

M1990 / January 2011

ELISA

Human Mannose Binding Lectin ELISA kit



M1990



- ELISA kit for in vitro quantitative analysis of mannose binding lectin in human serum (or plasma) (en)

	Ab	BLANC	BUF	CAL	COAT	CONJ
en	Antibodies against	Blanco	Buffer	Calibrator	Coat	Conjugate

	CONTROL	DIL	HUM	MANNAN	MOU	SPE	STOCK
en	Control	Dilution	Human	Mannan	Mouse	Sample	Stock

	WASH	≥≤
en	Wash	Range

I. Introduction

Mannose binding lectin (MBL), also named mannose- or mannan-binding protein, is an important factor in the innate immunity. MBL belongs to the class of collectins in the C-type lectin superfamily, whose function appears to be pattern recognition in the first line of defense in the pre-immune host.

MBL recognizes carbohydrate patterns, found on the surface of a large number of pathogenic micro-organisms, including bacteria, viruses, protozoa and fungi (1,2,3).

Binding of MBL to a micro-organism results in activation of the classical complement route, long before specific antibodies are formed. MBL has an oligomeric structure (400-700 kDa), built of subunits that contain three identical peptide chains of 32 kDa each. Although MBL can form different oligomers, there are indications that dimers and trimers are not biologically active and at least a tetramer form is needed for activation of complement.

The peptide chains consist of a collagen-like part and a lectin part. The lectin part binds via 'carbohydrate recognition domains' (CRDs) at different sugar groups like mannose, maltose, N-acetylglucosamine, N-acetylgalactosamine, fucose and glucose. This binding has low affinity ($K_d 10^{-3}$) and it is essential that more lectin parts of one MBL molecule attach simultaneously to obtain a functional binding (4).

In the circulation MBL forms a functional complex with MASPs (MBL-associated serine protease) 1, 2 and 3, that becomes enzymatically active, after binding with a micro-organism. MASP-2 is identical to C1-esterase in the classical complement route and able to split C4 and C2, whereupon C4b2a is formed.

C4b2a functions as C3-convertase and splits C3, whereupon C3b is formed, that causes opsonisation of the microbe (2).

Besides, there are indications that MBL binds directly to collectin receptors on the surface of phagocytes, and it is suggested that MBL binds to the C1q receptor on neutrophil granulocytes, monocytes and macrophages. Binding to effector cells may stimulate the production of pro-inflammatory cytokines.

MBL is an acute phase protein and synthesized by hepatocytes. Immediately after parturition the MBL plasma concentration rises from 1000 ng/mL to a maximum of 2500 ng/mL within some weeks, after which the concentration falls to a level of 1700 ng/mL in adults. Individual MBL concentrations vary considerably and may rise twentyfold during acute phase reaction.

As part of the innate immune system MBL is important in the period following parturition, when maternal antibody concentrations fall and the own IgG production has to start. Furthermore, MBL plays an important role at any age in the primary contact with micro-organisms, before IgM concentrations rise.

The clinical relevance of MBL

The incidence of MBL deficiency is relatively high, compared to other deficiencies of the complement system, in about 20-25% of total population.

MBL deficiency is associated with increased susceptibility to infections, such as otitis media, pneumonitis, gastro-enteritis, meningitis, osteomyelitis and sepsis.

In children, a heterozygote MBL gen mutation doubles the risk of hospitalisation due to an infectious disease, compared to children with normal MBL levels.

In case of homozygote gen mutation the risk of infections is even greater and the course of the disease is worse than in patients with normal MBL levels.

Cystic fibrosis patients with a MBL-gen mutation are more susceptible to infections with *Pseudomonas aeruginosa* or *Burkholderia cepacia* and have a shorter life expectancy than cystic fibrosis patients without the mutation (5).

Oncological patients, receiving cancer chemotherapy and developing neutropenia as a consequence, are more susceptible to long periods of fever if they have low plasma concentrations of MBL.

In children under the age of one year, suffering from Kawasaki's disease, MBL deficiency is a risk factor for aneurysms of the coronary veins.

In HIV positive patients the progression to AIDS occurs more rapidly if they are MBL deficient.

MBL deficiency is associated with repeated spontaneous abortions, possibly due to intra-uterine infections.

MBL deficiency is associated with autoimmune diseases such as Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (RA).

Correlation between MBL deficiency and the incidence of both infections and autoimmune diseases has not only led to an increase of MBL-diagnostics, but also demonstrated the need for therapeutic applications of purified MBL.

II. Technical principle

The assay is an ELISA format, performed in microwells coated with mannan.

MBL present in a measured volume of sample or calibrator will bind to the coated mannan on the microtitre plate.

Non-bound material is removed by washing.

Subsequently, a horseradish peroxidase (HRP) conjugated antibody to MBL (anti-human-MBL/1-HRP) is added.

After removal of non-bound HRP conjugate by washing, substrate solution is added to the wells. The colour that develops is proportional to the amount of MBL present in sample or calibrator.

The enzymatic reaction is stopped chemically and the colour intensity is read at 450nm in an ELISA reader.

From the absorbance of the samples and those of a calibration curve, the concentration of MBL can be determined by interpolation.

III. Storage and stability

The MBL ELISA kit should be stored at -18°C to -32°C. The performance of the kit is guaranteed until the expiration date shown on the case label. Transport conditions may differ from storage conditions.

Do not freeze-thaw more than three times.

IV. Contents of the kit

The MBL ELISA kit contains material sufficient for 288 tests (three plates), including calibrators, control and blank. See the Table at the end of this package insert.

The mannan to be used as a coat is purified from *Saccharomyces cerevisiae*.

The monoclonal antibody was purified from tissue culture medium, using column chromatography (ion exchange and affinity chromatography).

The calibrator and control are human sera.

V. Additional materials required

Additional materials:

- Distilled water for dilution of wash, dilution and substrate buffers.
- Beakers, flasks, cylinders necessary for preparation of reagents.
- Pipetting devices for accurate delivery of volumes.

- A standard ELISA washer or a 500 mL plastic squirt bottle for automatic or manual washing of the strips.
- A standard ELISA reader for measuring absorbance at 450 nm.
- Log linear paper.

Additional buffers and solutions:

Coating buffer : 0.1 M carbonate/bicarbonate buffer pH 9.6

Buffers and solutions for enzymatic colour development:

Substrate buffer: 0.11 M acetate buffer pH 5.5

Dissolve 15.0 g sodium-acetate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$) in 800 mL distilled water. Adjust pH to 5.5 with glacial acetic acid, add distilled water to a volume of 1 liter.

Do not add any preservative (e.g. merthiolate, sodium azide) since this may affect the quality of the enzymatic colour development.

3,5,3',5'-tetramethylbenzidine (TMB) stock solution:

6 mg/mL TMB in DMSO.

Dissolve 30 mg 3,5,3',5'-tetramethylbenzidine (TMB) in 5 mL dimethylsulfoxide (DMSO).

Hydrogen peroxide stock solution:

3% H_2O_2 solution in distilled water.

Stop solution:

1.8 M H_2SO_4 solution in distilled water.

For one microtitreplate prepare 12 mL substrate solution by adding together:

12 mL substrate buffer

200 μL TMB stock solution

12 μL H_2O_2 stock solution

VI. Test sample handling

Blood specimens should be collected aseptically. Serum is the preferred specimen, but heparin or EDTA anti coagulated plasmas are also suitable for use in the assay.

In case of plasmas, 10 U/mL heparin should be added to the dilution buffer.

The samples should be as fresh as possible or stored frozen.

VII. Assay protocol

- On the day of testing thaw all reagents, bring to room temperature (18-25°C) and mix gently. Avoid bubbles or foam.
- 5-fold concentrated dilution buffer can be placed in 37°C water bath and mixed regularly.
- Calibrator and control can be thawed handheld.
- Centrifuge all vials before use (1 minute at 3000g).
- It is advised to test all samples, control and calibrator dilutions in duplicate.
- See table 4 of enclosed leaflet for proposed plate plan

Microtitreplate

The kit contains three microtitreplates. Depending on the amount of samples to be tested one or more plates should be coated with mannan, the day before the actual test.

Mannan is provided as a 100-fold concentration in this kit.

Coating buffer should be prepared fresh. Per microtitreplate, add 120 μL of the mannan to 12 mL coating buffer.

Add 100 μL to all wells, cover micro titre plate(s) with lid and incubate overnight at room temperature (18 – 25°C).

Buffers

Calculate the quantity of wash buffer required and prepare a working strength solution by diluting the buffer 20-fold in distilled water (one plate requires approximately 300 mL). The diluted wash buffer must be stored at 2-8°C and remains stable for 1 week.

Dilution buffer: Calculate the quantity of dilution buffer required and prepare a working-strength solution by diluting the buffer 5-fold in distilled water.

Preparation of the calibrator and control (*in duplo*)

For concentrations see Table 2 and 3 of the enclosed information leaflet.

Thaw the calibrator and the control by holding in your hands and mix gently.

Label four tubes, two for the control (*in duplo*) and two for the highest (350 ng/mL) calibration point (*in duplo*).

Calibrator: See table 1 of the enclosed information leaflet for preparation of the highest calibration point.

Label eight tubes, one tube for each calibration curve dilution (*in duplo*): 140; 56; 22.4 and 9.0 ng/mL.

Subsequently, pipette 150 μL of dilution buffer into these tubes. Transfer 100 μL from the highest calibration point tube into the second tube, labelled 140 ng/mL and mix well. Repeat the serial dilution three more times by adding 100 μL of the previous tube of diluted calibrator to the 150 μL of diluent in the labelled tubes.

The calibration curve will contain 350; 140; 56; 22.4; 9.0 ng/mL (*in duplo*).

Use dilution buffer as blank value (0 ng/mL).

Control : Pipette 380 μL working-strength dilution buffer into the tubes for the control (*in duplo*), add 20 μL of the control and mix well.

Preparation of samples

Dilute test samples 1:20 in dilution buffer, similar to the control serum. For results that lie outside the given ranges (see Table 1 of the enclosed information leaflet), the test should be repeated with a different sample dilution.

1. First wash step

Aspirate the mannan solution from the wells of the microtitreplate(s)

and completely fill the wells ($\geq 300 \mu\text{L}$) with washing buffer and discard, repeat this procedure three times. Finally, the wells should be completely empty. Subsequent reagent should be added immediately, do not let the wells stand dry for an extended period of time.

2. First incubation step
Add 100 µL of the diluted calibrator, control and samples into the appropriate wells. Cover plate with adhesive seal, gently agitate by tapping the edge of the microtitreplate for a few seconds to mix contents of each well.
Incubate for 1 hour at room temperature (18–25 °C).
Just before washing, prepare next incubation reagent as described in point 4.
 3. Second wash step
Aspirate supernatant from the wells and wash the plate as described in point 1.
 4. Incubation with anti-human MBL-HRP.
Dilute the conjugate 1:100. Per microtitreplate, add 120 µL HRP conjugate to 12 mL dilution buffer.
Add 100 µL to each well. Cover the plate with adhesive seal, gently agitate by tapping the edge for a few seconds to mix the contents of each well.
Incubate for 1 hour at room temperature (18–25 °C).
Just before washing prepare next incubation reagent TMB-substrate as described in V (Additional materials required).
 5. Third wash step
Aspirate supernatant from the wells and wash the plate as described in point 1.
 6. Incubation with TMB-substrate
Add 100 µL of TMB-substrate solution to all wells.
Gently agitate by tapping the edge of the microtitreplate for a few seconds to mix the contents of each well.
Incubate for max. 30 minutes at room temperature (18-25 °C) in a dark place.
- Note : The speed of enzymatic colour development is influenced by many factors, including temperature and quality of the used TMB.
7. Stop enzymatic reaction
Add 100 µL of stop solution to all wells.
 8. Plate read-out
Read *within 30 minutes* at 450 nm in an ELISA reader.

VIII. Results and interpretation of data

- Record the absorbance at 450 nm for each well and average the duplicate values.
- For each sample, duplicates should not differ more than 15% from the mean value. If duplicates vary more, the assay should be repeated.
- Plot the average absorbances of the calibrators (y-axis) versus the related MBL in ng/mL (x-axis) on log-linear paper and draw the best fitting curve.
- The MBL levels of the control should lie within the range(s) given in Table 3 of the enclosed information leaflet.
- Interpolate the average absorbances value for each sample on the reference curve.
- Test samples which show a mean absorbance outside the range of the reference curve dilutions, should be diluted appropriately.

IX. Assay ranges

Consult Table 1 of the enclosed information leaflet for the kit specific assay ranges.

X. Reference ranges

Measurements in a group of normal healthy blood donors in the Netherlands (n = 244), yielded following MBL values:

10% percentile	<0.04 µg/mL
25% percentile	0.4 µg/mL
50% percentile	1.1 µg/mL
75% percentile	2.0 µg/mL
90% percentile	3.3 µg/mL

As reference ranges are subject to many influencing variables which may differ for each population investigated, each laboratory should establish its own reference range.

XI. Specific performance characteristics

Reproducibility

Intra-assay variation: Two different dilutions of one serum sample were each measured eleven times in one assay.

Inter-assay variation: In six up to ten different assays the amount of MBL was measured in three different serum samples.

Intra-assay	Average	Variation
Dilution 1 (1:10)	1.46 µg/mL	6.0%
Dilution 2 (1:50)	1.53 µg/mL	6.1%

Inter-assay	Average	Variation
Sample 1	1.52 µg/mL	7.9%
Sample 2	1.49 µg/mL	4.8%
Sample 3	1.60 µg/mL	11.2%

Note:

The values cited for specific performance characteristics of the test represent typical results and are not to be viewed as specifications for this kit.

Sensitivity

The kit has a minimum detection level of 9.0 ng/mL and a measurable concentration range of 9.0 to 350 ng/mL.

Note: The values cited for sensitivity of the test represent typical results and are not to be regarded as specific for this kit.

Specificity

The absence of cross-reaction with other plasma or serum components is supported by the fact that readings indistinguishable from zero are obtained from some MBL-deficient but otherwise normal donors.

Expected values

See X (Reference ranges).

XII. Restrictions

1. User should be trained and familiar with ELISA assays and test procedure.
2. Grossly haemolysed or lipaemic samples should not be used. Unexpected results may be obtained for samples containing rheumatoid factor, high bilirubin levels, or other circulating immune complexes. These samples should be analysed by another method.
3. Out of range samples should be repeated using different dilutions.
4. The finding of a decreased level of MBL can never provide a definite diagnosis, but should rather be considered as an indication of a disturbance of the immune system, requiring further diagnostic investigation.
5. The control serum should always be used to check the validity of the calibration curve. When the control is out of range, the results of the test samples are not reliable. The test should be repeated.
6. Reagents from different batches are not interchangeable.
7. Reminders of reagents (e.g. dead volume) should not be mixed with contents of freshly opened vials.
8. Caps and vials are not interchangeable. Caps should be replaced on the corresponding vials.
9. Although the human calibrator and control sera have been tested for the markers of specific disease transmitting agents in accordance with current EU guidelines to GMP and found to be nonreactive, all components of human origin should be considered potentially infectious.
10. Preservative: merthiolate 0.001%.
11. Use new plate seals for each incubation/fixation step in the ELISA-experiment to avoid cross contamination. Do not use aluminium foil.
12. Use disposable pipette tips for each transfer to avoid cross contamination.
13. Each time the kit is used, fresh dilutions of calibrators, conjugate and buffers should be made.
14. Do not use other reagents and microtitre plates than those supplied with the kit.
15. Sodium azide inactivates HRP, do not use solutions containing sodium azide, nor add sodium azide to the supplied buffers. Do not use merthiolate since this may affect the quality of the enzymatic colour development
16. The waste-disposal should be performed according to your laboratory regulations.
17. Do not allow wells to stand uncovered or dry for extended periods between incubation steps.

XIII. References

1. Turner, M.W. (1996) Immunol Today 17:532-540
2. Gadjeva, M. Thiel, S. Jensenius, J.C. (2001) Curr Opin Immunol 13:74-78
3. Klabunde, J. et al (2002) Parasitol Res 88(2):113-117
4. Thielens, N.M. et al (2001) J Immunol 166:5068-5077
5. Garred, P. (1999) J Clin Invest 104:431-437

ELISA									
Human Mannose Binding Lectin ELISA kit									
1	375 µl	COAT	MANNAN			STOCK		1:100	
1	500 µl	CAL							
1	200 µl	CONTROL							
1	375 µl	HRP	MOU	Ab	HUM	MBL	CONJ	1:100	
1	50 mL	BUF	DIL	STOCK	1:5				
1	60 mL	BUF	WASH	STOCK	1:20				
3	12x8	WELL							
10		SEALS							