



Designation: D 6499 – 03

Standard Test Method for The Immunological Measurement of Antigenic Protein in Natural Rubber and its Products¹

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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:

D 4483 Practice For Determining Precision For Test Method Standards in the Rubber and Carbon Black Industries²

D 5712 Test Method for Analysis of Aqueous Extractable Protein in Natural Rubber and its Products Using the Modified Lowry Method³

3. Terminology

3.1 Definitions:

¹ This specification 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 Annual Book of ASTM Standards, Vol 09.01.

³ Annual Book of ASTM Standards, Vol 09.02.

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

3.1.2 *antibody*—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*—any substance that provokes an immune response when introduced into the body.

3.1.4 *background absorbance*—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*—a non-reactive protein solution used to prevent nonspecific antibody adsorption.

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

3.1.7 *concentration range*—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)*—an immunological test method to quantify antigen or antibody levels using an enzyme as the detection mechanism.

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

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

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

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

3.1.13 *secondary antibody*—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*—the preparation of standard analyte used as a reference to which the unknown sample being measured is compared.

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

3.1.16 *titer*—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).⁴ 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 (recommnd Corning #25880-96, or equivalent).

7.3 *Multichannel Pipettors*.

7.4 *Analytical Balance*.

7.5 *Centrifuge*, (capable of $1000 \times g$) 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 ₂ CO ₃	0.795g
NaHCO ₃	1.465g
NaN ₃	0.1 g

Dissolve above in distilled H₂O 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 ₂ PO ₄ · H ₂ O	5.125 g
Na ₂ HPO ₄ · 7H ₂ O	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).

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 on request.

⁴ The boldface numbers given in parentheses refer to a list of references at the end of the text.