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FITkit®

FITkit® Hev b 6.02

Instructions for use of FITkit® Hev b 6.02 in quantitative determination of Hev b 6.02 (hevein) in natural rubber latex products

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FITkit®

Contents

1. Introduction 4
2. Principle of method 5
3. Contents of kit 5
4. Storage conditions 6
5. Materials and equipment required but not supplied 7
6. Precautions and notes 7
7. Preparation of samples 8
8. Procedure of test 9
   8.1 Preparation of reagents and equipment 9
   8.2 Test procedure 9
   8.3 Summary - test procedure 10
   8.4 Calculation of results 12
9. Expected values 14
10. Performance characteristics 15
    10.1 Detection limit 15
    10.2 Precision 15
    10.3 Recovery 16
    10.4 Linearity (dilution test) 17
    10.5 Specificity 17
11. Additional information 19
12. Literature 19

1. Introduction

Products containing natural rubber latex (NRL) from the rubber tree *Hevea brasiliensis* are widely used due to the economical price and advantageous processing properties of natural rubber, although adverse reactions against a number of allergenic proteins contained in the NRL are well known and documented. The NRL-containing products used by, e.g., healthcare personnel, such as surgical gloves and various other devices (like catheters, tubes, masks, etc.) contribute to the major portion of these adverse reactions. In healthcare, the NRL-based medical devices exhibit a potential danger not only to the personnel but also to the patients undergoing an examination or a surgery. Additionally, even the general population comes into daily contact with diverse NRL-containing products, such as household gloves, condoms and balloons, manufactured by the dipping procedure, and also with tubes, tires, erasers and the like.

Currently, latex allergy is recognized as a serious world-wide health problem: up to 15 % of health care workers and approximately 1 % of the entire population are allergic to NRL. The clinical manifestations of latex allergy range from mild contact urticaria to fatal anaphylaxis and the seriousness of the condition is accentuated by the fact that the first sign of sensitization can manifest as a life-threatening reaction.

Latex allergens are proteins or polypeptides eluting from the manufactured products upon contact with skin, mucous membranes or other tissues. According to the current allergen nomenclature system maintained by the International Union of Immunological Societies (IUIS) under the WHO, thirteen latex allergens, which have been characterized at the primary structure level and are contained in the official allergen list, are named as Hev b 1, Hev b 2, Hev b 3, Hev b 4, Hev b 5, Hev b 6 (6.01, 6.02, 6.03), Hev b 7, Hev b 8, Hev b 9, Hev b 10, Hev b 11, Hev b 12 and Hev b 13. Not all previously listed allergens are able to come through the production process. At present, four of these allergens (Hev b 1, Hev b 3, Hev b 5 and Hev b 6.02) have been mostly detected in manufactured latex products. According to the current literature sum quantity of these four allergens is highly dominant in determining the allergenic potential of NRL products.

FITkit® Hev 6.02 test is the first commercial quantitative test to measure Hev b 6.02 immunologically in NRL products. By the use of specific monoclonal antibodies, sensitivity and specificity stay high even in the presence of other proteins or chemical substances derived from the manufacturing process of NRL products.

FITkit® Hev 6.02 test is produced in accordance with ASTM International standard D7427-08 (www.astm.org/Standards/D7427.htm)

2. Principle of method

FITkit® Hev b 6.02 test is based on the immunoenzymetric assay technique. Microtiter wells are coated with Hev b 6.02-specific monoclonal capture antibody that during the first incubation binds Hev b 6.02 molecules from the sample extract. After incubation, unbound material is removed by washing the wells. During the second incubation, horseradish peroxidase (HRP) labelled Hev b 6.02-specific monoclonal detection antibody binds to a different epitope of Hev b 6.02 molecules. As such, the two antibodies are a matched pair that forms a “sandwich” around the target Hev b 6.02 allergen. After washing, HRP substrate is added and the intensity of the colour produced is directly proportional to the Hev b 6.02 concentration of the sample.

3. Contents of kit

The kit contains the reagents listed below, sufficient for 96 wells.

3.1 FITkit® Hev b 6.02 Microwell Plate
Cat F3-300-001
96 wells coated with mouse monoclonal Hev b 6.02 antibody, packed in a aluminium vacuum bag. The microwell plate is ready for use.

3.2 FITkit® Hev b 6.02 Assay Buffer, 15 mL
Cat 300-041
The Assay Buffer contains phosphate, sodium chloride, EDTA, bovine plasma albumin (BPLA), mouse antibodies, detergent and preservative Proclin 300®. Ready for use. Coloured pink.

3.3 FITkit® Hev b 6.02 Calibrators
Cat F3-300-031…036
Each vial contains 0.5 mL Hev b 6.02 calibrator in a stabilized buffer. The calibration is based on the analysis of Hev b 6.02 in reversed phase chromatography and N-terminal sequencing. The calibrator values are 0, 5, 15, 50, 100 and 200 µg/L. Ready for use.

3.4 FITkit® Hev b 6.02 Control
Cat F3-300-081
The control is made from field latex in a stabilized solution containing BPLA, detergents and preservatives. Reconstitute the lyophilized control with 500 µL of distilled water.

3.5 FITkit® Hev b 6.02 Enzyme Conjugate, 15 mL
Cat F3-300-016
Mouse monoclonal Hev b 6.02 antibody conjugated to horse radish peroxidase (HRP) in a buffered solution containing stabilizers, BPLA, detergent and preservative Proclin 300®. Ready for use.

3.6 FITkit® PBS Wash Concentrate, 50 mL
Cat F3-300-042
Before use dilute to 500 mL (1:10) with distilled water. Occasionally crystals may be present at +2…+8 °C, but they dissolve upon dilution and at room temperature.

3.7 FITkit® HRP Substrate Solution, 15 mL
Cat F3-300-043
ABTS (2.2´-azino-di[3-ethyl-benzthiazoline-6-sulphonate]) Peroxidase Substrate. Ready for use. Colourless to light green.

3.8 FITkit® Stopping Solution, 15 mL
Cat F3-300-044
1 % Sodium dodecyl sulphate (SDS). SDS precipitates at low temperatures, but redissolves upon warming to room temperature. Ready for use.

4. Storage conditions

The kit should be stored at +2…+8 °C.

The unopened kit is stable until the expiry date, printed on the kit label. The expiry date of each unopened component is printed on the label of the individual component.

Once opened, the microwell plate and liquid components are stable for eight weeks at +2…+8 °C.

After reconstitution, the control should be used during the same working day.

After dilution of the PBS Wash Concentrate, the washing solution is stable for eight weeks at room temperature (RT).

5. Materials and equipment required but not supplied

- Pipette with disposable plastic tips: 25 µL (for calibrators and samples), 500 µL (for the reconstitution of the control).
- Multichannel pipette with disposable plastic tips: 100 µL (assay buffer, enzyme conjugate, substrate, stopping solution).
- PBS (phosphate buffered saline) for sample preparation.
- Lid or sealing tape for microwell plate.
- Reagent troughs.
- Microwell plate shaker.
- Aspiration device or microwell washer.
- Photometer (microwell plate or strip reader), 414 nm or 405 nm.

6. Precautions and notes

- Wear only synthetic disposable gloves during the assay. Do not use any gloves or other materials containing natural rubber latex.
- Always use the polypropylene low protein binding vessels and tubes in every step. Do not use glass tubes because of the possible loss of proteins by adsorption to the inner surface of the tube walls.
- Protect the microwell plate from draught, strong light or direct sunlight during the test procedure.
- Each microwell plate should include a standard curve on the same plate and at the same time as control and samples.
- Careful aspiration of the washing solution is essential for good assay precision. It is recommended that the washing procedure mode is checked to get the best precision.
- Timing of the incubation steps is important to the performance of the assay. Pipetting of calibrators, control and samples should be done without interruption. Pipetting of the calibrators and samples should not exceed 10 minutes to avoid assay drift.
- Adding of substrate starts a kinetic reaction that is terminated by dispensing the stopping solution. Keep the incubation times for each well the same by adding the reagents at timed intervals.
- Absorbance values are stable for 60 minutes if protected from light.
- Microwell plate readers measure absorbance vertically. Do not touch the bottoms of the wells.
- A wavelength of 405 nm can be used if 414 nm is not available. Absorbances are slightly lower at 405 nm than at 414 nm.

7. Preparation of samples

7.1 **A.** For solid-state material:
Weigh the entire natural rubber product and record the total weight per product so that the allergen content can be later reported as micrograms of allergen per gram of product weight. Clearly non-NRL parts may be removed*.

** If non-NRL parts were removed from the product, weigh, calculate and report only the analysed NRL part of the product. For example, if the product is a baby pacifier with a soft nipple and stiff plastic base, remove the plastic base, weigh, extract and analyse only pacifier nipple pieces without the plastic base. Report the result in μg/g with the additional claim that the result value is per partial product (pacifier nipple) not per entire product.*

B. For solutions- Proceed to Step 7.6.

7.2 Cut the product into pieces using clean and completely dry scissors.

7.3 Place the pieces of test specimen into an extraction vessel. Extract in PBS (phosphate buffered saline) using volume to weight ratio 5:1 (5mL PBS per 1g of NRL product). All product surfaces should be evenly exposed to PBS.

7.4 Extract the specimen pieces at room temperature (21-23°C) for 2 hours on an end-over-end or an orbital shaker. The extraction vessel should be continuously rotated to ensure even exposure to the PBS. Avoid foaming.

7.5 Remove the test specimen pieces from the extraction buffer.

7.6 Centrifuge the supernatant 2000 g for 15 min at RT.

7.7 Collect the supernatant liquid and assay immediately or store at +4°C up to 4 hours. If Hev b 6.02 determination is performed later, the samples should be kept frozen (-20°C). Avoid repeated freeze thaw cycles.

8. Procedure of test

8.1 Preparation of reagents and equipment

Allow all reagents to reach room temperature (21-23°C) before use.
Reconstitute the control (Step p 8.3.2) and let it dissolve for 60 min, mix gently (or vortex for 1-2 seconds).
Dilute the PBS Wash Concentrate to make washing solution (Step 8.3.3).
Take the required number of microwell plate strips and place the remaining strips back into the aluminium vacuum bag. Close the bag tightly again.

8.2 Test procedure

8.2.1 Dispense 100 μL of Assay Buffer into each well.

8.2.2 Pipette 25 μL of calibrator, control and sample into appropriate wells in duplicate.

8.2.3 Cover the microwell plate. Incubate the microwell plate for 60 minutes at room temperature on a microwell plate shaker (100-200 rpm).

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

8.2.5 Dispense 100 μL of Enzyme Conjugate into the wells in duplicate.

8.2.6 Cover the microwell plate. Incubate the microwell plate for 30 minutes at room temperature on a microwell plate shaker (100-200 rpm).

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

8.2.8 Add 100 μL of HRP Substrate Solution at fixed time points into each well.

8.2.9 Cover the microwell plate. Incubate the microwell plate for 15 minutes at room temperature on a microwell plate shaker (100-200 rpm).

8.2.10 Stop the reaction by adding 100 μL of Stopping Solution into each well at the same fixed time points as in Step 8.2.8 so that exactly the same substrate reaction time is achieved. Shake the microwell plate for 1-2 minutes to mix the solutions.

8.2.11 Measure the absorbance at 414 nm using a microwell plate or strip reader, preferably immediately but no more than 60 minutes after stopping the reaction. If the microwell plate is not read immediately, protect the microwell plate from light.

8.3 Summary - test procedure

Preparative steps

1.

Bring all reagents to room temperature.

2.

Reconstitute Control with 500 µL of distilled water. Let it stand for 60 min. Mark the wells to be used.

3.

Dilute 50 mL Wash Concentrate with 450 mL of distilled water.

Test procedure

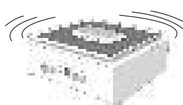
4.

Dispense 100 µL Assay Buffer

5.

Add 25 µL calibrators, controls and product extracts into appropriate wells.

6.



Incubate for 60 min with shaking at RT.

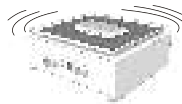
7.

Wash 4 times.

8.

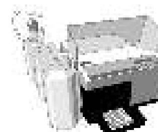
Dispense 100 µL Enzyme Conjugate.

9.



Incubate for 30 min with shaking at RT.

10.

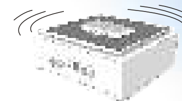


Wash 4 times.

11.

Dispense 100 µL Substrate Solution.

12.



Incubate for 15 min with shaking at RT.

13.

Dispense 100 µL Stopping Solution.

14.

Shake for 1-2 minutes and measure at 414 nm (or 405 nm). Fit the curve and read off results.

8.4 Calculation of results

Calculate the mean absorbance for each duplicate. Subtract blank values (Calibrator A) from the mean absorbances.

Plot the absorbances against the respective Hev b 6.02 concentrations on a log-log scale. Software that fits the standard curve can be used to calculate results of samples. Cubic spline fit type is recommended.

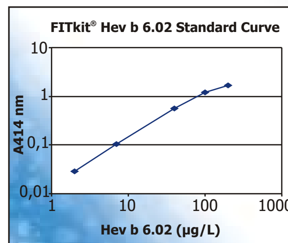
Read off the concentrations of the controls and samples (µg/L). Samples showing lower concentrations of Hev b 6.02 compared to Calibrator B (5 µg/L) are considered as undetectable. Samples showing higher concentrations of Hev b 6.02 compared to Calibrator F (200 µg/L) must be diluted further to obtain result. If samples have been diluted, multiply the result with the dilution factor.

Report Hev b 6.02 protein content in microgram of Hev b 6.02 per gram (µg/g) of product. To convert Hev b 6.02 concentration from µg/L to µg/g, use following equation:

$$C1\ (\mu g/g) = \frac{C2\ (\mu g/L) * V1\ (L)}{W1\ (g)}$$

C1 - Hevb 6.02 content in (µg/g)
C2 - Hevb 6.02 concentration (µg/L)
V1 - Volume of PBS used for sample extraction (L)
W1 - Weight of the product (g) determined in Step 7.1.A

Worksheet and standard curve of typical assay (Not to be used for calculation of actual test results)



FITkit® Hev b 6.02 test

Wells	Identity	Conc. µg/L	A414 nm	$A_{414\,nm}$-blank	Conc. µg/L
A1-A2	Calibr. A	0	0,062		
B1-B2	Calibr. B	5	0,117	0,055	
C1-C2	Calibr. C	15	0,290	0,228	
D1-D2	Calibr. D	50	1,033	0,971	
E1-E2	Calibr. E	100	1,974	1,912	
F1-F2	Calibr. F	200	2,994	2,932	
G1-G2	Sample 1	unknown	0,263	0,201	13,5
H1-H2	Sample 2	unknown	0,665	0,604	33
A3-A4	Sample 3	unknown	0,882	0,820	43

Quality control

Each kit contains FITkit® Hev b 6.02 Control, which should give results within the specified range given in a separate certificate of analysis enclosed in the kit.

9. Expected values

Results obtained from 13 different glove brands extracted to PBS (1 g glove/5 mL PBS) in 2 hours at room temperature are given below in Table 1.

Table 1. Hev b 6.02 content of different glove brands extracted in PBS

Extract	Type	Glove Material	Hev b 6.02 µg/L
1	Examination	Latex (powdered)	474
2	Examination	Latex (powder free)	27
3	Examination	Latex (powder free)	<5
4	Examination	Latex (powder free)	7700
5	Examination	Latex (powdered)	5629
6	Surgical	Latex (powder free)	<5
7	Surgical	Latex (powder free)	<5
8	Surgical	Synthetic (powdered)	<5
9	Examination	Nitrile (protein free)	<5
10	Examination	Vinyl	<5
11	Surgical	Latex (powdered)	185
12	Surgical	Latex (powdered)	16415
13	Surgical	Latex (powder free)	<5

10. Performance characteristics

10.1 Detection limit

Detection limit of Hev b 6.02 test was defined by the minimum Hev b 6.02 concentration deviating by 2 SD from that of the Calibrator A. The test was performed by using 16 replicate determinations of Calibrator A and Calibrator B. On the basis of this test the detection limit of Hev b 6.02 assay is 0.1 µg/L.

10.2 Precision

Repeatability (intra-assay variation) and reproducibility (inter-assay variation) were determined by analysing three samples containing a low, medium and high concentration of Hev b 6.02. The results are given in Table 2 and 3.

Table 2. Repeatability

Sample	Number of replicates	Mean (µg/L)	SD (µg/L)	CV%
1	16	13	0.6	4.6
2	16	33	1.3	4.1
3	16	43	2.5	5.8

Table 3. Reproducibility

Sample	Number of assays	Mean (µg/L)	SD (µg/L)	CV%
1	5	2.5	1.14	5.6
2	5	33	1.4	4.3
3	5	44	3.0	6.9

10.3 Recovery

5, 50 and 200 µg/L concentration of purified Hev b 6.02 calibrator was added to equal volume of three samples containing a low (2.6 µg/L), medium (14 µg/L) and high concentration (46 µg/L) of Hev b 6.02. Determination of Hev b 6.02 was done using unspiked samples and samples spiked with Hev b 6.02 calibrators. The theoretical concentration and the recovered concentrations were calculated. The results are shown in Table 4.

Table 4. Recovery

Sample	Added conc. (µg/L)	Expected conc. (µg/L)	Obtained conc. (µg/L)	Recovery %
Low	0		2.6	100
	5	3.8	4.4	116
	50	26	29	110
	200	101	110	109
Medium	0		14	100
	5	9	9	99
	50	32	33	104
	200	107	105	98
High	0		46	100
	5	25	24	94
	50	48	46	96
	200	123	130	106

10.4 Linearity (dilution test)

Two samples containing 13.5 µg/L and 43 µg/L of Hev b 6.02 were diluted with PBS 1:2, 1:5 and 1:10, if appropriate. The concentration of each diluted and original sample was measured. The results given as the percentage of the original concentration corrected with the dilution factors are given in Table 5.

Table 5. Linearity

Sample	Dilution	Conc. (µg/L)	%
1	undiluted	13.5	100
	1:2	12.5	93
	1:5	12.5	93
2	undiluted	43	100
	1:2	48	110
	1:5	40	92
	1:10	44	102

10.5 Specificity

Cross-reactions

Cross-reactions with Hev b 1, Hev b 3, Hev b 5 and Hev b 7 were tested using concentrations on a weight-to-weight basis. No cross reactions were detected, thus the cross reactivity is less than 0.02 %, 0.02 %, 0.02 % and 0.002 %, respectively. The results are given in Table 6.

Table 6. Cross-reactions

Substance	Concentration tested	Cross-reaction (w/w)
Hev b 1	10 000 µg/L	< 0.02 %
Hev b 3	10 000 µg/L	< 0.02 %
Hev b 5	10 000 µg/L	< 0.02 %
Hev b 7	86 000 µg/L	< 0.002 %

Interfering substances

Several accelerators, antioxidants and detergents used in the rubber industry were tested using the interfering substance in PBS and in the solution containing Hev b 6.02. The test was performed in the presence and absence of each substance.

Substances tested: 0.1 %, 0.02 % and 0.005 % (w/v), solutions of pure ZDEC, ZDBC, SDBC, AS100, Arbestab Z, ZMBT, P25, BKF, Ralox LC, MB2, Setsit 104 and 0.1 %, 0.02 % and 0.005 % (v/v) solutions of ZDEC, ZDBC, ZMBT, MBT, TMTD, DPTT, ZnO, Sulphur, TiO_2, Wingstay made from emulsions thereof.

Solutions containing 0.1 %, 0.02 % and 0.005 % of Triton X114, Surfynol TG. Surfynol DF37, Foamaster VL, Sodium Caprylate, Darvan #1, Cellosize, Igepal CA630 and Algene N40 were tested in the same way.

No substance alone gave any background in the assay. No interference was observed when tested as 0.1 % solution in PBS containing Hev b 6.02. The recovered values fell within three standard deviations from the control.

11. Additional information

Current FITkit® Hev b 6.02 is one of four FITkits available. There are specific kits to measure Hev b 1, Hev b 3 and Hev b 5.
Additional information about FITkits and for kit users (troubleshooting, frequently asked questions, microwell plate layout print version, etc.) is available on the web page www.icosagen.com.

12. Literature

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