EBNA IgM

Enzyme ImmunoAssay for the qualitative determination of IgM antibodies to Epstein Barr Virus Nuclear Antigen (EBNA) in human plasma and sera

- for “in vitro” diagnostic use only -

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REF EBNM.CE
96 Tests

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A. INTENDED USE
Enzyme ImmunoAssay (ELISA) for the qualitative determination of IgM class antibodies to Epstein Barr Virus Nuclear Antigen (EBNA) in human plasma and sera.
The kit is intended for the classification of the viral infective agent and the follow-up of EBV infected patients.
For "in vitro" diagnostic use only.

B. INTRODUCTION
Epstein Barr Virus or EBV is the principal etiological agent of infectious mononucleosis, as well as a contributory factor in the etiology of Burkitt's lymphoma and nasopharyngeal carcinoma, or NPC.
A member of the family Herpesviridae, it has a worldwide distribution, such that 80 to 90% of all adults have been infected. Primary infections usually occur during the first decade of life. While childhood infections are mostly asymptomatic, 50 to 70% of young adults undergoing primary EBV infections show mild to severe illness.
EBV may cause a persistent, latent infection which can be reactivated under immunosoppression or in AIDS affected patients. As humoral responses to primary EBV infections are quite rapid, the level and class of antibodies raised in most cases allow classification as to whether the patient is still susceptible, has a current or recent primary infection, had a past infection or may be having reactivated EBV infection.
The detection of EBV-specific IgG, IgM and IgA antibodies to its major immunodominant antigens has become therefore an important and useful determination for the monitoring and follow-up of EBV infected patients.

C. PRINCIPLE OF THE TEST
Microplates are coated with affinity purified native EBNA.
The solid phase is first treated with the diluted sample and anti-EBNA IgM are captured, if present, by the antigens.
After washing out all the other components of the sample, in the 2nd incubation bound anti-EBNA IgM are detected by the addition of anti hIgM antibody, labelled with peroxidase (HRP).
The enzyme captured on the solid phase, acting on the substrate/chromogen mixture, generates an optical signal that is proportional to the amount of anti-EBNA IgM antibodies present in the sample.
Interferences due to IgG and RF in samples are blocked directly into the well by a Neutralizing Reagent.

D. COMPONENTS
Each kit contains sufficient reagents to carry out 96 tests.

1. Microplate: MICROPLATE
12 strips x 8 breakable wells coated with affinity-purified native EBNA and sealed into a bag with desiccant. Allow the microplate to reach room temperature before opening; reseal unused strips in the bag with desiccant and store at 4°C.

2. Negative Control: CONTROL -
1x4ml. Human serum base not reactive for anti-EBNA IgM antibodies. It contains 0.2 mg/ml gentamicine sulphate and 0.1% Kathon GC as preservatives. The Negative Control is pale yellow colour coded.

3. Positive Control: CONTROL +
1x2ml. Human serum base reactive for anti-EBNA IgM antibodies. It contains 0.2 mg/ml gentamicine sulphate and 0.1% Kathon GC as preservatives. The Positive Control is green colour coded.

4. Wash buffer concentrate: WASHBUF 20X
1x60ml/bottle. 20x concentrated solution.
Once diluted, the wash solution contains 10 mM phosphate buffer pH 7.0+/-0.2, 0.05% Tween 20 and 0.1% Kathon GC.

5. Enzyme conjugate: CONJ
1x16ml/vial. Ready to use and red colour coded. It contains Horseradish peroxidase conjugated polyclonal antibodies to human IgM, 5% BSA, 10 mM Tris buffer pH 6.8+/-0.1, 0.1% Kathon GC and 0.02% gentamicine sulphate as preservatives.

6. Specimen Diluent: DILSPE
2x60.0 ml/vial. Buffered solution for the dilution of samples. It contains 2% casein, 0.2 M Tris buffer pH 6.0+/-0.1, 0.2% Tween 20, 0.1% Kathon GC and 0.09% sodium azide as preservatives. The component is blue color coded.

7. Neutralizing Reagent: SOLN NEUT
1x8ml. Proteic solution for the neutralization of IgG and RF in samples. It contains a detergent, proteic stabilizers, 0.1% sodium azide and 0.1% Kathon GC as preservatives.

8. Chromogen/Substrate: SUBS TMB
1x16ml/vial. Contains a 50 mM citrate-phosphate buffered solution at pH 3.5-3.8, 0.03% tetra-methyl-benzidine or TMB and 0.02% hydrogen peroxide of H_2O_2.
Note: To be stored protected from light as sensitive to strong illumination.

9. Sulphuric Acid: H_2SO_4 O.3 M
1x15ml/vial. Contains 0.3 M H_2SO_4 solution.
Attention: Irritant (Xi R36/38; S2/26/30)

10. Plate sealing foils n°2

11. Package insert n°1

E. MATERIALS REQUIRED BUT NOT PROVIDED
1. Calibrated Micropipettes in the range 10-1000 ul and disposable plastic tips.
2. EIA grade water (double distilled or deionised, charcoal treated to remove oxidizing chemicals used as disinfectants).
3. Timer with 60 minute range or higher.
4. Absorbent paper tissues.
5. Calibrated ELISA microplate thermostatic incubator (dry or wet) set at +37°C.
6. Calibrated ELISA microwell reader with 450nm (reading) and if possible with 620-630nm (blanking) filters.
7. Calibrated ELISA microplate washer.
8. Vortex or similar mixing tools.

F. WARNINGS AND PRECAUTIONS
1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible of the laboratory.
2. All the personnel involved in performing the assay have to wear protective laboratory clothes, talc-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
3. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.
4. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-born microbial

agents, when opening kit vials and microplates and when performing the test. Protect the Chromogen/Substrate (TMB/H_2O_2) from strong light and avoid vibration of the bench surface where the test is undertaken.

5. Upon receipt, store the kit at +2..8°C into a temperature controlled refrigerator or cold room.
6. Do not interchange components between different lots of the kits. It is recommended that components between two kits of the same lot should not be interchanged.
7. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures.
8. Avoid cross-contamination between serum/plasma samples by using disposable tips and changing them after each sample.
9. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.
10. Do not use the kit after the expiration date stated on external (primary container) and internal (vials) labels.
11. Treat all specimens as potentially infective. All human serum specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
12. The use of disposable plastic labware is recommended in the preparation of the washing solution or in transferring components into other containers of automated workstations, in order to avoid contamination.
13. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from the washing procedure, from residuals of controls and from samples has to be treated as potentially infective material and inactivated. Suggested procedures of inactivation are treatment with a 10% final concentration of household bleach for 16-18 hrs or heat inactivation by autoclave at 121°C for 20 min..
14. Accidental spills have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.
15. The Sulphuric Acid is an irritant. In case of spills, wash the surface with plenty of water.
16. Other waste materials generated from the use of the kit (example: tips used for samples and controls, used microplates) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.

G. SPECIMEN: PREPARATION AND RECOMMANDATIONS

1. Blood is drawn aseptically by venepuncture and plasma or serum is prepared using standard techniques of preparation of samples for clinical laboratory analysis. No influence has been observed in the preparation of the sample with citrate, EDTA and heparin.

2. Avoid any addition of preservatives to samples; especially sodium azide as this chemical would affect the enzymatic activity of the conjugate.

3. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results. When the kit is used for the screening of blood units, bar code labeling and electronic reading is strongly recommended.

4. Haemolysed (red) and visibly hyperlipemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.

5. Sera and plasma can be stored at +2°..8°C for up to five days after collection. For longer storage periods, samples can be stored frozen at –20°C for several months. Any frozen samples should not be frozen/thawed more than once as this may generate particles that could affect the test result.

6. If particles are present, centrifuge at 2.000 rpm for 20 min or filter using 0.2-0.8u filters to clean up the sample for testing.

H. PREPARATION OF COMPONENTS AND WARNINGS

A study conducted on an opened kit has not pointed out any relevant loss of activity up to 6 re-uses of the device and up to 3 months.

Microplate:
Allow the microplate to reach room temperature (about 1 hr) before opening the container. Check that the desiccant has not turned dark green, indicating a defect in storing.
In this case, call Dia.Pro's customer service.
Unused strips have to be placed back inside the aluminum pouch, with the desiccant supplied, firmly zipped and stored at +2°..8°C.
***Important Note**: After first opening, remaining strips are stable until the humidity indicator inside the desiccant bag turns from yellow to green.*

Controls
Ready to use. Mix well on vortex before use.

Wash buffer concentrate:
The whole content of the 20x concentrated solution has to be diluted with bidistilled water up to 1200 ml and mixed gently end-over-end before use. During preparation avoid foaming as the presence of bubbles could impact on the efficiency of the washing cycles.
***Note:** Once diluted, the wash solution is stable for 1 week at +2..8°C.*

Specimen Diluent
Ready to use. Mix on vortex before use.

Neutralizing Reagent
Ready to use. Mix on vortex before use.

Chromogen/Substrate:
Ready to use. Mix well on vortex before use.
Avoid contamination of the liquid with oxidizing chemicals, air-driven dust or microbes. Do not expose to strong light, oxidizing agents and metallic surfaces.
If this component has to be transferred use only plastic, and if possible, sterile disposable container.

Sulphuric Acid:
Ready to use. Mix well on vortex before use.
Attention: Irritant (Xi R36/38; S2/26/30)
Legenda: R 36/38 = Irritating to eyes and skin.
S 2/26/30 = In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

I. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. Micropipettes have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (70% ethanol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample or the components of the kit. They should also be regularly maintained in order to show a precision of 1% and a trueness of ±2%.
2. The ELISA incubator has to be set at +37°C (tolerance of ±0.5°C) and regularly checked to ensure the correct temperature is maintained. Both dry incubators and water baths are suitable for the incubations, provided that the

instrument is validated for the incubation of ELISA tests and the right temperature of +37°C is assured to the mi croplate.

3. The ELISA washer is extremely important to the overall performances of the assay. The washer must be carefully validated and correctly optimized using the kit controls/calibrator and reference panels, before using the kit for routine laboratory tests. Usually 4-5 washing cycles (aspiration + dispensation of 350 ul/well of washing solution = 1 cycle) are sufficient to ensure that the assay performs as expected. A soaking time of 20-30 seconds between cycles is suggested. In order to set correctly their number, it is recommended to run an assay with the kit controls/calibrator and well characterized negative and positive reference samples, and check to match the values reported below in the section "Internal Quality Control". Regular calibration of the volumes delivered and maintenance (decontamination and cleaning of needles) of the washer has to be carried out according to the instructions of the manufacturer. Manual washing is nor recommended, in case, pay extremely attention to avoid cross contamination of wells.
4. Incubation times have a tolerance of $\pm$5%.
5. The ELISA microplate reader has to be equipped with a reading filter of 450nm and ideally with a second filter (620-630nm) for blanking purposes. Its standard performances should be (a) bandwidth $\leq$ 10 nm; (b) absorbance range from 0 to $\geq$ 2.0; (c) linearity to $\geq$ 2.0; repeatability $\geq$ 1%. Blanking is carried out on the well identified in the section "Assay Procedure". The optical system of the reader has to be calibrated regularly to ensure that the correct optical density is measured. It should be regularly maintained according to the manufacturer 's instructions.
6. When using an ELISA automated work station, all critical steps (dispensation, incubation, washing, reading, shaking, data handling) have to be carefully set, calibrated, controlled and regularly serviced in order to match the values reported in the section "Internal Quality Control". The assay protocol has to be installed in the operating system of the unit and validated as for the washer and the reader. In addition, the liquid handling part of the station (dispensation and washing) has to be validated and correctly set. Particular attention must be paid to avoid carry over by the needles used for dispensing samples and for washing. This must be studied and controlled to minimize the possibility of contamination of adjacent wells due to strongly reactive samples, leading to false positive results. The use of ELISA automated work stations is recommended for blood screening and when the number of samples to be tested exceed 20-30 units per run.
7. Dia.Pro's customer service offers support to the user in the setting and checking of instruments used in combination with the kit, in order to assure full compliance with the requirements described. Support is also provided for the installation of new instruments to be used with the kit.

L. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label (primary container). Do not use if expired.
2. Check that the liquid components are not contaminated by visible particles or aggregates.
3. Check that the Chromogen/Substrate is colorless or pale blue by aspirating a small volume of it with a sterile plastic pipette.
4. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box (primary container).
5. Check that the aluminum pouch, containing the microplate, is not punctured or damaged.
6. Dilute all the content of the 20x concentrated Wash Solution as described above.
7. Allow all the other components to reach room temperature (about 1 hr) and then mix gently on vortex all liquid reagents.
8. Set the ELISA incubator at +37°C and prepare the ELISA washer by priming with the diluted washing solution, according to the manufacturers instructions. Set the right number of washing cycles as found in the validation of the instrument for its use with the kit.
9. Check that the ELISA reader is turned on or ensure it will be turned on at least 20 minutes before reading.
10. If using an automated work station, turn on, check settings and be sure to use the right assay protocol.
11. Check that the micropipettes are set to the required volume.
12. Check that all the other equipment is available and ready to use.
13. In case of problems, do not proceed further with the test and advise the supervisor.

M. ASSAY PROCEDURE

The assay has to be carried out according to what reported below, taking care to maintain the same incubation time for all the samples in testing.

1. Dilute samples 1:101 dispensing 1 ml Specimen Diluent into a disposable tube and then 10 ul sample; mix on vortex before use. Do not dilute the Controls as they are ready-to-use.
2. Place the required number of strips in the microplate holder. Leave A1 well empty for the operation of blanking. Store the other strips into the bag in presence of the desiccant at 2..8°C, sealed.
3. Dispense 50 µl Neutralizing Reagent in all the sample wells; do not dispense in A1 and in the Controls wells.
4. Pipette 100 µl Negative Control in triplicate and 100 µl Positive Control in single into appropriate wells. Then dispense 100 µl of samples into the appropriate sample wells.
5. Incubate the microplate at **+37°C for 60 min** .

Important note: *Strips have to be sealed with the adhesive sealing foil only when the test is performed manually. Do not cover strips when using ELISA automatic instruments.*

6. Wash the microplate as reported in section I.3.
7. In all the wells, except A1, pipette 100 µl Conjugate. Check that the reagent has been correctly added. Incubate the microplate at **+37°C for 60 minutes.**

Important note: *Be careful not to touch the inner surface of the well with the pipette tip when dispensing the Conjugate. Contamination might occur.*

8. Wash the microplate as described in section I.3.
9. Pipette 100 µl Chromogen/Substrate in each well, the blank well A1 included. Check that the reagent has been correctly added. Then incubate the microplate at **room temperature for 20 minutes**.

Important note: *Do not expose to strong direct light as a high background might be generated.*

10. Stop the enzymatic reaction by pipetting 100 µl Sulphuric Acid into each well and using the same pipetting sequence as in step 9.
11. Then measure the color intensity with a microplate reader at 450nm (reading) and possibly at 620-630nm (blanking), blanking the instrument on A1.

Important general notes:

1. *If the second filter is not available, ensure that no finger prints are present on the bottom of the microwell before reading at 450nm. Finger prints could generate false positive results on reading.*
2. *Reading has should ideally be performed immediately after the addition of the Stop Solution but definitely no longer than 20 minutes afterwards. Some self oxidation of the chromogen can occur leading to a higher background.*

N. ASSAY SCHEME

Neutralizing Reagent Controls Samples diluted 1:101	50 ul 100 ul 100 ul
1st incubation	**60 min**
Temperature	+37°C
Enzyme Conjugate	100 ul
2nd incubation	**60 min**
Temperature	+37°C
TMB/H_2O_2 mix	100 ul
3rd incubation	**20 min**
Temperature	r.t.
Sulphuric Acid	100 ul
Reading OD	450nm & 620nm

An example of dispensation scheme is reported below:

Microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	S4										
B	NC	S5										
C	NC	S6										
D	NC	S7										
E	PC	S8										
F	S1	S9										
G	S2	S10										
H	S3	S11										

Legenda: BLK = Blank // NC = Negative Control
PC = Positive Control // S = Sample

O. INTERNAL QUALITY CONTROL

A validation check is carried out on the calibrators and control serum any time the kit is used in order to verify whether the performances of the assay are as qualified.
Control that the following data are matched:

Parameters	Requirements
Blank well	< 0.100 OD450nm
Negative Control (NC)	< 0.150 OD450nm after blanking
Positive Control (PC)	> 0.500 OD450nm

If the results of the test match the requirements stated above, proceed to the next section.

If they do not, do not proceed any further and perform the following checks:

Problem	Check
Blank well > 0.100 OD450nm	1. that the Chromogen/Substrate solution has not become contaminated during the assay
Negative Control OD450nm > 0.150	1. that the washing procedure and the washer settings are as validated in the pre qualification study; 2. that the proper washing solution has been used and the washer has been primed with it before use; 3. that no mistake has been done in the assay procedure when the dispensation of Controls is carried out; 4. that no contamination of the Control or of the wells where it was dispensed has occurred due to spills of positive samples or Conjugate; 5. that micropipettes have not become contaminated with positive samples or with the Conjugate; 6. that the washer needles are not blocked or partially obstructed.
Positive Control OD450nm < 0.500	1. that the procedure has been correctly performed; 2. that no mistake has occurred during its distribution (ex.: dispensation of a wrong Control); 3. that the washing procedure and the washer settings are as validated in the pre qualification study; 4. that no external contamination of the Control has occurred.

P. RESULTS

If the test turns out to be valid, results are calculated from the mean OD450nm value of the Negative Control (NC) by means of a cut-off value (Co) determined with the following formula:

Cut-Off = NC + 0.250

Important Note: *When the calculation of results is performed by the operating system of an ELISA automated workstation, ensure that the proper formulation is used to generate the correct interpretation of results.*

Q. INTERPRETATION OF RESULTS

Test results are interpreted as the ratio of the Sample OD450nm value (S) and the Cut-Off value (Co), or S/Co, according to the following table:

S/Co	Interpretation
< 1.0	Negative
1.0 – 1.2	Equivocal
> 1.2	Positive

A negative result indicates that the patient has not developed IgM antibodies to EBV.
Any patient showing an equivocal result should be retested on a second sample taken 1-2 weeks after the initial sample.
A positive result is indicative of an ongoing EBV infection and therefore the patient should be treated accordingly.

Important notes:

1. *EBNA IgM results alone are not enough to provide a clear diagnosis of EBV infection. Other tests for EBV (supplied by Dia.Pro Diagnostic BioProbes s.r.l. at code n° EBNG.CE, EAG.CE, EAM.CE, VCAG.CE, VCAM.CE and RealTime PCR assay for EBV), should be carried out.*
2. *Interpretation of results should be done under the supervision of the laboratory supervisor to reduce the risk of judgment errors and misinterpretations.*
3. *When test results are transmitted from the laboratory to another facility, attention must be paid to avoid erroneous data transfer.*
4. *Diagnosis has to be done and released to the patient by a suitably qualified medical doctor.*

An example of calculation is reported below.

The following data must not be used instead or real figures obtained by the user.

Negative Control: 0.100 – 0.120 – 0.080 OD450nm
Mean Value: 0.100 OD450nm
Lower than 0.150 – Accepted
Positive Control: 1.000 OD450nm
Higher than 0.500 – Accepted
Cut-Off = 0.100+0.250 = 0.350
Sample 1: 0.080 OD450nm
Sample 2: 1.800 OD450nm
Sample 1 S/Co < 1.0 = negative
Sample 2 S/Co > 1.2 = positive

R. PERFORMANCE CHARACTERISTICS
Evaluation of Performances has been conducted in an external clinical center on panels of negative and positive samples with reference to a commercial kit.

1. Limit of detection
No international standard for EBNA IgM Antibody detection has been defined so far by the European Comunity.
In its absence, an Internal Gold Standard (or IGS), derived from a patient in the acute phase of mononucleosis infection, has been defined in order to provide the device with a constant and excellent sensitivity.

2. Diagnostic Sensitivity and Specificity:
The **diagnostic sensitivity** was studied on 88 positive samples, pre-tested with the reference kit of European origin in use at the laboratory. Positive samples were collected from patients undergoing acute mononucleosis infection.
The **diagnostic specificity** was determined on 352 negative samples from normal individuals classified negative with the reference kit.
Moreover a panel of potentially interfering samples (RF+, unrelated virus infections, et.c) were tested with no false results.
Both plasma, derived with different standard techniques of preparation (citrate, EDTA and heparin), and sera have been used to determine the specificity. No false reactivity due to the method of specimen preparation has been observed.
Frozen specimens have also been tested to check whether samples freezing interferes with the performance of the test. No interference was observed on clean and particle free samples.
The Performance Evaluation provided the following values :

Sensitivity	> 98 %
Specificity	> 98 %

3. Reproducibility:
Data obtained from a study conducted on three samples of different EBNA IgM reactivity, examined in 16 replicates in three separate runs showed in general CV% values lower than 15%, depending on the OD450nm readings.
The variability shown in the tables did not result in sample misclassification.

S. LIMITATIONS
False positivity has been assessed as less than 2 % of the normal population, mostly due to high titers of Rheumatoid Factor.
Frozen samples containing fibrin particles or aggregates may generate false positive results.

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