Designation: D7427 – 16

Standard 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[1]

This standard is issued under the fixed designation D7427; 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 known as an immunoenzymetric assay to quantify the amount of 4 principal *Hevea brasiliensis [Hev b]* allergenic proteins [Hev b 1, Hev b 3, Hev b 5 and Hev b 6.02] in Hevea natural rubber and its products[2] derived from latex using monoclonal antibodies specific for epitopes on these proteins. Since these assays quantify the levels of only 4 of the known 14 officially acknowledged allergens potentially present in Hevea natural rubber latex containing products, the sum of the four allergen levels shall be viewed as an indicator of the allergen burden and not as a measure of the total allergen content that can be released from the product.

1.2 For the purpose of this test method, the range of allergenic protein will be measured in terms of nanogram to microgram quantities per gram or unit surface area of a Hevea natural rubber containing product.

1.3 The test method is not designed to evaluate the potential of Hevea natural rubber containing materials to induce or elicit Type I (IgE-mediated) hypersensitivity reactions.

1.4 This test method should be used under controlled laboratory conditions to detect and quantify the level of 4 allergenic proteins found in Hevea natural rubber containing products. It should not be used to describe, appraise or assess the hazard or risk of these Hevea natural rubber containing materials or products under actual in use conditions.

1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.6 *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:*[3]

D1193 Specification for Reagent Water

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

D4678 Practice for Rubber—Preparation, Testing, Acceptance, Documentation, and Use of Reference Materials

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 The Immunological Measurement of Antigenic Protein in Natural Rubber and its Products

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

3. Terminology

3.1 *Definitions:*

3.1.1 *accepted reference value (ARV)*—value that serves as an agreed upon reference for comparison and which is derived as *(1)* a theoretical or established value, based on scientific principles, *(2)* an assigned or certified value, based on experimental work of some national or international organization, or *(3)* a consensus or certified value, based on collaborative experimental work under the auspices of a scientific or engineering group.

3.1.1.1 *Discussion*—ARV is an average industrial reference material (IRM) property or parameter value established by way of a specified test program. In this standard, the ARV as defined in the IRMs for the reference antigens and capture and detection antibodies is determined by analyzing a high and low

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[2] This procedure has not been validated for condoms, particularly lubricated condoms, which could contain surfactants or other ingredients that could interfere with the assay.

[3] 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.

control in an inter-laboratory study and using the assigned values of these high and low controls to verify that the assay is in control and that the reagents are performing properly.

3.1.2 *accuracy*—the closeness of agreement between a test result and an accepted reference value.

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

3.1.4 *analyte*—any element, ion, compound, substance, factor, infectious agent, cell, organelle, activity (enzymatic, hormonal, or immunological), or property the presence or absence, concentration, activity, intensity, or other characteristics of which are to be determined.

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

3.1.5.1 *Discussion*—Any of numerous Y-shaped protein molecules produced by B lymphocytes as a primary immune response, each molecule and its clones having a unique binding site that can combine with the complementary site of an antigen, as on a virus or bacterium, thereby signaling other immune responses. (See *monoclonal antibody*.)

3.1.6 *antigen*—any substance that can stimulate the production of antibodies within an organism and combine specifically with them.

3.1.7 *background absorbance*—the absorbance reading in the solution resulting from non-specific interactions caused by the presence of chemicals, ions, etc., other than the analyte being measured.

3.1.8 *binding capacity—within the context of this document*, refers to the number of Hev b allergen molecules that a primary capture antibody can bind reproducibly under standardized assay conditions (pH, ionic strength, protein matrix, time, temperature).

3.1.9 *blocking solution*—a non-reactive protein solution used to prevent nonspecific antibody adsorption and to reduce background absorbance.

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

3.1.11 *calibration material/calibrator*—a material (for example, solution) of known quantitative/qualitative characteristics (for example, concentration, activity, intensity, reactivity) used to calibrate, graduate, or adjust a measurement procedure or to compare the response obtained with the response of a test specimen/sample.

3.1.12 *concentration range*—the recommended analyte concentration range in nanograms per mL to micrograms per mL that produces an absorbance reading from 0.1 to 2.0–3.0 units (depending on the instrument).

3.1.13 *data reduction algorithm*—a mathematical process that converts assay-response data (for example, absorbance units) into interpolated dose results.

3.1.13.1 *Discussion*—The dose–response relationship in the assay is defined by the standard, reference, or calibration curve.

3.1.14 *detection limit//limit of detection*—the smallest quantity of an analyte that can be reproducibly and a statistically significant manner distinguished from the variance of the background, or a zero calibrator in a given assay system.

3.1.14.1 *Discussion*—It is usually defined at the 95 % confidence interval and has also been called the lower detection limit or positive threshold of the assay; this term is not synonymous with analytical sensitivity.

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

3.1.16 *epitope/determinant—(1)* the minimum molecular structure of the antigenic site that will react with an antibody; *(2)* any site on an antigen molecule at which an antibody can bind; the chemical structure of the site determining the specific combining antibody.

3.1.17 *IgE*—human IgE is an immunoglobulin of the approximate molecular weight of 190 000, which exists normally in monomeric form and constitutes approximately 0.0005 % of the total serum immunoglobulins.

3.1.17.1 *Discussion*—It (IgE) binds with high affinity to FcεR1 receptors on mast cells and basophils and FcεRII receptors on a number of cells. IgE mediates the release of vasoactive mediators following the binding of allergen.

3.1.18 *immunoenzymetric assay (IEMA)*—a two-site non-isotopic immunological test method that employs two antibodies, a primary antibody to capture and a secondary enzyme conjugated antibody to detect the analyte of interest.

3.1.19 *immunoglobulin*—a glycoprotein composed of two heavy and two light chains that functions as an antibody. Human immunoglobulins have been subdivided into different isotypes (IgM, IgG, IgA, IgD, IgE), each of which possess a unique set of antigenic markers, physiochemical properties, and each of which produce a different pattern of effector functions (receptor binding, complement activation, opsonization).

3.1.19.1 *Discussion*—All antibodies are immunoglobulins, but it is not certain that all immunoglobulins possess antibody function.

3.1.20 *industry reference materials (IRM)*—materials that have been prepared according to a specified production process to generate a uniform lot; the parameters that define the quality of the lot are evaluated by a specified measurement program.

3.1.20.1 *Discussion*—IRMs are divided into two types according to the production process for generating the material.

3.1.21 *linearity*—the ability (within a given range) of an assay to provide results that are directly proportional to the concentration [amount] of the analyte in the test sample.

3.1.22 *monoclonal antibody*—antibody produced by cells created through the fusion of an antibody producing cell (B-lymphocyte) with immortal cancer cells.

3.1.22.1 *Discussion*—This fusion process produces a hybrid (hybridoma) that expresses properties of both cells. The cells are all identical since they derive from a single cell and are called “monoclonal.”

3.1.23 *parallelism*—extent to which the dose–response relationship between two materials (that is, calibrator versus unknown specimens) is constant for the examined range of concentrations.

3.1.23.1 *Discussion*—Parallelism is a property (and a requirement) of quantitative immunoassays in which the calibrator and test sera produce parallel dose–response curves.

3.1.24 *precision*—the closeness of agreement between independent test results obtained under prescribed conditions; agreement between replicate measurements.

3.1.24.1 *Discussion*—Precision has no numerical value but is expressed in terms of imprecision—the standard deviation (SD) or the coefficient of variation (CV: SD/mean) of the results in a set of replicate measurements.

3.1.25 *precision profile*—the precision of an assay across the analyte concentration range of interest.

3.1.25.1 *Discussion*—A precision profile is constructed by determining the standard deviation (or coefficient of variation) of replicate measurements (within assays, between assays, or between specimen dilutions within an assay) spanning the entire analyte concentration range, albeit without the exact knowledge of the true analyte concentration that is contained in the serum specimens. When the CV_{dose} (*Y*-axis) is graphed against the dose (*X*-axis), a precision profile plot is generated. The precision profile is also referred to as the "imprecision profile" by some investigators.

3.1.26 *primary antibody*—the antibody used first in an assay sequence that is specific for the antigen and is sometimes referred to as the capture antibody that binds the analyte of interest from a biological specimen.

3.1.27 *proficiency testing (PT)*—an independent (non-manufacturer sponsored) program in which challenge specimens are sent to participating laboratories to be evaluated in assays that measure a spectrum of analytes.

3.1.28 *qualitative assay*—an assay system that produces an indication of the presence or absence of an analyte but does not provide a precise estimate of the concentration of that analyte.

3.1.28.1 *Discussion*—A positive test result implies only that the assay signal exceeds the analytical threshold or positive cutoff point that has been set to obtain an arbitrary combination of diagnostic sensitivity and specificity.

3.1.29 *quantitative assay*—an assay system that produces an accurate and reproducible estimate of the concentration of an analyte in the test specimen.

3.1.29.1 *Discussion*—Its (quantitative assay) analysis involves interpolation from a calibration curve, which is referenced to a readily available standard reference preparation.

3.1.30 *quality control response*—level of analyte produced by an assay for a quality control specimen that has a previously defined analyte concentration range as defined by the manufacturer.

3.1.30.1 *Discussion*—Assay performance was evaluated by determining the agreement in Hev b 1, 3, 5 or 6.02 levels obtained for two quality control extracts containing a high or low level of each Hev b allergen, following analysis in multiple laboratories participating in the multi-center study.

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

3.1.32 *relative standard deviation (RSD)*—the coefficient of variation which is the standard deviation divided by the mean.

3.1.33 *repeatability*—precision under conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time.

3.1.34 *repeatability limit (r)*—the value below which the absolute difference between two individual test results obtained under repeatability conditions may be expected to occur with a probability of approximately 0.95 (95 %).

3.1.34.1 *Discussion*—The repeatability limit is 2.8 (~1.96 · square root of 2) times the repeatability standard deviation. This multiplier is independent of the size of the inter-laboratory study.

3.1.35 *reproducibility*—precision obtained under conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment.

3.1.36 *reproducibility limit (R)*—the value below which the absolute difference between two test results obtained under reproducibility conditions may be expected to occur with a probability of approximately 0.95 (95 %).

3.1.36.1 *Discussion*—The reproducibility limit is 2.8 (~1.96 · square root of 2) times the reproducibility standard deviation. This multiplier is independent of the number of laboratories participating.

3.1.37 *secondary antibody—in an IEMA*, it is the enzyme conjugated antibody used second in a sequence that is specific for the analyte or interest and that completes the sandwich of the analyte.

3.1.38 *standard solution*—the preparation containing a standard analyte that is used as a reference to which the unknown sample being measured is compared.

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

3.1.40 *titer*—the strength of an antibody in solution that takes into consideration its concentration and affinity.

4. Summary of Test Method

4.1 This standard defines a general laboratory method called the immunoenzymetric assay. The first step in the method involves the extraction of a latex-containing product using a procedure previously described in Test Methods D5712 and D6499. In brief, the latex product is extracted for 2 h in an aqueous buffer, typically at 5 to 10 mL per gram with agitation such as rotation or shaking. Following the extraction process, the extract is recovered and the level of 4 Hev b allergens is quantified using a two-site, non-competitive binding immunoenzymetric assay (IEMA) (**1**).[4]

4.2 The general IEMA design is based on the use of a monoclonal antibody which is specific for a single Hev b allergenic protein that is attached to a microtiter plate by adsorption. This "capture" antibody has also been called the primary antibody in the assay system. Following blocking of the microtiter plate, the "unknown or test" extracts of the latex

[4] The boldface numbers in parentheses refer to the list of references at the end of this standard.

TABLE 1 Characterized Latex Allergens

NOTE 1—Hev b 1, 3, 5. and 6.02 have been set in boldface because these are the allergenic proteins that are quantified by the immunoenzymetic assays covered in this standard.

Name	Description	MW (kD)	Plant Family	Crossreactivity to Food
Hev b 1	**Rubber elongation factor**	**58/14.6**	**—**	**Papain, fig**
Hev b 2	Beta 1/3 glucanase	34–36	PR-2[A]	—
Hev b 3	**Prenyltransferase**	**24–27**	**—**	**—**
Hev b 4	Microhelix	110/115	—	—
Hev b 5	**Acidic protein**	**16**	**—**	**Kiwi**
Hev b 6.01	Hevein preprotein (Prohevein)	20	PR-3	Avocado, banana, chestnut
Hev b 6.02	**Hevein protein (Mature hevein)**	**4.7**	**PR-3**	**Avocado, banana, chestnut**
Hev b 7	Patatin homologue	43–46	—	Potato
Hev b 8	Hevea Profilin	14–14.2	Profilin	Pollens, celery
Hev b 9	Hevea Enolase	51	—	Molds
Hev b 10	Mn Superoxide Dismutase	22–26	—	Molds
Hev b 11	Class I Chitinase	33	PR-3	Banana, avocado
Hev b 12	Lipid Transfer Protein	9.4	PR-14	Peach, stone fruit
Hev b 13	Esterase	42	—	—
Hev b 14	Hevamine	30	—	—

[A] PR = pathogenesis-related.

product are pipetted into replicate wells in the plate. In a different part of the plate, calibrators with pre-defined levels of the relevant Hev b allergenic protein are pipetted in replicate to produce a calibration curve. These calibrators can be either reference proteins or working calibrators that have been cross-validated against the reference proteins. Additionally, two control extracts with pre-defined ranges are pipetted in replicates into their wells in the plate and these serve as positive controls to verify that the assay is in control. Accepted reference values are assigned by the manufacturer to these two control preparations. These controls permit the assessment of assay agreement between laboratories by determining the range and coefficient of variation of the quality control response (see 3.1.30). Following the incubation of the extract with the insolubilized capture antibody, the plate is washed and a horseradish peroxidase enzyme-labeled secondary or "detection" antibody is pipetted into all wells in the plate. This detection antibody binds to a second determinant or epitope on the Hev b allergenic protein that is already bound to the primary capture antibody. As such, the two antibodies are a matched pair that form a "sandwich" around the target Hev b allergen. Following the conjugated second antibody incubation, the plate is washed to remove unbound labelled antibodies and substrate is pipetted into all wells in the plate. The clear substrate is enzymatically converted to a colored product. The extent or intensity of the color is assessed in a microtiter plate reader at an appropriate wavelength. The final measured optical density in each well is proportional to the amount of enzyme-labeled detect antibody bound, which in turn is proportional to the amount of the Hev b allergenic protein bound. Interpolation of the unknown optical density from the calibration curve permits determination of the concentration of the Hev b allergenic protein in the test extracts.

4.3 This standard describes the IEMA generic procedure. There are in fact 4 separate IEMAs which are covered by this standard. These are IEMAs that quantify Hev b 1, Hev b 3, Hev b 5 and Hev b 6.02 in extracts, each with their own unique primary capture and secondary detection antibodies and calibrators.

5. Significance and Use

5.1 IgE-mediated allergic reactions to protein allergens in Hevea natural rubber latex derived from the *Hevea brasiliensis* tree emerged in the 1990s as a concern with occasional allergic manifestations. Symptoms encompassing hives, uriticaria, rhinitis, asthma and anaphylaxis have all been reported in latex allergic individuals exposed to products derived from Hevea natural rubber latex.

5.2 Since no safe level of Hevea latex allergen exposure is known, avoidance is the primary mode of treating latex allergy.

5.3 As a result of investigations conducted by many scientists across the world, fourteen latex allergens have so far been identified and categorized by the Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies (IUIS) as Hev b 1 to Hev b 13 (Table 1) (see Specification D1193). Reported sensitization rates for these allergenic Hev b proteins vary among the many reports as a result of differences in the study populations, IgE antibody assay methods and the quality of the Hev b allergens used as calibrators and quality control reagents in the analysis. Most studies, however, agree that Hev b 1 and Hev b 3 are important allergens for individuals (for example, children with spina bifida) who are exposed through mucosal contact as a result of multiple surgeries or latex catheter use for an extended period of time. Additionally, investigators performing sensitization studies also agree that Hev b 5 and Hev b 6.02 are important allergens that may elicit sensitization in genetically-predisposed individuals who are exposed to Hevea natural rubber latex (**2-4**). On the basis of these clinical studies, assays for these four allergenic proteins (that is, Hev b 1, Hev b 3, Hev b 5 and Hev b 6.02) have been developed and they are thus the subject of this standard. Adoption of immunoenzymetric assay reagents and standard proteins needed to quantify other latex allergens (other than Hev b 1, 3, 5, and 6.02) in extracts of Hevea natural rubber latex products will require separate documentation and validation.

5.3.1 From the historical context, a number of assays have been developed to quantify the level of protein, antigen and

allergen in Hevea natural rubber latex containing products (see Practices D4483 and D4678).

5.3.2 The *modified Lowry* assay for total protein, Test Method D5712, was the first assay of this type. It assesses the level of total protein as an indirect indicator of allergenicity of latex-containing products. This assay does not discriminate between the allergenic and non-allergenic proteins.

5.3.3 The second assay to be developed involved the use of human latex-specific IgE antibody in a *competitive inhibition immunoassay* format to estimate the overall allergenic potency of a Hevea natural rubber product extract (**5, 6**). The extract is incubated with human serum containing latex-specific IgE antibody and then this mixture is incubated with a solid phase latex allergosorbent. Latex allergenic proteins, if they are present, bind to the latex-specific IgE antibody in solution and they thus inhibit IgE antibody binding onto the latex allergosorbent. Allergosorbent bound IgE is then quantified and the extent of competitive inhibition of IgE binding is a measure of latex allergens. While this assay provides an estimate of the allergenicity or level of Hevea natural rubber allergens extractable from a product, difficulty in procuring reproducible lots of latex specific IgE containing human serum has precluded widespread use of this assay. For this reason, this assay has not been put forth as an ASTM standard.

5.3.4 A third assay design is similar to the human IgE based competitive inhibition immunoassay, but it employs rabbit antiserum instead of human serum containing IgE anti-latex. The *competitive inhibition enzyme linked immunosorbent assay* (ELISA) has been adopted as Test Method D6499. It measures latex proteins that elicit immune responses, but it cannot distinguish between latex allergens (IgE inducing) from non-allergenic antigens (non-IgE inducing).

5.3.5 The most recent assay, which is the subject of this standard, is the *two-site immunoenzymetric assay (IEMA)* which uses an insolubilized capture antibody to bind one of Hev b allergenic proteins from a latex product extract, and a second enzyme labeled detection antibody to detect bound allergens. Optical density responses are interpolated from reference curves constructed with known allergens. The performance characteristics of the reagents used in immunoenzymetric assays for Hev b 1, 3, 5 and 6.02 were investigated in the international collaborative study associated with the development of this standard and results are provided in Sections 15 through 17.

6. Interferences

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

7. Apparatus

7.1 *96 Well Microtiter Assay Plate* (recommended high protein binding plates for optimal binding of capture antibody).

7.2 *Dilution Tubes,* low protein binding 12 × 75 mm (recommended polypropylene) test tubes for sample dilution.

7.3 *Mulitchannel Pipettors and Plastic Tips.*

7.4 *Analytical Balance.*

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

7.6 *Microtiter Plate Reader* and computer for data analysis.

7.7 *Microtiter Plate Sealing Tape.*

7.8 *Microtiter Plate Shaker.*

7.9 *Aspiration Device or a Microtiter Plate Washer.*

7.10 *End-Over-End Shaker.*

7.11 *Polypropylene Vessels,* for sample extraction.

8. Reagents and Materials

8.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available.[5] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of determination.

8.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water as defined by specifications in Specification D1193.

8.3 *Buffers*—The buffers and solutions should be prepared before beginning the protocol. Preferably, all solutions containing protein are made in polypropylene tubes through the assay.

8.3.1 The Anti-Hev b coating buffer is a phosphate buffer of pH 6.0 to 9.0. The following buffer solutions may be used: sodium phosphate buffer or phosphate buffered saline or the equivalent of sufficient buffering capacity (at least 25 mM) to maintain the pH between 6.0 and 9.0. The procedure for phosphate buffered saline (PBS) is provided below:

Phosphate Buffered Saline (PBS), pH 7.4; 1X Stock
1.5 mM KH_2PO_4—0.2 g
2.7 mM KCl—0.2 g
0.137 M NaCl—8.06 g
8.0 mM $Na_2HPO_4{\cdot}2H_20$—1.42 g

8.3.1.1 Dissolve ingredients in 800 mL of distilled water, adjust the pH to 7.4 if necessary and fill to 1000 mL. Alternatively, PBS can be purchased from a commercial source.

8.3.2 *Blocking Buffer (PBS-BSA)*—Add 0.5 g of BSA (bovine serum albumin) or BPLA (bovine plasma albumin) to 100 mL of PBS or the equivalent of sufficient buffering capacity (at least 25 mM) to maintain the pH between 6.0 and 9.0. Let the protein dissolve under gentle stirring at 25 ± 5°C. The amount needed for blocking one plate is approximately 30 mL. Store at 4 ± 3°C for a maximum of 2 weeks.

8.3.3 *Hev b 1 and Hev b 3 Assay Buffer*—The procedure for Hev b 1 and Hev b 3 Assay buffer is provided below:

[5] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For Suggestions on the testing of reagents not listed by the American Chemical Society, see *Annual Standards for Laboratory Chemicals,* BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary,* U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

10 mM Titriplex II—1.46 g
0.3 % Albumin BPLA1—1.5 g
0.03 % Tween20—0.15 mL
0.1 % Proclin300—0.5 mL
0.0001 % PyroninY—0.5 mL of 0.1 % PyroninY

8.3.3.1 Dissolve ingredients in 450 mL of distilled water, adjust the pH to 7.4 if necessary and fill to 500 mL. Alternatively, Hev b 1 and Hev b 3 Assay buffer can be purchased from a commercial source. Store at 4 ± 3°C.

8.3.4 *Hev b 5 Assay Buffer*—The procedure for Hev b 5 Assay buffer is provided below:

1X PBS, pH 7.4
10 mM Titriplex II—1.46 g
0.3 % Albumin BPLA1—1.5 g
0.03 % Tween20—0.15 mL
0.1 % Proclin300—0.5 mL
0.0001 % PyroninY—0.5 mL of 0.1 % PyroninY

8.3.4.1 Dissolve ingredients in 450 mL of distilled water, adjust the pH to 6.0 if necessary and fill to 500 mL. Add Mab Hev b 5 (clone 10-004) to final concentration 1.0 µg/mL. Alternatively, Hev b 5 Assay buffer can be purchased from a commercial source. Store at 4 ± 3°C.

8.3.5 *Hev b 6.02 Assay Buffer*—The procedure for Hev b 6.02 Assay buffer is provided below:

1X PBS, pH 7.4
10 mM Titriplex II—1.46 g
0.3 % Albumin BPLA1—1.5 g
0.03 % Tween20—0.15 mL
0.1 % Proclin300—0.5 mL
0.0001 % PyroninY—0.5 mL of 0.1 % PyroninY

8.3.5.1 Dissolve ingredients in 450 mL of distilled water, adjust the pH to 7.4 if necessary and fill to 500 mL. Add Mab Hev b 6.02 (clone 11-002) to final concentration 1.5 µg/mL. Alternatively, Hev b 6.02 Assay buffer can be purchased from a commercial source. Store at 4 ± 3°C.

8.4 *Wash Buffer (PBS-Tween)*—Add 0.5 mL of Tween 20 to 1 liter of 1X PBS (0.05 %) and mix well.

8.5 *Stop Solution* (1%SDS [sodium dodecyl sulfate or sodium lauryl sulfate]).

8.6 *Horseradish Peroxidase Substrate* (ABTS [2,2'-azino-di[3-ethylbenzthiazoline sulfonic acid]]).

8.7 *Monoclonal Antibody Pairs,* primary (capture) and secondary (enzyme-conjugated detection) antibodies.[6]

8.7.1 *Capture Monoclonal Antibodies*—These capture antibodies can be coated directly on microtiter plates (diluted to 5.0 µg/mL in the anti-Hev b coating buffer, 0.1 mL per well) or antibody-coated strips or plates that can be purchased from a commercial source.[6]

8.7.1.1 MAbs Hev b 1 specific towards latex protein Hev b 1 (clone 07-005, IRM 915).

8.7.1.2 MAbs Hev b 3 specific towards latex protein Hev b 3 (clone 09-005, IRM 918).

TABLE 2 IEMA Working Ranges and Calibrator Concentrations

Description	Hev b 1 (ng/mL)	Hev b 3 (ng/mL)	Hev b 5 (ng/mL)	Hev b 6.02 (ng/mL)
Calibrator A	0	0	0	0
Calibrator B	10	10	5	5
Calibrator C	50	50	10	15
Calibrator D	200	200	25	50
Calibrator E	500	500	50	100
Calibrator F	1000	1000	100	200

8.7.1.3 MAbs Hev b 5 specific towards latex protein Hev b 5 (clone 10-004, IRM 921).

8.7.1.4 MAbs Hev b 6.02 specific towards latex protein Hev b 6.02 (clone 11-002, IRM 924).

8.7.2 *Secondary (Detection) Monoclonal Antibodies*—These detection antibodies should be conjugated with horseradish peroxidase or HRP-conjugated antibodies can be purchased from a commercial source.[6]

8.7.2.1 MAbs Hev b 1 specific towards latex protein Hev b 1 (clone 07-001, IRM 916).

8.7.2.2 MAbs Hev b 3 specific towards latex protein Hev b 3 (clone 09-010, IRM 919).

8.7.2.3 MAbs Hev b 5 specific towards latex protein Hev b 5 (clone 10-011, IRM 922).

8.7.2.4 MAbs Hev b 6.02 specific towards latex protein Hev b 6.02 (clone 11-003, IRM 925).

8.8 *Reference Reagents:*

8.8.1 *Reference Antigens,* IRM 917 for Hev b 1, IRM 920 for Hev b 3, IRM 923 for Hev b 5, and IRM 926 for Hev b 6.02.[6] These reference preparations are lyophilized and they contain known amounts of purified Hev b 1, Hev b 3, Hev b 5 or Hev b 6.02 proteins, respectively. Follow the steps provided in appropriate IRMs documents (Section 10) to prepare these antigens for use in their respective immunoenzymetric assay.

8.8.1.1 *Commercially-Available Liquid Calibrators,* containing known amounts of purified Hev b 1, Hev b 3, Hev b 5 or Hev b 6.02 proteins are available[6] and can be used to construct the standard curve used in each of the four immunoenzymetric assays.

8.8.1.2 *Working Calibrators*—Calibrators may be prepared and used instead of commercially-available reference antigens to construct standard curves in the IEMA.[7] These calibrators must, however, meet the following criteria:

(1) It is assumed that the working calibrator will be prepared from an extract of a Hevea natural rubber latex product, a liquid Hevea natural rubber *Hevea brasiliensis* latex or purified recombinant protein (with proved DNA sequence) expressed in *E. Coli.*

(2) The working calibrator must contain a sufficient amount of Hev b 1, Hev b 3, Hev b 5 and/or Hev b 6.02 protein to cover the targeted working range of each of the 4 assays as indicated in Table 2. For instance, the working calibrator for

[6] Monoclonal antibody pairs, liquid calibrators, and reference antigens are available from Icosagen AS, Eerika tee 1, Ülenurme vald, 61713, Tartumaa, Estonia. Monoclonal antibody pairs and reference antigens are also distributed by Scripps Laboratories, 6838 Flanders Drive, San Diego, CA 92121. These are the only two sources of supply for these products known to the committee at this time. 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.

[7] Hev b antigens are available from Biomay, Vienna, Austria, http://www.biomay.at/. Recombinant Hev b antigens (Hev b 1, 2, 3, 5, 6.01, 6.02, 8, 9, and 11, as MBP fusion proteins) are now available also from Phadia, www.phadia.com. These are the only two sources of supply for these products known to the committee at this time. 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.

the Hev b 1 IEMA needs to contain at least 1000 ng/mL of Hev b 1 to allow it to construct the lesser calibrator concentrations (for example, 1000, 500, 200, 50, and 10 ng/mL of Hev b 1). Likewise, the working range for the Hev b 3 IEMA should be 1000 to 10 ng/mL of Hev b 3; the working range for the Hev b 5 IEMA should be 100 to 5 ng/mL of Hev b 5; and the working range of the Hev b 6.02 IEMA should be 200 to 5 ng/mL.

(3) The working calibrator must be cross-validated against its appropriate reference antigen IRM 917 (Hev b 1), IRM 920 (Hev b 3), IRM 923 (Hev b 5) or IRM 926 (Hev b 6.02) respectively. Cross calibration (cross-validation) should be performed using the procedure provided in 11.2 of this document.

9. Hazards

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

10. Sampling, Test Specimens, and Test Units

10.1 Sample extraction is designed to be compatible with Test Methods D5712 and D6499 to allow total protein, antigenic protein and allergenic protein levels to be determined for the same sample extract.

10.2 An aqueous buffer of pH 7.4 and a minimum of 25 mM salt (for example, PBS) must be used as an extraction medium. Phosphate buffered saline is recommended.

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

10.4 The entire Hevea natural rubber product should be weighed and the total weight per product recorded so the allergen content can be reported as micrograms of allergen per gram of product weight. When it is required to report results in micrograms of allergen per dm^2 of surface area, the surface area of the product 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 the 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 Hevea natural rubber material. The ratio of extraction volume to the weight of the Hevea 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 final aqueous extracts should be used immediately, or stored up to one day at 2 to 4°C or stored for longer than one day at or below –20°C. Since Hev b 1 and Hev b 3 allergens can be unstable when extracted from product, it is recommended that prepared extracts are analyzed on the same day, without a freezing step.

11. Calibration and Standardization

11.1 *Equipment: Microtiter Plate Spectrophotometer Warm-Up*—Under normal operation, switch "on" the spectrophotometer and allow it to warm up following the manufacturer's recommendations. Zero the instrument as required in the manufacturer's manual prior to use.

11.2 *Standardization of the Hev b Immunoenzymetric Assays*—Each immunoenzymetric assay uses a calibration curve as defined in 8.8.1 of this document to standardize the assay and permit interpolation of unknown response (optical density) data into concentrations of Hev b proteins. The calibration curves in the four IEMAs may be constructed by analyzing reference antigens as defined in IRM 917 for Hev b 1, IRM 920 for Hev b 3, IRM 923 for Hev b 5 and IRM 926 for Hev b 6.02. Alternatively, working calibrators can be prepared that meet the criteria defined in 8.8.1.2 of this document. The working calibrators need to be cross-validated against the Hev b reference antigens defined in 8.8.1.1 using the following procedure:

11.2.1 Simultaneously analyze at least 6 dilutions of the reference antigen (for example, Hev b 1-IRM 917) and at least 6 appropriate dilutions of the proposed working calibrator in the same IEMA. Analyze these in triplicate in 3 assays, each performed on a separate day. The working range of the IEMA should span the desired assay working range as defined in Table 2.

11.2.2 Separately interpolate the mean optical density of each working calibrator dilution from the calibration curve constructed with the IRM reference antigen of known concentrations. Correct each interpolated result obtained with each working calibrator dilution for that dilution. For instance if a 1:100 dilution of the proposed working Hev b 5 calibrator interpolates off the Hev b 5 reference antigen based calibration curve as 15 ng/mL of Hev b 5, then the working preparation contains 15 ng/mL × 100 = 150 ng/mL of Hev b 5 following correction to neat (undiluted).

11.2.3 Compute the inter-dilution percentage coefficient of variation (%CV = standard deviation/mean × 100) for the working calibrators Hev b concentrations following correction for dilution as defined in 11.2.2. The inter-dilutional %CV should be ≤ 25 % and each analysis should include at least 3 dilutions of the proposed working calibrators. This analysis confirms parallelism/linearity of each assay run.

11.2.4 Repeat this cross-validation analysis (11.2.1 – 11.2.3) on 3 separate days. Compute the Hev b concentration grand mean as determined using the mean Hev b protein value determined for each of these 3 assay runs. The inter-assay

(inter-day) coefficient of variation should be ≤ 20 %. This defines the concentration of the Hev b protein in the working (working) calibrator.

12. Procedure

12.1 The procedure for performing each of the Hev b 1, Heb 3, Hev b 5 and Hev b 6.02 IEMAs are identical except for the specificity of the capture and detection reagents and the calibrators. This general assay procedure is provided and where necessary, specific details for each of the 4 IEMAs is provided.

Day 1

12.2 *Coating the Assay Plate:*

12.2.1 Prepare the coating antibody at 5.0 µg/mL in the anti-Hev b coating buffer.

12.2.2 To coat 3 microtiter plates for the Hev b 1 IEMA, mix 150 µg of Hev b 1 specific monoclonal antibody (clone 07-005) into 30 mL of anti-Hev b coating buffer. Mix end over end at least 10 min. Dispense the monoclonal antibody solution to 3 plates, 100 µL per each well. Cover the plates by a lid or a sealing tape. Mark the plates as 1, 2 and 3.

12.2.3 Incubate overnight standing at 25 ± 5°C.

NOTE 1—The D7427 testing temperature meets the temperature tolerance 25 ± 5°C and the data is on record with ASTM. However, at temperatures above 25°C the colored product may develop much more rapidly and the incubation time may need to be shortened. This is the responsibility of the lab doing the assay. For best results it is suggested that the incubations be carried out between 20 and 23°C.

Day 2

12.2.4 Empty the plates and wash four times with PBS-0, 05% Tween 20.

12.2.5 Block plates by adding 300 µl of the blocking buffer in each well and incubating at 25 ± 5°C for 1 h.

12.2.6 Cover the plates with the blocking buffer still in the wells and store the plates at 4 ± 3°C for no more than 1 week until use.

12.2.7 Before use, remove the blocking buffer from the strips to be used and tap them dry on a paper towel.

NOTE 2—*Wash Procedure*—Plate washing can be performed using a variety of methods. Washing using a multiple fluid dispenser or automatic plate washer is recommended. Manual hand washing can be done but it is discouraged.

12.2.8 To coat plates for the Hev b 3, Hev b 5 and Hev b 6.02 IEMAs, use the procedures specified in 12.2.1 to 12.2.7, except the capture antibody is different. Use anti-Hev b 3 (clone 09-005) for plates used in the Hev b 3 IEMA, anti-Hev b 5 (clone 10-004) for plates used in the Hev b 5 IEMA and anti-Hev b 6.02 (clone 11-002) for plates used in the Hev b 6.02 IEMA.

12.3 Perform Hev b 1, 3, 5 and 6.02 IEMAs using antibody-coated plates or strips. Alternatively, use ready-made plates that can be purchased from a commercial source (see 8.7.1).

12.3.1 Thaw all standards, quality control extracts and unknown extracts if frozen. Bring all reagents to room temperature (25 ± 5°C).

12.3.2 Dispense 100 µL of Hev b 1/3/5/6.02 Assay buffer in each well of the microtiter plate.

12.3.3 Pipette 25 µL of Hev b 1/3/5/6.02 calibrators A-F, controls and unknowns into appropriate wells in duplicate.

12.3.4 Cover the plate. Incubate the plate for 60 min at 25 ± 5°C on a plate shaker (200 to 300 rpm).

12.3.5 Aspirate and wash the wells 4 times with 300 µL of washing solution.

12.3.6 Dispense 100 µL of the appropriate Hev b 1/3/5/6.02 enzyme conjugate into the wells of the Anti-Hev b 1/3/5/6.02 antibody coated microtiter plate.

12.3.7 Cover the plate. Incubate the plate for 30 min at 25 ± 5°C on a plate shaker (200 to 300 rpm).

12.3.8 Aspirate and wash the wells 4 times with 300 µL of washing solution.

12.3.9 Add 100 µL of HRP Substrate Solution (ABTS) into each well.

12.3.10 Cover the plate. Incubate for 15 min at 25 ± 5°C on a plate shaker (200 to 300 rpm).

12.3.11 Stop the reaction by adding 100 µL of stop solution (1% SDS) into each well so that exactly the same substrate reaction time is achieved. Shake the plate for 1 to 2 min to mix the solutions.

12.3.12 Measure the absorbance of each wells at 405 or 414 nm with a plate or strip reader. Read the plate immediately. If the plate is not read immediately, cover the plate, protect from light and read the plate within 60 min after stopping the reaction.

12.3.13 To insure that substances such as detergents or surfactants in the specimen do not interfere in the IEMA, analyze the specimen at a dilution to verify the same result is obtained.

13. Calculation

13.1 The absorbance readings of the test extracts are converted to nanograms or micrograms of allergenic protein per millilitre using the appropriate calibration curve for the respective Hev b IEMA. The average absorbance readings for each duplicate of the calibrators minus the absorbance of the background (Zero Calibrator = Cal A) are plotted against the concentration of the reference Hev b protein in that particular calibrator using a log-log scale. A software package that fits the standard curve (for example, cubic spline smoothed) can be used to interpolate Hev b allergen results in the samples.

14. Report

14.1 The laboratory should maintain a record of all lot numbers of reagents and specimens used as well as all the observations and calculations derived from the data and the finalized test reports to allow the test to be satisfactorily repeated.

14.2 The report shall include a description of the Hevea natural rubber latex containing product including the lot number where appropriate. The concentration of Hev b allergenic protein may be expressed as nanograms or micrograms per gram of weight or per dm^2 of surface area.

14.3 It is recommended that the Hev b 1, Hev b 3, Hev b 5 and Hev b 6.02 levels as measured in an extract of a Hevea

natural rubber latex containing product (see 14.2) be reported both individually and as a sum of the four allergen levels. Since all Hev b allergens are not measured by these assays, the sum of the four allergen levels shall be viewed as an indicator of the allergen burden and not as a measure of the total allergen content that can be released from the product.

14.4 The minimum detectable concentration or limit of the assay detection is not defined for any of the assays because assay sensitivity should be determined by the laboratory if they perform the assay with laboratory made coated plates. Laboratories that use complete kits from a commercial source will be provided with a target analytical sensitivity and minimal detectable concentration.

15. Precision and Bias[8]

15.1 The inter-laboratory program for determining precision was conducted according to Practices D4483 and E691.

15.2 This inter-laboratory study documents the precision, variation in the quality control response and linearity of the IEMAs. This study involved the evaluation of multiple extracts of Hevea natural rubber latex products in 2 laboratories in Estonia and the United States of America using each of the four IEMAs that quantify Hev b 1, Hev b 3, Hev b 5 and Hev b 6.02 levels, respectively. Inter-laboratory reproducibility and intra-laboratory repeatability of the 4 individual IEMAs followed the general protocol used in previous ASTM inter-laboratory studies (see Practices D4483, D4678 and Test Methods D5712, D6499). This study was designed to assess precision and variation in the quality control response, but it was not designed to assess accuracy. Moreover, determination of the relationship between the level of the Hev b allergens in an extract of a latex product and the relative hazard of that product to induce an allergic reaction in a latex allergic individual was not assessed in this study. The reader should note, however, that correlation between the measured level of Hev b 1, Hev b 3, Hev b 5 and Hev b 6 in extracts using analogous assays and the level of these allergens as assessed by a Radioallergosorbent Test (RAST) inhibition assay and a ELISA inhibition assays using human IgE anti-latex has been previously reported (**2, 7**). For this reason, no additional cross-method comparisons were performed in the inter-laboratory study documenting the performance of the Hev b 1, Hev b 3, Hev b 5 and Hev b 6.02 IEMAs.

15.3 The results in this section give an estimate of the precision of the four individual Hev b IEMAs using extracts of Hevea natural rubber latex containing products as described in the inter-laboratory report as defined below. The precision parameters should not be used for acceptance or rejection testing of any group of materials.

15.4 Procedure for the determination of the precision of the immunological measurement of allergenic latex (Hev b) proteins.

15.4.1 Repeatability is the precision of independent test results obtained under repeatability conditions, that is, where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time. Reproducibility is the precision of test results obtained under reproducibility conditions, that is, where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment.

15.4.2 A test result is a mean value as specified by this test method that was obtained on duplicate determinations of the test extracts.

15.4.3 A total of 16 extracts were used in the precision component of the study. Nine extracts were analyzed by 3 laboratories in 3 countries in each of the 4 Hev b IEMAs (specimens 8-16: Hev b 1 IEMA, specimens 1-5, 8-10, and 14: Hev b 3 IEMA, specimens 1, 2, 4-5, 7-10, and 14: Hev b 5 IEMA, specimens 1, 2, and 4-10: Hev b 6.02 IEMA). The extracts were prepared powdered examination and surgeon gloves and other latex containing products.

15.4.4 The inter-laboratory program was conducted in November, 2006.

15.4.5 The results of the precision calculations for repeatability and reproducibility are presented in Table 3 and Fig. 1 for the Hev b 1 IEMA, Table 4 and Fig. 2 for the Hev b 3 IEMA, Table 5 and Fig. 3 for the Hev b 5 IEMA, and Table 6 and Fig. 4 for the Hev b 6.02 IEMA. Figs. 1-4 present the relative standard deviation (RSD) of the dose which is also known as the coefficient of variation as a function of the mean measured concentration of Hev b allergen. These plots are also know as precision profiles.

15.4.6 The repeatability and reproducibility standard deviations appeared to be proportional to the mean concentrations in all four assays, so the precision estimates are stated as relative standard deviations, as tabulated below.

Protein	Repeatability	Reproducibility
Hev b 1	15.3 %	22.1 %
Hev b 3	13.9 %	19.0 %
Hev b 5	7.3 %	12.8 %
Hev b 6.02	5.9 %	7.3 %

16. Variability in the Quality Control Response

16.1 The difference between an average test value and the reference (true) test property value is a measure of assay performance.

16.2 While absolute reference values do not exist, four quality control extracts with defined target ranges were analyzed triplicate in three assays on three days in the analyses.

16.3 Table 7 presents the mean and standard deviation of the Hev b 1, Hev b 3, Hev b 5 and Hev b 6.02, Kit Control, low, medium, and high control specimens as measured triplicate in three assays on three days. The 95 % confidence limit target ranges that were determined in advance of the study for the Kit Control, low, medium, and high controls are also presented in Table 7.

17. Linearity/Parallelism

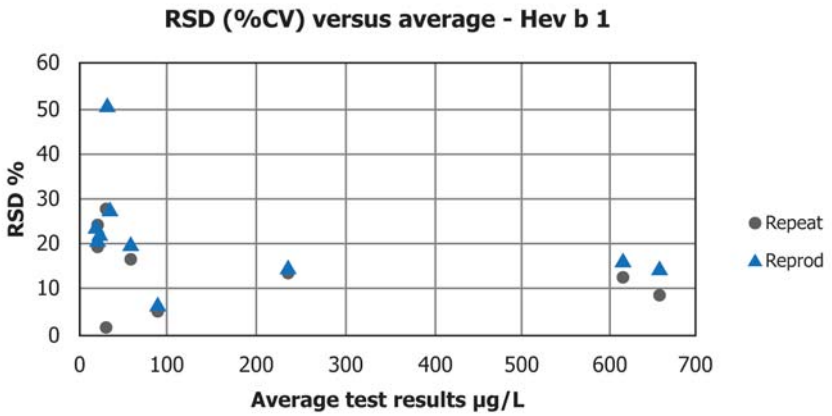
17.1 Linearity is the ability (within a given range) of an assay to provide results that are directly proportional to the concentration (amount) of the analyte in the test sample. This is also known as parallelism which is the extent to which the

[8] Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR:D11-1098. Contact ASTM Customer Service at service@astm.org.

TABLE 3 Immunological Measurement of Allergenic Hev b 1 Protein in Hevea Natural Rubber by IEMA

NOTE 1—This table contains the statistics for the Hev b 1 Immunoenzymetric assay (IEMA) for each specimen extract: the number of data, the average test result, the standard deviations (SD) and Relative SD or RSD (as %) for repeatability and reproducibility. The RSD is synonymous with coefficient of variation (SD/mean × 100). The standard deviations appeared to increase with the averages but the CVs (RSDs) remain fairly constant over the working range of the assay. Fig. 1 presents a summary of these data in pictorial format as the RSD versus the average Hev b 1 data for 2 participating laboratories.

				Repeatability		Reproducibility	
Specimen Number	n	df	Hev b 1 Average	Std Dev	RSD	Std Dev	RSD
KitControl	9	9	88.0	4.9	5.5	5.9	6.7
Sample 8	9	9	656.6	63.6	9.0	100.6	15.3
Sample 9	9	9	616.1	82.4	12.9	101.8	16.5
Sample 10	9	9	19.6	3.8	19.7	4.2	21.3
Sample 11	9	9	21.1	4.6	21.4	4.7	22.3
Sample 12	9	9	56.6	9.1	16.8	11.4	20.2
Sample 13	9	9	235.3	31.5	13.7	35.9	15.3
Sample 14	9	9	18.4	4.7	24.7	4.5	24.3
Sample 15	9	9	30.8	5.4	27.9	15.7	51.0
Sample 16	9	9	33.1	0.6	1.6	9.3	28.1
						Medians	
		90		21.1	15.3	29.4	22.1



NOTE 1—Fig. 1 presents the relative standard deviation of the dose as a function of the average Hev b 1 dose obtained for 2 participating laboratories in the inter-laboratory study (ILS). Note that the relative standard deviation or RSD is also known as the coefficient of variation which is the standard deviation divided by the mean.

FIG. 1 Intra-Laboratory Repeatability and Inter-Assay Reproducibility as a Measure of the Hev b 1 IEMA Precision

dose–response relationship between two materials (that is, calibrator versus unknown specimens) is constant for the examined range of concentrations Parallelism is a property (and a requirement) of quantitative immunoassays in which the calibrator and test sera produce parallel dose–response curves.

17.2 Table 8 presents the inter-dilutions mean, standard deviation and coefficients of variation for 2 to 4 dilutions of 4 specimens analyzed in each of the four IEMAs. Latex extracts 1–4 were analyzed in the Hev b 1 IEMA, extracts 5–8 were analyzed in the Hev b 3 IEMA; extracts 9–12 were analyzed in the Hev b 5 IEMA; and extracts 13–16 were analyzed in the Hev b 6.02 IEMA.

17.3 An inter-dilutional % coefficient of variation < 15 % for 2 or more dilutions of a given extract which fall on the working range of the IEMA dose response curve is considered acceptable for clinical assays.

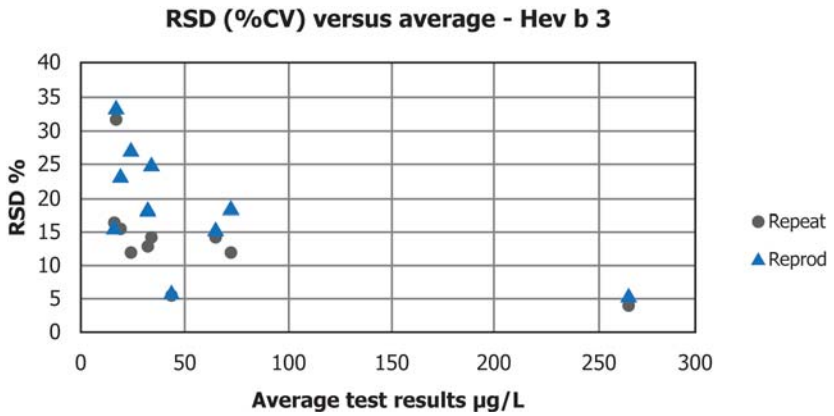
18. Keywords

18.1 allergens; Hevea brasiliensis; IEMA; immunoenzymetric assay; Hevea natural rubber latex; proteins

TABLE 4 Immunological Measurement of Allergenic Hev b 3 Protein in Hevea Natural Rubber by IEMA

NOTE 1—This table contains the statistics for the Hev b 3 Immunoenzymetric assay (IEMA) for each specimen extract: the number of data, the average test result, the standard deviations (SD) and Relative SD or RSD (as %) for repeatability and reproducibility. The RSD is synonymous with coefficient of variation (SD/mean × 100). The standard deviations appeared to increase with the averages but the CVs (RSDs) remain fairly constant over the working range of the assay. Fig. 2 presents a summary of these data in pictorial format as the RSD versus the average Hev b 3 data for 2 participating laboratories.

				Repeatability		Reproducibility	
Specimen Number	n	df	Hev b 3 Average	Std Dev	RSD	Std Dev	RSD
KitControl	9	9	265.2	10.5	4.0	14.9	5.6
Sample 1	9	9	32.3	4.4	13.0	5.9	18.4
Sample 2	9	9	43.4	2.5	5.7	2.6	6.0
Sample 3	9	9	18.5	3.1	15.5	4.3	23.4
Sample 4	9	9	16.7	5.7	31.9	5.5	33.7
Sample 5	9	9	65.0	9.3	14.4	10.1	15.6
Sample 8	9	9	72.0	9.2	11.8	13.4	18.6
Sample 9	9	9	33.8	5.3	14.5	8.5	25.1
Sample 10	9	9	23.6	3.3	12.1	6.5	27.5
Sample 14	9	9	15.8	2.6	16.2	2.5	15.9
						Medians	
		90		5.6	13.9	7.4	19.0



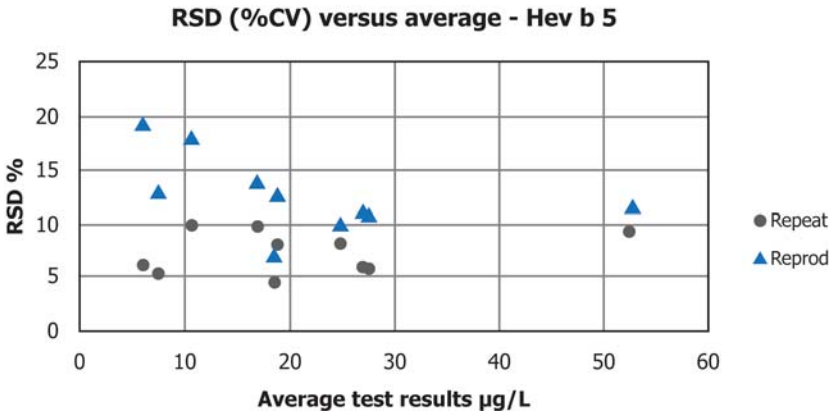
NOTE 1—Fig. 2 presents the relative standard deviation of the dose as a function of the average Hev b 3 dose obtained for 2 participating laboratories in the inter-laboratory study (ILS). Note that the relative standard deviation or RSD is also known as the coefficient of variation which is the standard deviation divided by the mean.

FIG. 2 Intra-Laboratory Repeatability and Inter-Assay Reproducibility as a Measure of the Hev b 3 IEMA Precision

TABLE 5 Immunological Measurement of Allergenic Hev b 5 Protein in Hevea Natural Rubber by IEMA

NOTE 1—This table contains the statistics for the Hev b 5 Immunoenzymetric assay (IEMA) for each specimen extract: the number of data, the average test result, the standard deviations (SD) and Relative SD or RSD (as %) for repeatability and reproducibility. The RSD is synonymous with coefficient of variation (SD/mean × 100). The standard deviations appeared to increase with the averages but the CVs (RSDs) remain fairly constant over the working range of the assay. Fig. 3 presents a summary of these data in pictorial format as the RSD versus the average Hev b 5 data for 2 participating laboratories.

				Repeatability		Reproducibility	
Specimen Number	n	df	Hev b 5 Average	Std Dev	RSD	Std Dev	RSD
KitControl	9	9	27.0	1.5	5.9	3.0	11.3
Sample 1	9	9	6.0	0.4	6.2	1.2	19.4
Sample 2	9	9	10.5	1.1	9.9	1.9	18.1
Sample 4	9	9	24.8	2.1	8.2	2.5	10.0
Sample 5	9	9	27.5	1.7	5.9	3.0	10.9
Sample 7	9	9	18.5	0.9	4.6	1.3	7.1
Sample 8	9	9	16.9	1.7	9.8	2.4	13.9
Sample 9	9	9	7.3	0.4	5.4	1.0	13.0
Sample 10	9	9	52.4	5.0	9.3	6.2	11.7
Sample 14	9	9	18.8	1.6	8.0	2.4	12.8
						Medians	
		90		1.6	7.3	2.5	12.8



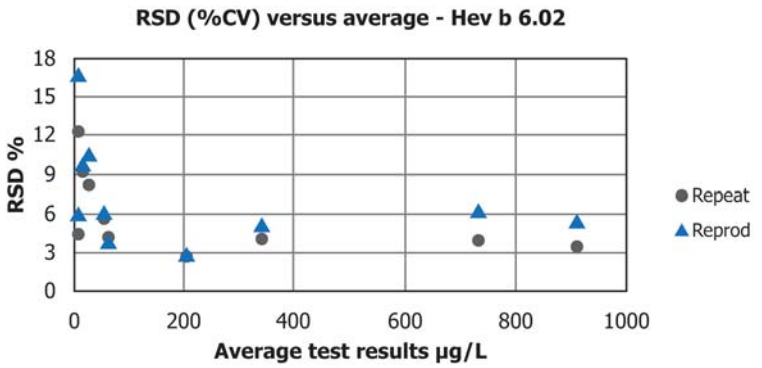
NOTE 1—Fig. 3 presents the relative standard deviation of the dose as a function of the average Hev b 5 dose obtained for 2 participating laboratories in the inter-laboratory study (ILS). Note that the relative standard deviation or RSD is also known as the coefficient of variation which is the standard deviation divided by the mean.

FIG. 3 Intra-Laboratory Repeatability and Inter-Assay Reproducibility as a Measure of the Hev b 5 IEMA Precision

TABLE 6 Immunological Measurement of Allergenic Hev b 6.02 Protein in Hevea Natural Rubber by IEMA

NOTE 1—This table contains the statistics for the Hev b 6.02 Immunoenzymetric assay (IEMA) for each specimen extract: the number of data, the average test result, the standard deviations (SD) and Relative SD or RSD (as %) for repeatability and reproducibility. The RSD is synonymous with coefficient of variation (SD/mean × 100). The standard deviations appeared to increase with the averages but the CVs (RSDs) remain fairly constant over the working range of the assay. Fig. 4 presents a summary of these data in pictorial format as the RSD versus the average Hev b 6.02 data for 2 participating laboratories.

				Repeatability		Reproducibility	
Specimen Number	n	df	Hev b 6.02 Average	Std Dev	RSD	Std Dev	RSD
KitControl	9	9	24.9	2.0	8.2	2.6	10.6
Sample 1	9	9	7.0	0.3	4.5	0.4	6.0
Sample 2	9	9	14.7	1.4	9.3	1.4	9.8
Sample 4	9	9	339.9	13.6	4.1	17.8	5.2
Sample 5	9	9	53.9	3.1	5.7	3.3	6.1
Sample 6	9	9	5.7	0.7	12.4	1.0	16.8
Sample 7	9	9	62.3	2.6	4.2	2.4	3.8
Sample 8	9	9	734.0	28.4	4.0	45.9	6.2
Sample 9	9	9	912.0	32.0	3.6	49.5	5.4
Sample 10	9	9	205.9	5.8	2.8	5.8	2.8
						Medians	
		90		9.0	5.9	13.0	7.3



NOTE 1—Fig. 4 presents the relative standard deviation of the dose as a function of the average Hev b 6.02 dose obtained for 2 participating laboratories in the inter-laboratory study (ILS). Note that the relative standard deviation or RSD is also known as the coefficient of variation which is the standard deviation divided by the mean.

FIG. 4 Intra-Laboratory Repeatability and Inter-Assay Reproducibility as a Measure of the Hev b 6.02 IEMA Precision

TABLE 7 Observed Hev b IEMA Quality Control Response (mean ± 2 SD and % coeffient of variation[A] [CV])

Control Specimen	Hev b 1 ng/mL	Hev b 3 ng/mL	Hev b 5 ng/mL	Hev b 6.02 ng/mL
Kit Control 95 Percentile Range	66–98	97–294	33–42	44–53
Kit Control Measured	82.6 ± 3.7 0.4 % CV	291.6 ± 21.0 7.2 % CV	6.6 ± 1.3 3.5 % CV	52.4 ± 3.2 6.1 % CV
Low Control 95 Percentile Range	11–22	39–60	13–17	13–18
Low Control Measured	22.6 ± 2.9 12.8 % CV	58.7 ± 5.2 8.8 % CV	15.4 ± 0.8 5.5 % CV	16.7 ± 2.4 14.2 % CV
Medium Control 95 Percentile Range	40–71	128–194	21–29	49–56
Medium Control Measured	63.8 ± 9.9 15.5 % CV	188.3 ± 13.5 7.2 % CV	24.1 ± 1.3 5.4 % CV	54.7 ± 3.2 5.8 % CV
High Control 95 Percentile Range	184–270	391–576	51–81	79–97
High control Measured	256.4 ± 32.7 2.7 % CV	496.1 ± 69.0 13.9 % CV	63.1 ± 7.5 11.8 % CV	97.4 ± 4.5 4.7 % CV

[A] The coefficient of variation or CV equals the RSD or relative standard deviation which equals the standard deviation/mean × 100.

TABLE 8 Inter-dilutional Mean, Standard Deviation and % Coefficients of Variation Data obtained in the Parallelism/Linearity Study of the Hev b 1, 3, 5 and 6.02 IEMAs Target (≤25 % ID CV)

Specimen	Allergen	Inter-Dilutional Mean	Inter-Dilutional SD	Inter-Dilutional CV	Number of Dilutions
sample 1	Hev b 1	172.0	9.6	5.6 %	4
sample 2	Hev b 1	219.8	14.0	6.4 %	4
sample 3	Hev b 1	252.6	16.6	6.6 %	4
sample 4	Hev b 1	146.2	3.3	2.3 %	4
sample 5	Hev b 3	313.1	5.9	1.9 %	4
sample 6	Hev b 3	232.7	4.3	1.8 %	4
sample 7	Hev b 3	617.6	16.3	2.6 %	4
sample 8	Hev b 3	83.5	7.5	9.0 %	4
sample 9	Hev b 5	56.7	8.8	5.6 %	3
sample 10	Hev b 5	47.4	3.4	7.3 %	3
sample 11	Hev b 5	27.5	2.0	7.2 %	3
sample 12	Hev b 5	57.0	1.7	3.0 %	4
sample 13	Hev b 6.02	156.3	30.1	19.2 %	4
sample 14	Hev b 6.02	78.5	41.4	23.2 %	4
sample 15	Hev b 6.02	54.1	8.4	15.6 %	4
sample 16	Hev b 6.02	9.2	10.7	10.8 %	4

APPENDIX

(Nonmandatory Information)

X1. Preparation of Recombinant and Native Hev b Proteins Used as Reference Materials and in Preparation of the Monoclonal Anti-Hev b 1, Hev b 3, Hev b 5 and Hev b 6.02 Capture and Detection Reagents

Industry Reference Materials

IRM 915 Mouse monoclonal antibody Hb1-5 (Clone 07-005) Industry Reference Material (IRM)
IRM 916 Mouse monoclonal antibody Hb1-1 (Clone 07-001) Industry Reference Material (IRM)
IRM 917 Hev b 1 Reference Protein Industry Reference Material (IRM)
IRM 918 Mouse monoclonal antibody Hb3-5 (Clone 09-005) Industry Reference Material (IRM)
IRM 919 Mouse monoclonal antibody Hb3-10 (Clone 09-010) Industry Reference Material (IRM)
IRM 920 Hev b 3 Reference Protein Industry Reference Material (IRM)
IRM 921 Mouse monoclonal antibody Hb5-4 (Clone 10-004) Industry Reference Material (IRM)
IRM 922 Mouse monoclonal antibody Hb5-11 (Clone 10-011) Industry Reference Material (IRM)
IRM 923 Hev b 5 Reference Protein Industry Reference Material (IRM)

Industry Reference Materials
IRM 924 Mouse monoclonal antibody Hb6.02-2 (Clone 11-002) Industry Reference Material (IRM)
IRM 925 Mouse monoclonal antibody Hb6.02-N3 (Clone 11-003) Industry Reference
IRM 926 Hev b 6.02 Reference Protein Industry Reference Material (IRM)

X1.1 Purified recombinant fusion proteins rMBP-Hevb1, rMBP-Hevb3, rMBP-Hevb5 and r-avidin-Hevb6.02 were used as antigens (Ag) for inducing the immune response and for boosting female Balb/c mice. Mice were injected with Ag in Complete Freund's Adjuvant (CFA) for initial immunization and with Ag in Incomplete Freund's Adjuvant (IFA) for the booster immunizations. Blood samples were collected and screened for specific antibody reactivity using an indirect ELISA with the homologous antigen insolublized on the microtiter plate. Animals with an antibody a titer of 1:5000 to 1:10 000 or greater were chosen for hybridization involving the mouse's spleen cells and a stock myeloma cell line. The spleen cells were derived from the spleen and fused with the Sp2/O-Ag-14 (abbr.Sp2) cells. The fused cells were suspended in RPMI medium supplemented with the selection agent HAT. Feeder cells were added to the suspension. Seven days later, RPMI with the growth supplement HT was added. Antibody positive clones were cross-checked for their reactivity with the recombinant fusion partner protein (MBP or avidin respectively), the same recombinant fusion protein used for immunization, the recombinant Hev b protein without fusion partner, Field Latex (FL) and glove extract antigens. Only clones that simultaneously reacted with the appropriate recombinant Hev b protein, recombinant fusion protein, FL and glove extracts were selected for further characterization. The isotypes (immunoglobulin classes) and subclasses of the selected murine monoclonal antibodies (Mabs) were defined using an ELISA. Mabs were further produced in large scale in ascites and purified by affinity chromatography with Protein A/G. Hybridomas were also cultured in semi-permeable membrane-based system ICL (*in vitro* production) and adapted to grow in RPMI medium with low (1 to 3 %) Fetal Bovine Serum (FBS) medium or FBS free medium. After the incubation phase, the entire cell suspension was removed. The antibodies were finally affinity purified from the cell culture supernatant using a Protein G adsorbent. Restrictions on quantity per order may apply when purchasing the current IRM-s from an ASTM approved institution.

X1.2 *Preparation of the Recombinant Hev b 1 Protein*—A Hev b 1 coding fragment was initially amplified by PCR and subcloned into the pMAL-c2x-plasmid. The sequence of the recombinant plasmid was confirmed by sequencing. This recombinant plasmid was introduced into the *E. coli* JM109 host cells. After cultivation, cells were lysed and the cell lysate was clarified. The supernatant was filtered through a 0,2 µm membrane filter. Fusion protein purification was based on amylose affinity chromatography. The purity and concentration of MBP-Hevb1 fusion protein was determined by reverse phase chromatography on a TSKgel TMS-250. Cleavage of the recombinant MBP-tag from Hevb1 protein was performed by Factor Xa protease. Separation of cleaved rHevb1 was performed by gel filtration in a Superdex 75HR column. The concentration of the rHevb1 was determined by reversed phase chromatography on a TSKgel TMS-250. The protein concentration was determined by peak integration and comparison of the peak integral to the peak integrals of bovine serum albumin and beta lactoglobulin. N-terminal sequence analysis of purified rHevb1 protein followed. The rHevb1 preparation was diluted (1:10) to the storage buffer and freeze-dried (lyophilized) into small vials. The lyophilized vials were cross-calibrated against unlyophilized ones. Unlyophilized proteins were kept at –20°C until thawed for calibration. As the process for preparation of the recombinant Hevb1 protein is costly and time-consuming, it is recommended that end-users do not use the current IRM 917 in end-user applications. Users of this material are recommended to prepare a "working" antigen reference material that has been validated against IRM 917 material instead. The validation process and parameters are described in 11.2. Restrictions on quantity per order may apply when purchasing the current IRM-s from an ASTM approved institution.

X1.3 *Preparation of the Recombinant Hev b 3 Protein*—The Hev b 3 coding fragment was amplified by PCR and subcloned into a pMAL-c2x-plasmid. The sequence of the purified recombinant plasmid was confirmed by sequencing. This recombinant plasmid was then introduced into the *E. coli* JM109 host cells. After cultivation, cells were lysed and the cell lysate was clarified. The supernatant was sterile filtered through a 0,2 µm membrane. Fusion protein purification was performed using amylose affinity chromatography. The purity and concentration of MBP-Hevb3 was determined by reversed phase chromatography on a TSKgel TMS-250. Cleavage of the recombinant MBP-tag from rHevb3 protein was performed by Factor Xa protease. Separation of cleaved rHevb3 was performed by gel filtration in a Superdex 75HR column. Concentration of rHevb3 was determined by reversed phase chromatography on a TSKgel TMS-250. The protein concentration was determined by peak integration and comparison of the peak integral to the peak integrals of bovine serum albumin and beta lactoglobulin. N-terminal sequence analysis of purified rHevb3 protein followed. The purified protein molecular mass was determined by MALDI-TOF mass spectrometry. The rHev b 3 preparation was diluted with storage buffer and freeze-dried (lyophilized) into small vials. Lyophilized vials were calibrated against unlyophilized vials. Unlyophilized proteins were maintained at –20°C until thawed for calibration. As the process of preparing the recombinant Hev b 3 protein is costly and time-consuming, it is recommended that end-users do not use this current IRM 920 strategy to prepare reference materials. Users are recommended to prepare "working" antigen reference materials that are validated against IRM 920 material instead. The validation process and parameters are described in 11.2. Restrictions on quantity per order may apply when purchasing the current IRM material from an ASTM approved institution.

X1.4 *Preparation of the Hev b 5 Protein*—Hev b 5 antigen was purified from Hevea Natural Rubber Latex C-serum by gel filtration on Bio-Gel P6DG column with buffer exchange at the same time. Cation exchange chromatography on MonoS HR column with a linear gradient of NaCl was then performed. Reversed phase (RP) chromatography on a TSKgel TMS-250 column followed. The purified Hev b 5 protein fraction was collected and the protein content determined using analytical reversed phase chromatography on a TSKgel TMS-250 column. The protein concentration was identified by peak integration and comparison of the peak integral to the peak integrals of bovine serum albumin and beta lactoglobulin that were used as external standards. The purified protein molecular mass was determined by MALDI-TOF mass spectrometry. Collected material was concentrated, diluted with storage buffer and freeze-dried (lyophilized) into small vials. Lyophilized vials were calibrated against unlyophilized vials. Unlyophilized proteins were kept at –20°C until being thawed for use in calibration. As the process for preparation of the Hev b 5 protein is costly and time-consuming, it is recommended that end-users do not use the current IRM strategy to prepare Hev b 5 for assay calibration. Users are recommended to prepare a "working" antigen reference material that has been validated against IRM 923 material instead. The validation process is described in 11.2. Restrictions on quantity per order may apply when purchasing the current IRM material from an ASTM approved institution.

X1.5 *Preparation of the Hev b 6.02 Protein*—Hev b 6.02 antigen was purified from Hevea Natural Rubber Latex by attachment of chitin-binding proteins to chitin. Hev b 6.02 protein was eluted from chitin matrix with 0.6M acetic acid. The collected eluates were applied to a reversed phase chromatography column. The collected Hev b 6.02 containing fractions were concentrated, diluted to storage buffer and freeze-dried into small vials. The purity and concentration of the purified Hev b 6.02 was analyzed by reversed phase chromatography on a C1 column (TSKgel TMS-250). The N-terminal sequence was determined by an Edman degradation with an Procise 494A Sequencer and using the standard protocol from the manufacturer. The molecular mass was determined by MALDI-TOF mass spectrometry. Lyophilized vials were calibrated against unlyophilized vials. Unlyophilized proteins were kept at –20°C until being thawed for use in calibration. As the process for preparation of the Hev b 6.02 protein is costly and time-consuming, it is recommended that end-users do not use the current IRM strategy to prepare Hev b 6.02 for assay calibration. Users are recommended to prepare a "working" antigen reference material that has been validated against this IRM 926 material instead. The validation process is described in 11.2. Restrictions on quantity per order may apply when purchasing the current IRM material from an ASTM approved institution.

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