.3 Wash the plate three times with T-PBS.

12.7.4 Add 100 µL of OPD substrate solution to each well of the plate.

12.7.5 Incubate the plate at room temperature for 5 to 30 min. It is recommended that the final O.D. should not exceed 2.0 units.

12.7.6 Add 50 µL of 4N sulfuric acid to each well to stop the reaction. The color in the wells will change from yellow to orange upon addition of the stop solution. It is recommended to wait 5 min before reading the plate to allow for color development to stabilize.

12.8 *Read the Optical Density of the Plate at 490 nm.*

12.9 *Sample Template for Inhibition Plates*—See Table 1.

NOTE 5—Temperature of T-PBS wash solution. ELISA assays can suffer from "edge effects" where the OD values of the wells on the perimeter of the plate are consistently higher or lower than the duplicate wells on the interior. One possible explanation for these results is the uneven heating and cooling of the plates if they are washed with a solution that is RT and then placed into an incubator at a higher T such as 37°C. To lessen the effects of uneven temperature distribution one can ensure that the wash buffer is the same temperature of that of the incubations.

13. Calculation

13.1 The absorbance readings of the test extracts are converted to µg protein/mL using a calibration curve. The average absorbancy readings of the standard protein solutions minus the absorbance of the background solution (no secondary antibody) are plotted against the concentration of the reference protein. This is most conveniently performed with computer software. Calculate the results with a curve-fitting computer program that uses either a quadratic polynomial approximation, a spline approximation, or a Semi-log transformation of the absorbance data. The concentration of the protein in the test extract is read from the calibration curve. Absorbance readings of at least two dilutions of the samples must fall within the linear range of the assay, preferably between 40 and 65 % inhibition. A test result is a mean value obtained on duplicate measurements of at least two different dilutions (four wells) but preferably three dilutions (six wells) of the sample extract. A standard curve must be prepared for every ELISA plate that is evaluated.

13.2 Alternatively, the OD readings are converted to percent inhibition and then to µg/mL protein determined using a calibration curve.

14. Report

14.1 The working laboratory should maintain a record of all observations, calculations, derived from the data and test reports to allow the test to be satisfactorily repeated.

14.2 The report shall include a description of the NRL device including lot number, when appropriate. The protein concentrations should be expressed in µg/g and µg/dm^2.

15. Precision and Bias[6]

15.1 The precision of this test method is based on an interlaboratory study of Test Method D6499 conducted in 2013. Four laboratories participated in this study. Each of the four labs was asked to report two replicates of eleven different materials, being tested for protein concentration. Every "test result" reported represents an individual determination. Except for the use of only four laboratories, Practice E691 was followed for the design and analysis of the data.

15.1.1 *Repeatability (r)*—The difference between repetitive results obtained by the same operator in a given laboratory applying the same test method with the same apparatus under constant operating conditions on identical test material within short intervals of time would in the long run, in the normal and correct operation of the test method, exceed the following values only in one case in 20.

15.1.1.1 Repeatability can be interpreted as the maximum difference between two results, obtained under repeatability conditions, that is accepted as plausible due to random causes under normal and correct operation of the test method.

15.1.1.2 Repeatability limits are listed in Table 2 and Table 3.

15.1.2 *Reproducibility (R)*—The difference between two single and independent results obtained by different operators applying the same test method in different laboratories using different apparatus on identical test material would, in the long run, in the normal and correct operation of the test method, exceed the following values only in one case in 20.

15.1.2.1 Reproducibility can be interpreted as the maximum difference between two results, obtained under reproducibility conditions, that is accepted as plausible due to random causes under normal and correct operation of the test method.

15.1.2.2 Reproducibility limits are listed in Table 2 and Table 3.

15.1.3 The above terms (repeatability limit and reproducibility limit) are used as specified in Practice E177.

15.1.4 Any judgment in accordance with statements 15.1.1 and 15.1.2 would normally have an approximate 95 % probability of being correct, however the precision statistics obtained in this ILS must not be treated as exact mathematical

[6] Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR:D11-1139. Contact ASTM Customer Service at service@astm.org.

TABLE 2 Protein Concentration (µg/gm)

	Average[A] $\bar{x}$	Repeatability Standard Deviation Sr	Reproducibility Standard Deviation SR	Repeatability Limit r	Reproducibility Limit R
Sample 1	6.545	1.391	2.885	3.896	8.078
Sample 2	3.758	0.629	2.543	1.763	7.120
Sample 3	3.936	1.946	1.946	5.449	5.449
Sample 4	2.036	0.332	1.293	0.928	3.621
Sample 5	3.484	0.834	2.359	2.334	6.605
Sample 6	3.334	1.419	2.402	3.972	6.726
Sample 7	2.608	0.532	1.668	1.489	4.670
Sample 8	5.028	1.401	2.982	3.923	8.350
Sample 9	4.229	0.955	2.260	2.674	6.329
Sample 10	3.891	0.451	1.722	1.264	4.823
Sample 11	3.688	0.761	2.388	2.130	6.687

[A] The average of the laboratories' calculated averages.

TABLE 3 Protein Concentration (µg/dm^2)

	Average[A] $\bar{x}$	Repeatability Standard Deviation Sr	Reproducibility Standard Deviation SR	Repeatability Limit r	Reproducibility Limit R
Sample 1	3.074	0.744	1.529	2.082	4.281
Sample 2	1.730	0.442	1.187	1.237	3.323
Sample 3	1.788	0.818	0.818	2.290	2.290
Sample 4	1.386	0.300	1.066	0.839	2.986
Sample 5	1.889	0.473	1.567	1.323	4.387
Sample 6	2.226	1.017	2.013	2.846	5.635
Sample 7	1.801	0.359	1.543	1.004	4.321
Sample 8	3.536	1.030	2.606	2.885	7.297
Sample 9	2.453	0.566	1.666	1.585	4.666
Sample 10	1.908	0.225	1.053	0.629	2.950
Sample 11	1.991	0.430	1.572	1.205	4.402

[A] The average of the laboratories' calculated averages.

quantities which are applicable to all circumstances and uses. The limited number of laboratories reporting results guarantees that there will be times when differences greater than predicted by the ILS results will arise, sometimes with considerably greater or smaller frequency than the 95 % probability limit would imply. The repeatability limit and the reproducibility limit should be considered as general guides, and the associated probability of 95 % as only a rough indicator of what can be expected.

15.2 *Bias*—At the time of the study, there was no accepted reference material suitable for determining the bias for this test method, therefore no statement on bias is being made.

15.3 The precision statement was determined through statistical examination of 154 results, from four laboratories, on eleven materials.

The materials tested were described as:

Sample 1:	Condom #1, 1:10 dilution
Sample 2:	Condom #2, 1:10 dilution
Sample 3:	Condom #3, 1:10 dilution
Sample 4:	Glove, 5:1 dilution
Sample 5:	Glove, 5:1 dilution
Sample 6:	Glove, 5:1 dilution
Sample 7:	Glove, 5:1 dilution
Sample 8:	Glove, 5:1 dilution
Sample 9:	Glove, 5:1 dilution
Sample 10:	Glove, 5:1 dilution
Sample 11:	Glove, 5:1 dilution

NOTE 6—To judge the equivalency of two test results, it is recommended to choose the sample type closest in characteristics to your test material.

16. Keywords

16.1 ELISA; natural rubber latex; proteins

APPENDIXES

(Nonmandatory Information)

X1. REFERENCE MATERIALS

X1.1 For NRL reference protein production, ammoniated latex was sampled immediately on arrival in the US. Collect lots of full ammonia (0.9 % ammonia) and low ammonia latex (0.4 % ammonia) from preferably three or more different latex suppliers. Centrifuge the latex at 100 000 × g for 2 h. The rubber layer is removed and the aqueous extract (C serum) passed through a 0.45 µm filter. The extract is dialyzed against 0.1 M carbonate buffer pH 9.6 using dialysis tubing of MWCO 1000 to remove the ammonia and other chemical compounds. The latex protein concentration is determined using Test Method D5712 and the protein was freeze-dried (lyophilized) into small vials. The lyophilized standard reference antigen (StAg) evaluated during development of this protocol will be supplied to the test users. Future consideration will be given for use of "in house" NRL protein reference material validated against the IRM once appropriate validation parameters are established.

X1.2 New Zealand white rabbits were immunized with the standard antigen. Rabbits were injected with StAg in Complete Freund's Adjuvant (CFA) for initial immunizations and StAg in Incomplete Freund's Adjuvant (IFA) for booster immunizations. Rabbits were bled to check titer followed by a final bleeding and exsanguination when titers were sufficiently high. A pool of sera from the three different laboratories was used for the final reference sera. The standard anti-NRL serum evaluated during development of this protocol will be supplied to the test users. Future consideration will be given for use of "in house" anti-NRL reference antisera validated against the IRM once appropriate validation parameters are established.

X2. MODIFICATION OF TEST METHOD D6499 TO INCLUDE PARTICULATES

X2.1 Scope

X2.1.1 This appendix outlines a modification of Test Method D6499 to measure the antigenic protein level in glove extracts that contain the particulate fraction. Only the steps requiring modification are described here; all other steps remain unchanged.

X2.1.2 Generally, protein attached to powder does not contribute a major portion of the final protein content generated by the Test Method D6499 assay. However, there is an inherent error to the measurement, and there is a potential for antigenic protein bound to the powder to contribute significantly to the final measurement, particularly for powdered gloves. If the concentration determined using Test Method D6499 with the centrifuged extract of the glove is at or above 8 µg/dm^2, it is recommended that the complete extract be assayed to ensure the total protein level is below 10 µg/dm^2 (see Table X2.1).

X2.2 Sample Extraction and Preparation

X2.2.1 Preparation of the extracts will follow Section 10 except for 10.7.

NOTE X2.1—It is important to avoid unnecessary manipulation of the glove samples prior to extraction to prevent loss of powder.

X2.2.2 Replace 10.7 as follows. Remove test specimen from the extract solution. Shake sample solution well to insure even distribution of the powder and transfer the solution containing the extractable protein and powder into a polypropylene tube. Do not centrifuge the tube.

X2.3 Inhibition Assay Procedure

X2.3.1 Replace 12.4.3 as follows: Add 200 µL of StAg (2 µg/mL) in wells A1-2. Mix the sample extracts thoroughly to re-suspend the powder and add 200 µL to well A3-12.

X2.3.2 Replace 12.4.8 with: Cover the plate with a plastic lid or sealing tape and place plates on a plate shaker. Incubate the plates for 2 h at room temperature with constant shaking. At the end of the incubation period, centrifuge the inhibition plates at 500 × g for 15 min. Alternatively, if you cannot centrifuge the plates, let them settle undisturbed for the last 30 min at room temperature prior to removing the samples for addition to the assay plates.

X2.3.3 Replace 12.5.2 with: Into the coated and blocked assay plate, transfer 100 µL/well of each sample from the centrifuged or settled inhibition plate in 12.4. Use a new pipette tip for each well. Take care not to pipette up any of the powder pellet.

X2.4 Precision and Bias

X2.4.1 An interlaboratory test program to determine precision for the modification of the test method was performed in 2006. Samples of 28 different brands of medical gloves were sent to four different laboratories and tested by six technicians. The gloves were purchased in June 2006 and included 2 powdered surgical (PS), 2 powder-free surgical (PFS), 4 powdered exam (PE), and 20 powder-free exam gloves (PFE). Glove types were chosen in proportion to an estimate of market share. Intact gloves from each brand were sent to the participating laboratories for extraction and performance of the modified test method.

X2.4.2 A test result is a mean value as specified by this test method obtained on duplicate measurements of at least two different dilutions of the glove extract. Both repeatability and reproducibility are short term; a period of minutes separates the replicate test results.

X2.4.3 The results of the precision calculations according to Practice E691 are presented in Table X2.2.

X2.4.4 Reference values do not exist for this test since the antigen level of the test is exclusively defined by the test method; bias therefore cannot be determined.

TABLE X2.1 Comparison of Test Method D6499 and the Modified Test Method D6499 Antigenic Protein Levels in 28 Brands of NRL Gloves[A]

Sample	Test Method D6499	Modified D6499
1 PS	2.0	3.6
2 PS	12.0	11.4
3 PFS	2.7	1.9
4 PFS	7.5	0.9
5 PE	8.4	17.5
6 PE	6.8	12.8
7 PE	40.5	19.3
8 PE	193.5	42.0
9 PFE	6.3	1.2
10 PFE	2.6	4.8
11 PFE	0.3	0.8
12 PFE	0.7	1.4
13 PFE	1.3	1.3
14 PFE	1.8	1.0
15 PFE	3.7	4.4
16 PFE	6.3	7.0
17 PFE	1.7	4.1
18 PFE	0.5	0.8
19 PFE	0.7	0.7
20 PFE	3.9	4.9
21 PFE	0.4	0.6
22 PFE	2.8	1.8
23 PFE	5.7	4.8
24 PFE	5.0	2.5
25 PFE	1.6	2.7
26 PFE	0.9	1.9
27 PFE	1.7	2.6
28 PFE	0.9	1.4

[A]Data represents calculations of antigenic protein in µg/g for NRL gloves purchased in June 2006 and tested within a single laboratory.
PS = powdered surgical glove; PFS = powder free surgical glove; PE = powdered exam glove; PFE = powder free exam glove.

TABLE X2.2 Antigenic Latex Protein Levels in Extracts from Natural Rubber Latex Gloves Tested Using the Modification to Include Any Particulates[A]

		Within Laboratories			Between Laboratories		
Sample	Mean Level	*Sr*	*r*	*(r)*	*SR*	*R*	*(R)*
1 PS	0.79	0.19	0.53	0.67	0.32	0.90	1.14
2 PS	3.05	0.53	1.48	0.48	1.97	5.51	1.81
3 PFS	0.29	0.07	0.21	0.71	0.15	0.41	1.38
4 PFS	0.20	0.07	0.20	1.03	0.12	0.34	1.70
5 PE	3.09	0.52	1.46	0.47	0.95	2.66	0.86
6 PE	1.92	0.31	0.87	0.45	0.85	2.39	1.24
7 PE	5.05	0.58	1.63	0.32	1.98	5.56	1.10
8 PE	9.89	1.32	3.70	0.37	4.37	12.23	1.24
9 PFE	0.27	0.05	0.14	0.53	0.13	0.36	1.37
10 PFE	0.77	0.11	0.29	0.38	0.30	0.85	1.10
11 PFE	0.16	0.03	0.09	0.54	0.07	0.19	1.17
12 PFE	0.21	0.05	0.13	0.65	0.10	0.28	1.36
13 PFE	0.21	0.06	0.17	0.81	0.12	0.33	1.60
14 PFE	0.28	0.06	0.16	0.58	0.20	0.57	2.01
15 PFE	1.10	0.20	0.55	0.50	0.49	1.38	1.25
16 PFE	1.23	0.17	0.48	0.39	0.55	1.53	1.25
17 PFE	0.64	0.11	0.32	0.50	0.28	0.78	1.21
18 PFE	0.13	0.01	0.04	0.31	0.08	0.22	1.72
19 PFE	0.11	0.01	0.04	0.35	0.07	0.19	1.74
20 PFE	0.83	0.18	0.50	0.60	0.34	0.94	1.13
21 PFE	0.12	0.02	0.06	0.50	0.07	0.18	1.50
22 PFE	0.36	0.09	0.25	0.70	0.15	0.42	1.19
23 PFE	0.80	0.15	0.42	0.53	0.34	0.96	1.21
24 PFE	0.87	0.21	0.60	0.69	0.55	1.55	1.77
25 PFE	1.00	0.20	0.56	0.56	0.73	2.03	2.04
26 PFE	0.38	0.07	0.19	0.48	0.12	0.32	0.84
27 PFE	0.67	0.16	0.45	0.67	0.39	1.08	1.62
28 PFE	0.31	0.05	0.15	0.47	0.08	0.22	0.72

[A] Data is presented in µg/mL and represents the mean of 6 determinations.
Sr = repeatability standard deviation
r = 2.8 × Sr
(r) = repeatability
SR = reproducibility standard deviation
R = 2.8 × SR
(R) = reproducibility
PS = powdered surgical gloves; PFS = powder free surgical gloves; PE = powdered exam gloves; PFE = powder free exam glove.

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