Designation: D8238 – 18 (Reapproved 2023)

Standard Test Method for Immunological Assay to Quantify Extractable Guayule Natural Rubber (GNR) Proteins[1]

This standard is issued under the fixed designation D8238; 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 protein in Guayule Natural Rubber (GNR) and its products using rabbit antisera specific for Guayule Natural Rubber latex proteins. This immunoassay procedure quantitatively measures the level of GNR proteins in solution using an inhibition format. The samples may include but are not restricted to, gloves or other GNR product extracts, and liquid GNR samples which have been collected in order to measure the protein levels.

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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.4 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

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

D6499 Test Method for Immunological Measurement of Antigenic Protein in Hevea Natural Rubber (HNR) and its Products

D7427 Test Method for Immunological Measurement of Four Principal Allergenic Proteins (Hev b 1, 3, 5 and 6.02) in Hevea Natural Rubber and Its Products Derived from Latex

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

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

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 OD 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.

[1] This test method is under the jurisdiction of ASTM Committee D11 on Rubber and Rubber-like Materials 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.

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.

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 test sample is extracted in an aqueous buffer. The extract is recovered, and the antigen levels are determined using inhibition Enzyme Linked ImmunoSorbent Assay (ELISA) technology.[3] The ELISA assay is based on polyclonal antiserum which recognizes GNR proteins. ELISA technology takes advantage of the specificity and sensitivity of the antibody-antigen reaction. As a variation of the ELISA method this inhibition ELISA has been developed for the detection and quantification of GNR protein antigens. In the inhibition ELISA, the GNR antigen is immobilized by absorption to the wells of a 96-well test plate. The sample extract is mixed with antibody specific for GNR protein in a dilution plate. Following a brief incubation to allow for antibody recognition of the relevant GNR antigens, the mixture is added to the immobilized antigen in the assay plate. Anti-GNR antibody which is not bound to the soluble GNR 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 GNR protein permits quantification. The assay is highly sensitive and can quantitate GNR proteins in the nanogram per millilitre range.

5. Significance and Use

5.1 NRL derived from *Hevea* contains over 200 proteins a number of which are allergens capable of eliciting Type 1 anaphylactic reactions. Guayule Natural Rubber derived from the *Parthenium argentatum* plant contains many fewer proteins and at much lower levels than Hevea NRL. While there have been no reports of allergic reactions to GNR proteins at present, they have been shown to induce antibody production in exposed individuals.[4] An immunological assay that tests for the presence of GNR has the advantage of providing the specificity and sensitivity that other ASTM standards such as Test Methods D5712 and D6499 do not offer.

5.2 This test method describes an immunological method for the quantitation of Guayule natural rubber proteins using rabbit anti-GNR serum. Rabbits immunized with GNR 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 GNR. This test method may be used to determine if a test item contains material derived from a natural source.

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,).

7.2 *Dilution Plate,* a low protein binding 96 well plate for sample dilution and antibody reaction (recommend Corning #9017, or equivalent).

7.3 *Multichannel Pipettors.*

7.4 *Analytical Balance.*

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

7.6 *Incubator,* capable of regulating the temperature at 37 °C ± 3 °C.

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

7.8 *ELISA Plate Sealing Tape,* or plastic lids for covering plates.

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 ± 0.1:

Na_2CO_3	0.795g
$NaHCO_3$	1.465g
NaN_3	0.1 g

8.1.1.1 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 2 °C to 8 °C. Alternatively, carbonate buffer capsules can be purchased from a commercial source.

8.1.2 *Phosphate-Buffered Saline (PBS),* pH 7.4 ± 0.1; 10X stock:

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

8.1.2.1 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.

[3] *Current Protocols in Molecular Biology*, eds. Ausbel, F., Brent, R.M., Kingston R.E. et al. Unit 11, J. Wiley and Sons, Inc., 1997.

[4] Robert G. Hamilton and Katrina Cornish, "Immunogenicity studies of guayule and guayule latex in occupationally exposed workers," *Industrial Crops and Products*, Volume 31, Issue 1, January 2010, pages 197-201.