

PRODUCT INFORMATION & MANUAL

Human Galectin-3 Platinum ELISA

BMS279/4 / BMS279/4TEN

Enzyme-linked Immunosorbent Assay for
quantitative detection of human Galectin-3.

For research use only.

Not for diagnostic or therapeutic procedures.



Human Galectin-3 Platinum ELISA

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1. Intended Use

The human Galectin-3 ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human Galectin-3. **The human Galectin-3 ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

2. Summary

Galectin-3 is a 26kDa β -galactoside-binding protein belonging to the Galectin family, which consists of more than ten members. Galectin-3 is composed of a carboxyl-terminal carbohydrate recognition domain (CRD) and amino-terminal tandem repeats. Galectin-3 normally distributes in epithelia of many organs and various inflammatory cells, including macrophages as well as dendritic cells and Kupfer cells. The expression of this lectin is up-regulated during inflammation, cell proliferation and cell differentiation and through transactivation by viral proteins. Its expression is also affected by neoplastic transformation: upregulation is found in certain types of lymphomas, and thyroid carcinoma, while it is down regulated in other types of malignancies such as colon, breast, ovarian and uterine carcinomas. The expression of Galectin-3 has a strong correlation with the grade and malignant potential of primary brain tumors. Increased Galectin-3 levels have also been noted in human atherosclerotic lesions. These findings suggest that Galectin-3 expression is affected during these physiological and pathological responses. Galectin-3 has been shown to function through both intracellular and extracellular actions. It is a component of heterogeneous nuclear ribonuclear protein (hnRNP), a factor in pre-mRNA splicing and has been found to control cell cycle and prevent T-cell apoptosis through interaction with the Bcl-2 family members. On the other hand, this protein, which is secreted from monocytes/macrophages and epithelial cells has been demonstrated to function as an extracellular molecule in activating various types of cells such as monocytes/macrophages, mast cells, neutrophils and lymphocytes. Galectin-3 has been shown to mediate cell-cell and cell-extracellular matrix interactions and acts as a novel chemoattractant for monocytes and macrophages.

For literature update refer to **www.eBioscience.com**

3. Principles of Test

An anti-Galectin-3 coating antibody is adsorbed onto microwells.

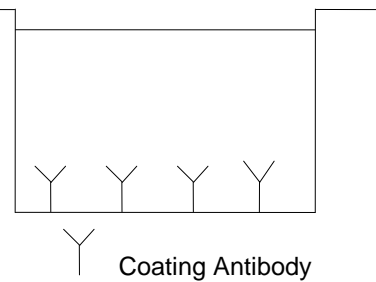
Galectin-3 present in the sample or standard binds to antibodies adsorbed to the microwells.

Following incubation unbound biological components are removed during a wash step and the HRP-conjugated anti-Galectin-3 antibody is added and binds to Galectin-3 captured by the first antibody.

Following incubation unbound HRP-conjugated anti-Galectin-3 antibody is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

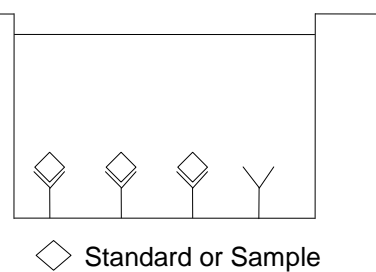
Coated Microwell

Figure 1



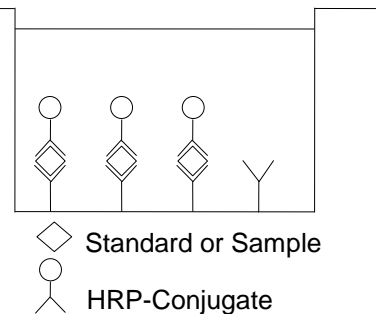
First Incubation

Figure 2



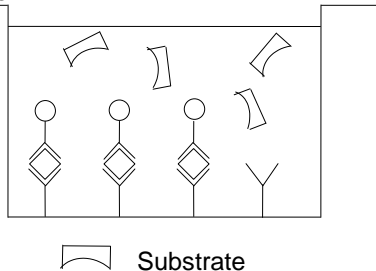
Second Incubation

Figure 3

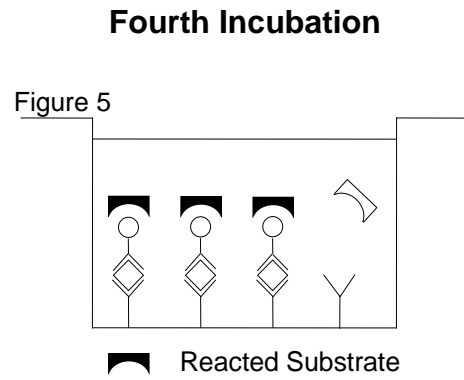


Third Incubation

Figure 4



A coloured product is formed in proportion to the amount of Galectin-3 present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 Galectin-3 standard dilutions and Galectin-3 sample concentration determined.



4. Reagents Provided

4.1 Reagents for human Galectin-3 ELISA BMS279/4 (96 tests)

- 1 aluminium pouch with **Microwell Plate coated** with monoclonal antibody to human Galectin-3
- 1 vial (120 µl) **HRP-Conjugate** anti-human Galectin-3 monoclonal antibody
- 2 vials human Galectin-3 **Standard** lyophilized, 60 ng/ml upon reconstitution
- 1 vial (12 ml) **Sample Diluent**
- 1 vial (5 ml) **Assay Buffer Concentrate 20x**
(PBS with 1% Tween 20 and 10% BSA)
- 1 bottle (50 ml) **Wash Buffer Concentrate 20x**
(PBS with 1% Tween 20)
- 1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)
- 1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)
- 1 vial (0.4 ml) **Blue-Dye**
- 1 vial (0.4 ml) **Green-Dye**
- 4 **Adhesive Films**

4.2 Reagents for human Galectin-3 ELISA BMS279/4TEN

(10x96 tests)

- 10 aluminium pouches with a **Microwell Plate coated** with monoclonal antibody to human Galectin-3
- 10 vial (120 µl) **HRP-Conjugate** anti-human Galectin-3 monoclonal antibody
- 10 vials human Galectin-3 **Standard** lyophilized, 60 ng/ml upon reconstitution
- 7 vials (12 ml) **Sample Diluent**
- 2 vials (5 ml) **Assay Buffer Concentrate 20x**
(PBS with 1% Tween 20 and 10% BSA)
- 3 bottles (50 ml) **Wash Buffer Concentrate 20x**
(PBS with 1% Tween 20)
- 10 vials (15 ml) **Substrate Solution** (tetramethyl-benzidine)
- 10 vials (15 ml) **Stop Solution** (1M Phosphoric acid)
- 6 vials (0.4 ml) **Blue-Dye**
- 6 vials (0.4 ml) **Green-Dye**
- 20 **Adhesive Films**

5. Storage Instructions – ELISA Kit

Store kit reagents between 2° and 8°C. Immediately after use reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels.

Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

6. Specimen Collection and Storage Instructions

Cell culture supernatants, serum and plasma (EDTA, citrate, heparin) are able for use in the assay. Remove the serum or plasma from the clot or red cells, respectively, as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human Galectin-3. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability to 13.5).

Avoid repeated freeze-thaw cycles. Prior to assay, frozen samples should be brought to room temperature slowly and mixed gently.

7. Materials Required But Not Provided

- 5 ml and 10 ml graduated pipettes
- 5 μ l to 1000 μ l adjustable single channel micropipettes with disposable tips
- 50 μ l to 300 μ l adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microplate shaker
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform linear regression analysis

8. Precautions for Use

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statements(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.

- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagents.
- Exposure to acids will inactivate the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

9. Preparation of Reagents

Buffer concentrates should be brought to room temperature and should be diluted before starting the test procedure.

If crystals have formed in the **Buffer Concentrates**, warm them gently until they have completely dissolved.

9.1 Wash Buffer (1x)

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** (20x) into a clean 1000 ml graduated cylinder. Bring final volume to 1000 ml with glass-distilled or deionized water. Mix gently to avoid foaming.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.

Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

9.2 Assay Buffer (1x)

Pour the entire contents (5 ml) of the **Assay Buffer Concentrate** (20x) into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming.

Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.

Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

9.3 HRP-conjugated antibody

Please note that the HRP-conjugated antibody should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **HRP-Conjugate** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	HRP-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

9.4 Human Galectin-3 Standard

Reconstitute **Galectin-3 standard** by addition of distilled water.

Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 60 ng/ml).

Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.

The standard has to be used immediately after reconstitution and cannot be stored.

Standard dilutions can be prepared directly on the microwell plate (see 10.c) or alternatively in tubes (see 9.4.1).

9.4.1 External Standard Dilution

Label 7 tubes, one for each standard point.

S1, S2, S3, S4, S5, S6, S7

Then prepare 2-fold serial dilutions for the standard curve as follows:

Pipette 225 μ l of Sample Diluent into each tube.

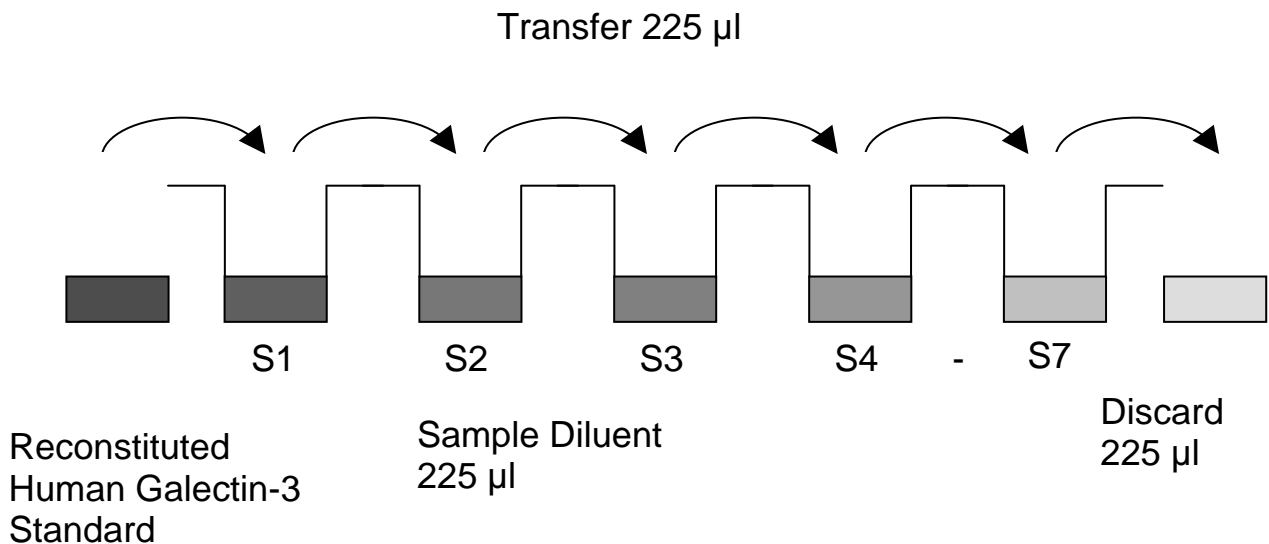
Pipette 225 μ l of reconstituted standard (concentration = 60 ng/ml) into the first tube, labelled S1 and mix (concentration of S1 = 30 ng/ml).

Pipette 225 μ l of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer.

Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 5).

Sample Diluent serves as blank.

Figure 5



9.5 Addition of Colour-giving Reagents: Blue-Dye, Green-Dye

In order to help our customers to avoid any mistakes in pipetting the eBioscience ELISAs, eBioscience offers a tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure.

This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.

Alternatively, the dye solutions from the stocks provided (**Blue-Dye, Green-Dye**) can be added to the reagents according to the following guidelines:

1. Diluent:

Before standard and sample dilution add the **Blue-Dye** at a dilution of 1:250 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of **Blue-Dye**, proceed according to the instruction booklet.

5 ml Sample Diluent	20 µl Blue-Dye
12 ml Sample Diluent	48 µl Blue-Dye
50 ml Sample Diluent	200 µl Blue-Dye

2. HRP-Conjugate:

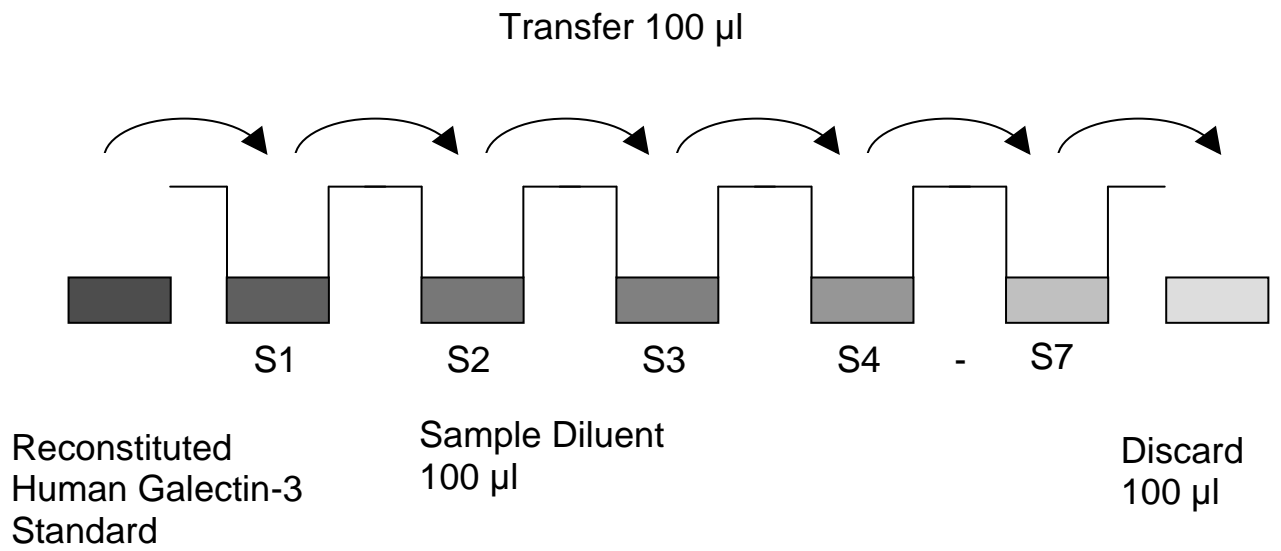
Before dilution of the concentrated HRP-Conjugate add the **Green-Dye** at a dilution of 1:100 (see table below) to the Assay Buffer (1x) used for the final conjugate dilution. Proceed after addition of **Green-Dye** according to the instruction booklet: Preparation of HRP-Conjugate.

3 ml Assay Buffer (1x)	30 µl Green-Dye
6 ml Assay Buffer (1x)	60 µl Green-Dye

10. Test Protocol

- a. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
- b. Wash the microwell strips twice with approximately 400 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells.
After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. **Do not allow wells to dry.**
- c. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes – see 0):
Add 100 µl of Sample Diluent in duplicate to all **standard wells**. Pipette 100 µl of prepared **standard** (see Preparation of Standard 9.4, concentration = 60 ng/ml), in duplicate, into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 30 ng/ml), and transfer 100 µl to wells B1 and B2, respectively (see Figure 6). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of Galectin-3 standard dilutions, ranging from 30 ng/ml to 0.47 ng/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 6



In case of an **external standard dilution** (see 0), pipette 100 µl of these standard dilutions (S1 – S7) in the standard wells according to Table 1.

Table 1

Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (30.00 ng/ml)	Standard 1 (30.00 ng/ml)	Sample 1	Sample 1
B	Standard 2 (15.00 ng/ml)	Standard 2 (15.00 ng/ml)	Sample 2	Sample 2
C	Standard 3 (7.50 ng/ml)	Standard 3 (7.50 ng/ml)	Sample 3	Sample 3
D	Standard 4 (3.75 ng/ml)	Standard 4 (3.75 ng/ml)	Sample 4	Sample 4
E	Standard 5 (1.88 ng/ml)	Standard 5 (1.88 ng/ml)	Sample 5	Sample 5
F	Standard 6 (0.94 ng/ml)	Standard 6 (0.94 ng/ml)	Sample 6	Sample 6
G	Standard 7 (0.47 ng/ml)	Standard 7 (0.47 ng/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

- d. Add 100 µl of **Sample Diluent** in duplicate to the **blank wells**.
- e. Add 50 µl of **Sample Diluent** to the **sample wells**.
- f. Add 50 µl of each sample in duplicate to the **sample wells**.
- g. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 1 hour, on a microplate shaker set at 400 rpm. **Shaking is absolutely necessary for an optimal test performance.**
- h. Prepare **HRP-conjugated antibody** (see Preparation of HRP-conjugated antibody 9.3).
- i. Remove adhesive film and empty wells. **Wash** microwell strips 4 times according to point b. of the test protocol. Proceed immediately to the next step.
- j. Add 100 µl of diluted **HRP-conjugated antibody** to all wells, including the blank wells.
- k. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 1 hour, on a microplate shaker set at 400 rpm. **Shaking is absolutely necessary for an optimal test performance.**
- l. Remove adhesive film and empty wells. **Wash** microwell strips 4 times according to point b of the test protocol. Proceed immediately to the next step.
- m. Pipette 100 µl of **TMB Substrate Solution** to all wells.
- n. Incubate the microwell strips at room temperature (18° to 25°C) for 30 minutes. Avoid direct exposure to intense light.

The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable.

Determination of the ideal time period for colour development has to be done individually for each assay.

It is recommended to add the stop solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

- o. Stop the enzyme reaction by quickly pipetting 100 μ l of **Stop Solution** into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.
- p. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

Note: Shaking is absolutely necessary for an optimal test performance.

11. Calculation of Results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the Galectin-3 concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating Galectin-3 for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding Galectin-3 concentration.
- **If instructions in this protocol have been followed samples have been diluted 1:2, the concentration read from the standard curve must be multiplied by the dilution factor (x 2).**
- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human Galectin-3 levels. Such samples require further external predilution according to expected Galectin-3 values with Sample Diluent in order to precisely quantitate the actual Galectin-3 level.**
- It is suggested that each testing facility establishes a control sample of known Galectin-3 concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 7. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 7

Representative standard curve for human Galectin-3 ELISA. Human Galectin-3 was diluted in serial 2-fold steps in Sample Diluent. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

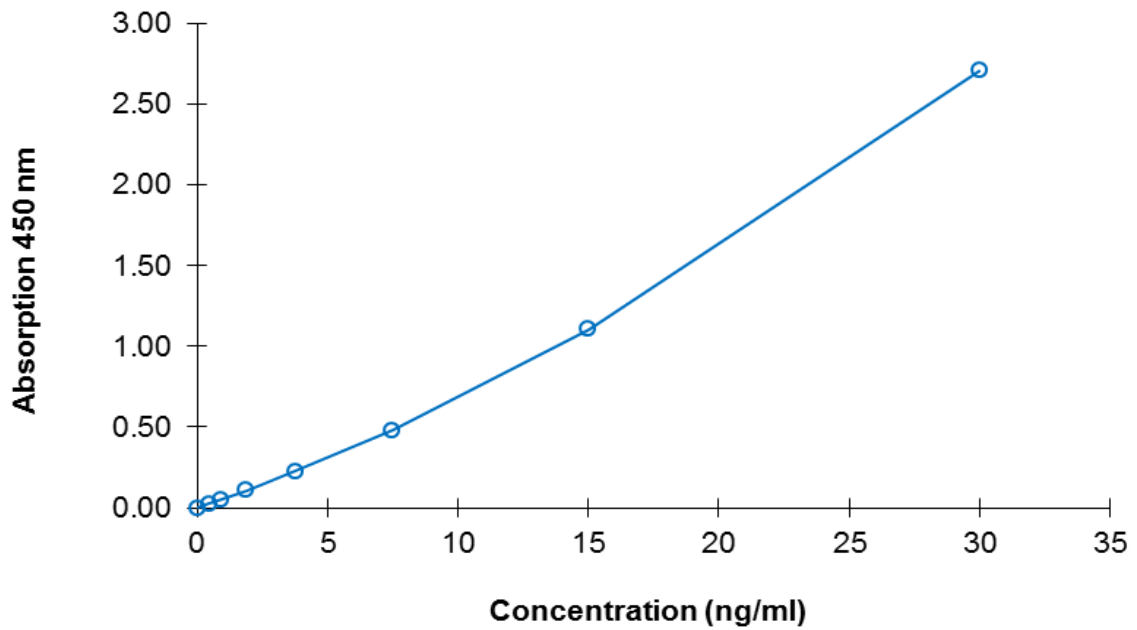


Table 2

Typical data using the human Galectin-3 ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Human Galectin-3 Concentration (ng/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	30.00	2.751 2.748	2.749	0.1
2	15.00	1.151 1.146	1.148	0.3
3	7.50	0.520 0.520	0.520	0.1
4	3.75	0.273 0.268	0.270	1.4
5	1.88	0.150 0.149	0.149	0.6
6	0.94	0.094 0.089	0.092	3.6
7	0.47	0.067 0.064	0.065	4.0
Blank	0.0	0.041 0.043	0.042	2.9

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

12. Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergent before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

13. Performance Characteristics

13.1 Sensitivity

The limit of detection for Galectin-3 defined as the analyte concentration resulting in an absorption significantly higher than the absorption of the dilution medium (mean plus two standard deviations) was determined to be 0.29 ng/ml (mean of 6 independent assays).

13.2 Reproducibility

13.2.1 Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 samples containing different concentrations of Galectin-3. 2 standard curves were run on each plate. The calculated overall intra-assay coefficient of variation was 7.5%.

13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 samples containing different concentrations of Galectin-3. 2 standard curves were run on each plate. The calculated overall inter-assay coefficient of variation was 5.4%.

13.3 Spike Recovery

The spike recovery was evaluated by spiking 3 levels of Galectin-3 into serum, plasma (EDTA, heparin, citrate) and cell culture supernatant. Recoveries were determined with 6 replicates each. The amount of endogenous Galectin-3 in unspiked samples was subtracted from the spike values. The recovery ranged from 80% to 120% with an overall mean recovery of 100%.

13.4 Dilution Parallelism

Serum, plasma (EDTA, citrate, heparin), cell culture supernatant samples with different levels of Galectin-3 were analysed at serial 2 fold dilutions with 4 replicates each. The recovery ranged from 80% to 120% with an overall mean recovery of 100%.

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

Aliquots of serum, plasma, cell culture supernatant samples (spiked or unspiked) were stored at -20°C and thawed 3 times, and the Galectin-3 levels determined. There was no significant loss of Galectin-3 immunoreactivity detected by freezing and thawing.

13.5.2 Storage Stability

Aliquots of serum, plasma, cell culture supernatant samples (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the Galectin-3 level determined after 24 h.

There was no significant loss of Galectin-3 immunoreactivity detected during storage under above conditions.

13.6 Specificity

The assay detects both natural and recombinant Galectin-3.

The cross reactivity and interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a Galectin-3 positive sample.

There was no cross reactivity or interference detected.

14 Ordering Information

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15 Reagent Preparation Summary

15.1 Wash Buffer (1x)

Add **Wash Buffer Concentrate** 20x (50 ml) to 950 ml distilled water.

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

15.2 Assay Buffer (1x)

Add **Assay Buffer Concentrate** 20x (5 ml) to 95 ml distilled water.

Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

15.3 HRP-Conjugate

Make a 1:100 dilution of **HRP-Conjugate** in Assay Buffer (1x):

Number of Strips	HRP-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

15.4 Human Galectin-3 Standard

Reconstitute lyophilized **human Galectin-3 standard** with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

16 Test Protocol Summary

1. Determine the number of microwell strips required.
2. Wash microwell strips twice with Wash Buffer.
3. Standard dilution on the microwell plate: Add 100 µl Sample Diluent, in duplicate, to all standard wells. Pipette 100 µl prepared standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells.
Alternatively external standard dilution in tubes (see 9.4.1): Pipette 100 µl of these standard dilutions in the microwell strips.
4. Add 100 µl Sample Diluent, in duplicate, to the blank wells.
5. Add 50 µl Sample Diluent to sample wells.
6. Add 50 µl sample in duplicate, to designated sample wells.
7. Cover microwell strips and incubate 1 hour at room temperature (18°-25°C) on a microplate shaker. **Shaking is absolutely necessary for an optimal test performance.**
8. Prepare HRP-Conjugate.
9. Empty and wash microwell strips 4 times with Wash Buffer.
10. Add 100 µl HRP-Conjugate to all wells.
11. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C) on a microplate shaker. **Shaking is absolutely necessary for an optimal test performance.**
12. Empty and wash microwell strips 4 times with Wash Buffer.
13. Add 100 µl of TMB Substrate Solution to all wells.
14. Incubate the microwell strips for about 30 minutes at room temperature (18° to 25°C).
15. Add 100 µl Stop Solution to all wells.
16. Blank microwell reader and measure colour intensity at 450 nm.

Note: If instructions in this protocol have been followed samples have been diluted 1:2, the concentration read from the standard curve must be multiplied by the dilution factor (x 2).