Designation: D 6499 – 07

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 D 6499; 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]

D 4483 Practice for Evaluating Precision for Test Method Standards in the Rubber and Carbon Black Manufacturing Industries

D 5712 Test Method for Analysis of Aqueous Extractable Protein in Natural Rubber and Its Products Using the Modified Lowry Method

E 691 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*.

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°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.

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).

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

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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°C. Aliquots, once thawed for use in the assay should be stored at 4°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°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°C until use.

8.4 *Substrate Development Solution*—A yellow colored reaction product is produced using o-phenylenediamine (OPD) and hydrogen peroxide. A 10 mg tablet of OPD 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 D 5712 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 D 5712).

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. 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 2 to 4°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.

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°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

[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.

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