NPC-REAAD™ Anti-EBV EA IgA ELISA FOR RESEARCH USE ONLY

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INTENDED USE

NPC-REAAD™ ELISA Anti-EBV EA IgA Test (**N**aso**p**haryngeal **C**ancer – **RE**combinant **A**ntigen-**A**ntibody **D**etection) is intended for qualitative and semi-quantitative detection of IgA antibodies in human serum. The test utilises Epstein Barr Virus (EBV) specific proteins to aid in the diagnosis of NPC by detecting a patient's EA IgA antibody response in serum samples. A number of publications, referenced below, have demonstrated a correlation with EBV antibody response, in particular an IgA response and NPC.

SUMMARY AND EXPLANATION OF TEST

Nasopharyngeal Cancer (NPC) is closely associated with the infection of Epstein Barr Virus (EBV), a virus belonging to the Herpes Virus family. The virus has also been implicated in diseases such as Infectious Mononucleosis (IM), Burkitt's lymphoma and Hodgkin's disease.

NPC-REAAD™ uses 4 highly NPC-specific polypeptides generated by DNA recombinant technology to detect the presence of EBV proteins known to be associated with NPC. These polypeptides are known as EA-D, RR-L RR-S and the other is a novel proprietary protein discovered by Restalyst during development of NPC-REAAD™. Studies have shown that their combined specificity to NPC patients' serum increased significantly, enabling discrimination of NPC from non-NPC patients. The Test uses an arbitrary unit of measurement known as REAAD™-units (RU) which can be used for determining the patients' NPC risk level.

A variety of commercial assays are available to detect acute or chronic EBV infections based on anti-EBV IgG, IgM or IgA antibodies against antigens such as VCA, EA and EBNA-1. These assays are helpful for the monitoring of convalescence and reactivated EBV infections. In addition they can also aid in the detection of Burkitt's lymphoma, NPC and other EBV-related diseases. All data from these assays, however at best offer supplementary information to clinicians and act as an aid to diagnosis only. It is important therefore that a diagnosis is made only in conjunction with other recommended clinical investigations.

PRINCIPLE OF THE PROCEDURE

Recombinant NPC-specific antigens are bound onto the microwells. Antibodies that are specific to these antigens, if present in diluted human serum, will bind to the antigens on the solid phase, forming antigen-antibody complexes. A combination of the 4 main polypeptides is used. This combination of the polypeptides has shown to be broadly reactive with NPC patient serum. Any excess antibody is removed through washing. Anti-human IgA conjugated with horseradish peroxidase then binds to the antigen-antibody complexes. Excess conjugate is removed by washing. Substrate Developer TMB (tetramethylbenzidine) forms a blue colour in the presence of the bound conjugate.

As the assay determination is based on enzymatic reaction, the reaction can be stopped by the addition of 1N H_2SO_4, which will turn the blue coloration to yellow. The wells can then be read on any suitable spectrophotometer or microwell ELISA plate reader. It is always recommended to read the wells at 450 nm against a 620-630 nm reference filter to eliminate any possible causes of interference.

MATERIALS PROVIDED

The amount of reagents is sufficient for 5 optimal runs.

Label	Reagent Constituents	Quantity
MICROPLATE	(Ready to use) 12 strips x 8 microwells coated with EA antigens	1 microplate
20X WASH BUFFER	NaCl (pH 7), 1% Tween 20	50 mL
POSITIVE CONTROL	Positive Serum. Preservative: 0.01% NaN_3	100 µL
NEGATIVE CONTROL	Negative Serum. Preservative: 0.01% NaN_3	100 µL
REFERENCE CONTROL	Reference Serum. Preservative: 0.01% NaN_3	120 µL
SAMPLE CONJUGATE BUFFER	Phosphate buffer, Bovine serum albumin. Preservative: 0.01% Thimerosal	40 mL
100X HRP CONJUGATE	Anti-IgA coupled with horseradish peroxidase in stabilizing buffer.	200 µL
TMB SUBSTRATE SOLUTION	(Ready-to-use) Solution with 3,3',5,5' tetramethylbenzidine	15 mL
STOP SOLUTION	(Ready-to-use) 1N H_2SO_4 solution	15 mL
	Reseal-able bag for unused microwells	1

PRECAUTIONS

1. This kit is for research use only and should not be used as a diagnostic tool.
2. Serum used for Calibrators and Controls have been excluded for antibody to Human Immunodeficiency Virus (HIV), Hepatitis C (HCV), and Hepatitis B. However, no test assay can offer complete assurance that HIV, HCV, Hepatitis B virus or other infectious agents are absent, specimens and reagents should be treated as if they are of infectious nature with all necessary precaution and safety procedures as set down by the regulating authority in your region.
3. Some countries may regulate this test to be handled at Biosafety Level 2.
4. All components in the test kit have been quality controlled and tested against a Master Lot Unit. Pooling of any component is strictly not recommended; if there

is sufficient balance to carry on testing, they should be use wholly on its own and never be pooled.

5. Reagents are only stable up till date of expiry and the manufacturer is not responsible for usage of expired reagents.
6. Assay set up must be carried out at room temperature. Any balance reagents that have been poured out should not be replaced into their original containers in case of cross contamination. All unused portions should be discarded appropriately.
7. Before opening the Calibrator and Control vials, tap the vials firmly to ensure that the liquids are at the bottom (of the vials).
8. Do not use tap water. Strictly only deionised water can be use whenever required.
9. After each wash, ensure that reagents are added immediately to avoid wells drying up.
10. To avoid cross contamination of reagents, recap containers immediately and change gloves if there are any spills. This is one major reason for false results.
11. Sodium Azide has been known to inhibit conjugate activity and thus any material that contains the chemical must not be present in the testing area.
12. Over or under washing can result in assay variation and will affect validity of results.

ADDITIONAL MATERIALS REQUIRED

1. Measuring containers for wash buffer and diluents.
2. Timer (up to 30 minutes)
3. Pipettes capable of dispensing 10-200 µL and 200-1000uL with less than 3%CV.
4. Deionised or distilled water.
5. Paper towels.
6. Wash bottle, semi-automated or automated wash equipment.
7. Microplate spectrophotometer with dual wave length. Actual reading at 450 nm with reference of 620~630 nm.
8. Dilution tubes
9. 37°C Dry-Heat incubator

STORAGE AND STABILITY

1. All reagents must be stored at 2 ~ 8°C. Refer to the package for kit expiry.
2. Unopened microwells must be sealed in the reseal-able bag provided and stored at 2 ~ 8°C.

[NOTE: Only with proper and constant storage will kit be stable for the dating period specified.]

SPECIMEN COLLECTION

1. Collect blood specimens and separate the serum.
2. Handle all blood samples as if of infectious nature.
3. Restalyst only warrants optimal performance if samples are freshly collected that are clear, non-haemolysed, non-lipemic and non-icteric.
4. Store all serum samples at 2 ~ 8°C if test within two days of collection; otherwise freeze in a frost-free freezer of -20°C for longer periods. Thawing and freezing affects sample integrity providing erroneous results.

PROCEDURE OF TEST

1. Place the desired number of strips into the microwell frame. Recommended to provide: one well each for reagent blank, Negative control and Positive control; two wells for Reference control/Calibrator. *If using software for automated system, please check for configuration setup or contact software manufacturer for alternative recommended configuration. Additionally, ensure system is adequately maintained and calibrated appropriately.*
2. Dilute REFERENCE CONTROL, POSITIVE CONTROL and NEGATIVE CONTROL together with patient samples at 20-fold dilution. (10µL of each to 190µL of SAMPLE CONJUGATE BUFFER).
3. Pipette 100µL of the respective into the designated well.
4. Incubate the microplate at 37°C for 30 minutes.
5. Meanwhile, prepare Diluted HRP Conjugate by diluting 1 volume of 100X HRP CONJUGATE with 100 volumes of SAMPLE CONJUGATE BUFFER. (1µL of HRP to 100µL of Conjugate buffer).
   Recommended to prepare Diluted HRP Conjugate 10 to 20 minutes before adding into well. For automated system, Diluted HRP Conjugate may be prepared immediately after Step 2.
   [CAUTION: too early or too late a preparation may yield unstable results]
6. When incubation time is up, wash the wells thoroughly either manually or by using a semi/fully automated washer. Use 300~350µL of Wash Buffer per well. A minimum of 3 wash cycles for manual wash is required while 5 cycles are required for semi/fully automated washers.
   [NOTE: Washing Buffer is prepared by diluting 50mL 20X WASH BUFFER with 950mL distilled water]
7. Ensure that plate is tapped dry; add 100µL of Diluted HRP Conjugate to each well.
8. Repeat steps 4 and 6.
9. When incubation time is up, add 100µL of ready-to-use TMB SUBSTRATE SOLUTION to each well and incubate at 37°C in the dark for 15 minutes.
10. Then add 100µL of STOP SOLUTION to each well and mix well by tapping gently on the sides.
11. Put the microplate in the OD reader to determine the optical density of each well, with primary filter at 450 nm and reference filter at 620~630 nm.

QUALITY CONTROL

For the assay to be valid:

1. The reagent blank control absorbance should be ≤ 0.200.
2. The Negative Control absorbance should be ≤ 0.300.
3. The Reference Control absorbance should be between 0.300 and 0.700.
4. The Positive Control absorbance should be ≥ 0.800.

CALCULATIONS

REAAD™-Units (RU) could be calculated as follow:

$$RU = \frac{OD_{Sample} - OD_{Blank}}{OD_{Reference} - OD_{Blank}} \times 100$$

INTERPRETATION OF RESULTS

Calculate the RU value for each test sample as described above (section "CALCULATIONS")
Results are interpreted as follows:

RU of sample <103 = "Negative"
RU of sample 103 – 127 (115.0 ± 10%) = "Equivocal"
RU of sample >127 = "Positive"

METHOD COMPARISON

A comparison study was undertaken between NPC-REAAD™ and Indirect Immunofluorescence Assay (IFA) using both positive and negative NPC patient samples. Positive samples chosen for the study were clinically diagnosed NPC patient samples which were both Anti-EA-IgA and Anti-VCA-IgA positive by IFA. 98.6% correlation was seen between both methods reflecting the intended use and design of NPC-REAAD™. To assess sensitivity and specificity characteristics at the chosen cut off for the range of sample types representative in the population (negative, range of positive types e.g. low titre /high titre) Receiver Operator Curve analysis was undertaken (ROC analysis). In ROC analysis the true positive rate (Sensitivity) is plotted as a function of the false positive rate (100-Specificity) for different cut-off points. NPC-REAAD™ was found to have a sensitivity of 96% and a specificity of 94%.

INTRA-ASSAY & INTER-ASSAY

Intra-assay and inter-assay precision were determined by testing a positive control and a negative control in replicates of 15 using 3 individual kit lots. The NPC-REAAD™ kit demonstrates an average Intra-assay %CV of 3.83% and 5.44% for positive and negative control respectively as well as an average Inter-assay %CV of 5.76% and 6.67% for positive and negative control respectively.

LIMIT OF BLANK & LIMIT OF DETECTION

Limit of blank was determined by testing a blank control in replicates of 21 using 3 individual kit lots. Limit of detection
was determined by testing a negative anti-EBV EA-IgA control in replicates of 21 using 3 individual kit lots. The
NPC-REAAD™ kit demonstrates an average Limit of blank of 3.18 RU as well as an average limit of detection of 7.36 RU.

LIMITATIONS

1. The values obtained from this assay are intended to be an aid to diagnosis only. Each physician must interpret the results in light of the patient's history, physical findings and other diagnostic procedures.
2. Results from children should be viewed with caution. This kit is designed to measure IgA antibody in patients' samples. Positive results in neonates must be interpreted with caution, since maternal IgA is transferred passively from the mother to the fetus before birth.
3. Results obtained from immuno-compromised individuals should be interpreted with caution.
4. Performing the assay outside the time and temperature ranges provided may produce invalid results. Assays not falling within the established time and temperature ranges must be repeated.
5. The performance characteristics have not been established for visual result determination.
6. There is a possibility of assay cross-reactivity with specimens containing anti-E.coli antibody.
7. This assay is not applicable for patient suffering from Selective IgA Deficiency.

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EFFECTIVE DATE: 02/01/2019

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