Enzyme-Immunossay for in-vitro detection of IgG antibodies against Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA-1) p72 in serum or plasma

**Intended use**

The Anti-EBV EBNA IgG ELISA is an in-vitro diagnostic kit designed for the detection of IgG antibodies against the EBV nuclear antigen 1 (p72) in serum or plasma. The enzyme reaction is stopped by adding a monoclonal antibody directed against human IgG. Non-specifically bound conjugate is unspecifically bound to the antigen p72 (4-6) coated to the microtiter plate. The test is used for the differentiation of primary and late stages of EBV infection and has a central role in EBV diagnosis.

**Principle of the assay**

The Anti-EBV EBNA IgG ELISA is a highly sensitive indirect enzyme immuno sorbent assay (ELISA) for the detection of EBV-specific antibodies in serum or plasma. During the first incubation step IgG antibodies of the sample will bind to the recombinant (rec) EBNA-1 antigen p72 (4-6) coated to the MTP. Unspecific material will be removed by washing. Non-specifically bound conjugate is removed by another washing step. For the last incubation the substrate solution (TMB, 3,3′,5,5′-Tetramethylbenzidine) is filled into the wells. The enzyme reaction is stopped by adding sulphuric acid (0.5 N). The optical density is measured with a spectrophotometer at 450 nm and a reference wavelength of 615-690 nm.

**Kit contains**

- Microplate
- Wash buffer concentrate
- Sample diluent
- Anti human IgG Conjugate (ready for use, human)
- EBV EBNA IgG negative control (ready for use, human)
- EBV EBNA IgG positive control (ready for use, human)
- Sample 1:21 with dilution directly in the plate
- Step solution (ready for use)
- Substrate solution (ready for use, human)
- Stop solution (ready for use, human)
- Storage bag
- Self-adhesive transparent foils for sealing the microtiter plate

**Preservatives:** Total concentration < 0.11%

**Materials required but not supplied with the kit**

- Microsyringe and pipettes, spectral photometer (450 nm, reference wavelength 615 – 690 nm), microplate washer (with bottom wash) and incubator (37°C) for MTP.

**Warning and precautions**

- Conjugate is irritant (Proclin-300).
- Do not incorporate reagents. Avoid contact with eyes and skin.
- All samples and reagents used for the test must be treated as being potentially infectious and appropriate safety precautions taken. The controls are negative for anti-HIV 1/2, anti-HCV, HBsAg, anti-lues and elevated transaminases. Do not pipet with mixing. According to good laboratory practice wear gloves, laboratory coat and safety glasses. Liquids and non-combustible materials should be decontaminated with sodium hypochlorite (final concentration: 3 %, activity time at least 30 minutes). Liquid waste which contains acids must be neutralised before disposal. Used MTP and all materials that are to be re-used must be autoclaved at least once and 30 minutes.
- Microplate is sensitive to light and has to be protected from light. The test must be performed by well-trained and authorised laboratory technicians. Testing is performed under aseptic and microbiologically controlled conditions.

**Sample preparation**

Fresh serum or plasma samples, free from haemolysis should be used. Highly lipaemic, icteric or microbially contaminated sera or plasma samples and concentrated immuno globulin preparations can lead to unreliable test results. Avoid repeated freezing and thawing of the samples. If samples are to be transported, they must be packed in accordance with legal requirements for the transportation of infectious materials. The samples should not be inactivated, as unspecific reactions may otherwise occur.

**Performance of test**

The protocol (see pipetting procedure) has to be followed strictly. Sample dilution 1:12 with predilution in tubes. Dilute PC, NC and samples 1:21 in a tube (e.g. 25 µl control or sample + 500 µl diluent). Mix well. Sample dilution 1:21 with dilution directly in plate:

- Pipet 200 µl [DIL] into every well. The dilution directly in the microtiter plate is particularly suitable for the use of automatic pipetting devices. If the dilution directly in the plate is performed manually it is important to avoid non-specific protein binding by observing the following steps: Pipet first 250 µl of DIL into the well and add 10 µl of sample or controls subsequently. Mix 5 up to 7 times when adding 10 µl sample or control.

**Washing procedure**

The wash procedure is critical. Insufficient washing will result in poor precision and unspecific reactions.

- W1: manual wash: wash five times with wash buffer. To do so, aspirate the content of the well and dispense 250 µl of wash buffer. Repeat this process five times.
- W2: automatic wash: wash three times with 250 µl of wash buffer using bottom wash program.

**Pipetting procedure of quantitative IgG determination (dilution tube)**

**Step 1**

All reagents to reach room temperature before use.

- The controls and the blank should be pipetted last. After pipetting the controls and samples immediately begin with incubation of the plate.

**Well [µl]**

- Blank
- NC
- PC
- SUB

**Step 2**

- Seal MTP with self-adhesive foils (not required in an ELISA processor).

**Well [µl]**

- Blank
- NC
- PC
- SUB

**Step 3**

- Seal MTP with self-adhesive foils (not required in an ELISA processor).

**Well [µl]**

- Blank
- NC
- PC
- SUB

**Step 4**

- Seal MTP with self-adhesive foils (not required in an ELISA processor).

**Well [µl]**

- Blank
- NC
- PC
- SUB

**Step 5**

- Seal MTP with self-adhesive foils (not required in an ELISA processor).

**Well [µl]**

- Blank
- NC
- PC
- SUB

**References**

- [REF]
- [UK]
- [IVD]
Calculation of qualitative determination
After measuring the extinction values at 450 nm in all wells (reference filter: 615-690 nm), the mean value of the blanks is subtracted from the extinction values of the controls and samples. Mean extinction value ≤ 0.00 OD. After subtraction of the blank, the control values must meet the following criteria of validity:
Mean OD-value of NC: 0.200; Mean OD-value of PC ≤ 0.800

Calculation of quantitative determination
The kit contains a standard conformity to the guidelines of the German Medical Assembly for the prevention of infection in laboratory diagnostics (RBKALD) for quantitative determination.
After measuring the extinction values at 450 nm in all wells (reference filter: 615 - 690 nm), the mean value of the blanks is subtracted from the extinction values of the controls and samples. Mean extinction of blanks ≤ 0.00 OD. After subtraction of the blank, the control values must meet the following criteria of validity:
Mean OD-value of NC: 0.200; Mean OD-value of STD: 20.800

Calculation of the cut-off-value and grey area
The cut-off-value is calculated from the mean OD value of the negative control (NC $\bar{X}$) plus 0.200. Cut-off value = $NC \bar{X} + 0.200$. The grey area extends between cut-off-value and cut-off-value minus 20%.

Interpretation of the result
Samples with an extinction value below the grey zone are considered to be negative. If a sample has an extinction value equal or greater than the grey zone, it is considered to be positive for the specific EBNA-specific IgG antibodies. If the OD-value of the retested sample is within the grey area (questionable result), we recommend to request for a follow up sample. For further interpretations of reactivity pattern (8) a complete interpretation scheme is available on request from Bio-Rad.

Assay procedure (quantification)
The kit contains a testpanel of 10 sera representing low, low-reactive and high reactive samples was tested on 10 different days. The inter-assay variability of these sera were 7.7% - 16.5%. The samples of the same panel were tested 5 times within one test run. The intra-assay variability of these sera was 4.8% - 15.4%.

Expected values
Ninety-nine serum specimens obtained from healthy, asymptomatic blood donors were tested with EBNA IgM/ELISA. Of the 99 specimens, 52 were found to be positive (52.53%) and 7 were found to be negative (7.06%). This is consistent with published rates for the prevalence of exposure to EBV in the adult population. Prevalence may vary depending on a variety of factors such as geographical location, age, socioeconomic status, race, type of test employed, specimen collection and handling procedures, clinical and epidemiological history (6, 7). Sera from patients after CMV (20), toxoplasma (20) and mycobacterial diseases (20) were analyzed. Using the EA IgM, EA IgA and EBNA IgG ELISA system 7, 17 and 29 samples could be identified with primary reactive- and past EBV infection (5).

Trouble Shooting Guide
1) Unexpected high rate of reactive results: Samples and controls were pipetted prior to pipetting of NC or mixing was insufficient.
2) Mean blank value higher than criteria of validity, ≥ 0.100 OD:
   a) SBT turned blue due to oxidation or contamination.
   b) Mixing fault: Perform 5x wash cycles/washing step. If using a manual washing de-
      vice, perform 7x wash cycles/washing step. Use Bio-Rad WB as contained in the kit.
   c) Incubation fault: Temperature too high, incubation time was exceeded or plate was
      incubated at a higher temperature or for a longer time.
   d) Contamination: Perform 7x wash cycles/washing step. Use Bio-Rad WB as contained in the kit.
3) Yellow coloration in all wells:
   (see 2a, 2b)
   a) [RB] contamination; Prepare new [WB].
   b) [CD] or [CN] contamination; Repeat test with reagents from unopened vials. Use reagents under less microbial conditions.
4) Mean value of PC $\bar{X}$ below ≤ 0.800 OD:
   a) Exceed of expire date.
   b) Temperature too low or fall below incubation time.
   c) Washing fault: Too intensive washing or mechanic contact of manifold and solid
      phase of the well.
   d) Contamination of PC $\bar{X}$ or [WB].
5) Mean value of PC $\bar{X}$ higher than ≥ 0.200 OD: (see 1 and 2a-d)
   a) NC was not pipetted subsequent to pipetting of samples; Pipet all samples prior to
      pipetting of blanks and controls.
   b) Contamination with the lid of the NC $\bar{X}$.
   c) Washing fault: Perform 5x wash cycles/washing step. If using a manual washing de-
      vice, perform 7x wash cycles/washing step. Use Bio-Rad WB as contained in the kit.

Mathematical determination:
The $\text{RU/mL}$ sample of a measured sample at 1:21 dilution is calculated according to the following formula:

$$\text{RU/mL sample} = \left(\frac{\text{OD cut-off} \times \text{STD}}{10} \right) \times \frac{\text{OD sample-cut-off}}{\text{STD} \times (1:21)}$$

For quantitative determination of a sample that has been diluted to more than 1:21, the value determined from the above calculation must be multiplied by the predilution factor (e.g. measured value x 10 for a dilution of 1:210).

Interpretation and information
The quantitative values determined are test-specific and are not comparable with values determined using the C-EBNA-1-IgG tests of other manufacturers. The definition of RU/mL is based on a Anti-EBNA-1-IgG specific serum standard used by Bio-Rad internally for standardizing the calibrator that is provided with the test kit. Reported results should point this out by means of an additional remark (e.g. ‘Bio-Rad Anti-EBNA-1-IgG units’).
However, consistency of the results of this quantitative method across the various test kit lots is ensured within normal manufacturing tolerances. The quantitative value does not correspond with the end-point titer of the sample. As a rough approximation, however, the quantitative value is proportional to the end-point titer. In the case of doubtful constellations, low Anti-EBNA-1-IgG quantitative values (<100 RU/mL) can support the diagnosis of past EBV infection. During immunosuppressive therapy or related to an acquired immunodeficiency (AIDS) original significant anti-EBNA IgG antibodies may strongly decline with time, sometimes it was shown, that a small portion (1.2%) of healthy individuals with previous (latent) EBV infection and a positive anti-EBNA IgG was not positive (negative IgG negative IgA). More rarely (<1%), there is apparent that healthy individuals in which EBNA antibodies can never be detected (EBNA non-

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