HCC-REAAD™ ERBB3 ELISA

CATALOG NO.: RUOHE0100ER3

FOR RESEARCH USE ONLY (RUO)

INTENDED USE

HCC-REAAD™ ERBB3 ELISA (**H**epato**c**ellular **C**arcinoma - **RE**combinant **A**ntigen-**A**ntibody **D**etection) is intended for qualitative and semi-quantitative detection of human epidermal growth factor receptor 3 (ERBB3) proteins in human plasma/serum samples. The test utilises sandwich ELISA using two layers of antibodies (i.e, capture and detection antibody) to determine a subject's ERBB3 proteins levels in plasma/serum samples.

SUMMARY AND EXPLANATION OF TEST

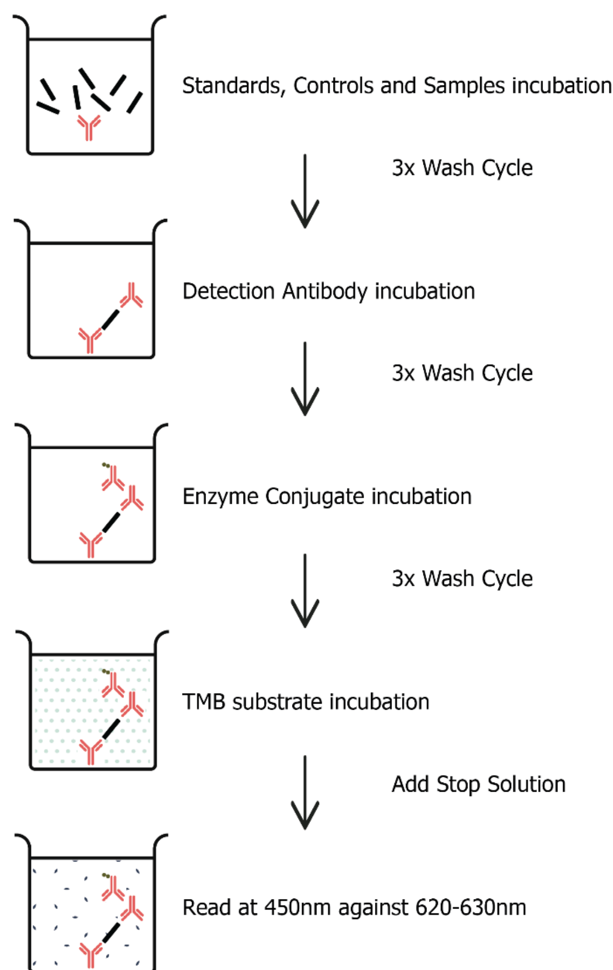
Liver Cancer is the sixth most common cancer malignancy in the world with 83% of cases occurring in less developed countries [1]. Liver Cancer is the second leading cause of cancer death worldwide. The highest estimated mortality rates are in Eastern and South-Eastern Asia [1,3]. Hepatocellular Carcinoma (HCC) is the most common type of liver cancer, accounting for at least 70% of the total liver cancer cases [3]. HCC was once prevalently only in Southeast Asia and Africa due to dietary intake of aflatoxin contaminated food grains. However, in recent years, an increase of HCC incidences is discovered in Europe and United States [4]. Higher levels of ERBB3 proteins were detected in subjects with HCC as compared to non-HCC subjects [2].

HCC-REAAD™ ERBB3 ELISA uses two layers of antibodies (i.e. capture and detection antibody) targeting at two different antigenic epitopes expressed by human ERBB3 protein capable of binding to the respective antibody to semi-quantify the level of human ERBB3 protein. The test uses an arbitrary unit of measurement known as REAAD™-units (RU).

HCC-REAAD™ ERBB3 ELISA incorporating the use of algorithm, is for hepatocellular carcinoma research. Combining the levels of ERBB3 protein determined using HCC-REAAD™ ERBB3 ELISA and levels of AFP (e.g. Elecsys AFP) together with the age of the individual into a mathematical algorithm (binary logistic regression), which enables as a better aid in the determination of HCC as comparing to using AFP alone.

PRINCIPLE OF THE PROCEDURE

Human ERBB3 Capture Antibody is first coated onto the wells of microplates. Samples, standards and controls containing ERBB3 proteins are pipetted into these wells. During the first incubation, the protein antigen binds to the capture antibody and form antigen-antibody complexes. After washing, Human ERBB3 Detection Antibody is added to the wells and binds to the immobilised protein captured during the first incubation. After removal of excess detection antibody, a Horseradish Peroxidase (HRP)-conjugated Streptavidin is added and binds to the detection antibody. After a third incubation and washing to remove the excess HRP conjugate, a TMB substrate solution (tetramethylbenzidine) is added and is converted by the enzyme to a detectable form (color signal). The enzymatic reaction will then be stopped by the addition of 1N Sulphuric acid, which will turn the blue coloration to yellow. The microwells can be read on any suitable spectrophotometer or microwell ELISA plate reader. It is always recommended to read the wells at 450nm against a 620-630 nm reference filter to eliminate any possible causes of interference. The intensity of this colored product is directly proportional to the concentration of antigen present in the original specimen.



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MATERIALS PROVIDED

The number of reagents is sufficient for 4 optimal runs.

Label	Reagent Constituents	Quantity
MICROPLATE	(Ready to use) 12 strips x 8 microwells coated with Human ERBB3 capture antibody	1 Microplate
20X WASH BUFFER	(20X Concentrate) Phosphate buffer (~pH 7) with 1% Tween 20	50 mL
DILUENT	(Ready to use) Phosphate Buffer (~pH 7) with 0.05% Tween 20, 1%BSA	50 mL
STANDARD	(Ready to use) Recombinant ERBB3 protein	1.2 mL
DETECTION ANTIBODY	(Ready to use) ERBB3 antibodies conjugated with biotin molecules	15 mL
100X ENZYME CONJUGATE	(100X Concentrate) Streptavidin conjugated with horseradish peroxidase in stabilizing buffer	150 µL
20X POSITIVE CONTROL	(20X Concentrate) Positive Control	100 µL
20X NEGATIVE CONTROL	(20X Concentrate) Negative Control	100 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 test is intended for research use only and should not be used for diagnostic purposes.
2. Some countries may regulate this test to be handled at Biosafety Level 2.
3. All components in the test kit have been quality controlled and tested against a Master Lot Unit. Pooling of any components is strictly not recommended; if there is sufficient balance to carry on testing, they should be used wholly on its own and never be pooled.
4. Reagents are only stable up till date of expiry and manufacturer is not responsible for usage of expired reagents.
5. 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.
6. Before opening the reagents, tap the vials firmly to ensure that the liquids are at the bottom of the vials.
7. Do not use tap water. Strictly only deionised water can be use whenever required.
8. After each wash, ensure that reagents are added immediately to avoid wells drying up.
9. 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.
10. Over or under washing can result in assay variation and will affect validity of results.

ADDITIONAL MATERIALS

1. Measuring containers for wash buffer and diluents.
2. Timer (up to 30 minutes).
3. Pipettes capable of dispensing 10-200µL and 200-1000µL 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 450nm with reference of 620~ 630nm.
8. Dilution tubes.
9. Plate shaker (approx. 350 rpm).

STORAGE AND STABILITY

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

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

SPECIMEN COLLECTION

We would like to emphasize that the quality of the specimen is extremely important. Restalyst only warrants optimal performance if samples used in experiment are freshly collected that are clear, non-haemolysed, non-lipemic and non-icteric nature.

Recommended Collection & Storage:

1. All blood samples should be handled as if of infectious nature. Plasma/serum samples used should be freshly collected and prepared. Follow recommendations for order of draw outlined by WHO [5].
2. All plasma/serum samples should be stored following recommendations set by WHO [6].
3. 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. Ensure that all reagents, except for 100X ENZYME CONJUGATE, are warmed up to room temperature. Prepare 1X WASH BUFFER by diluting 50mL of 20X WASH BUFFER with 950mL distilled water.
2. Place desired number of wells into microwell frame. Recommendations: 12 wells for STANDARD (including 1 well for Blank), 1 well each for POSITIVE CONTROL & NEGATIVE CONTROL, and 1 well each for respective sample. *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.*

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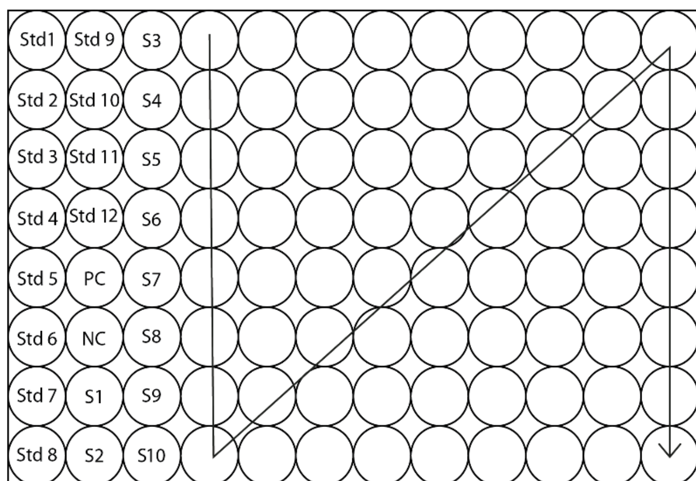

3. For STANDARD, no dilution have to be done for Standard #1. A 2-fold serial dilution will be needed for Standard #2-11 (150µL of previous standard point to 150µL of DILUENT).

Standard #1	300µL of STANDARD
Standard #2 - #11	150µL (Pervious standard) + 150µL DILUENT
Standard #12	150µL DILUENT

4. For 20X POSITIVE CONTROL, 20X NEGATIVE CONTROL, and subject samples, perform a 20-fold dilution (6µL of each to 114µL of DILUENT).
5. Pipette 100µL of mixture into respective wells.
6. Incubate the microplate on a plate shaker (approx. 350rpm) at room temperature for 30 minutes.
7. After incubation, wash the wells thoroughly either manually or by using a semi/fully automated washer. Use 300~350µL of 1X WASH BUFFER per well. Strictly 3 wash cycles for manual wash are required while 5 cycles may be required for semi/fully automated washers.
8. Ensure that plate is tapped dry; add 100µL of DETECTION ANTIBODY to each well.
9. Repeat Steps 6 and 7.
10. Meanwhile, perform a 100-fold dilution for 100X ENZYME CONJUGATE (1µL of reagent to 99µL of DILUENT).
11. Ensure that plate is tapped dry; add 100µL of diluted HRP into respective wells.
12. Repeat Steps 6 and 7.
13. Ensure that plate is tapped dry; add 100µL of TMB SUBSTRATE SOLUTION to each well and incubate at room temperature in the dark for 15 minutes.
14. Add 100µL of STOP SOLUTION to each well and mix well by tapping gently on the sides.
15. Put the microplate in the OD reader to determine the optical density of each well, with primary filter at 450nm and reference filter at 620~630nm.

QUALITY CONTROL

For the assay to be valid:

1. Blank: OD ≤ 0.200
2. Std #8: 0.300 ≤ OD ≤ 0.700
3. Positive Control: OD ≥ 1.000
4. Negative Control: OD ≤ 0.2
5. The R^2 of standard curve should be ≥ 0.99

[NOTE: If the absorbance obtained is below the expected value, it signifies that the reagents have deteriorated]

CALCULATION

REAAD™ Units (RU) is calculated using 4PL equation:

$$y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^b}$$

Plot a 4 Parameter Logistic Regression (4PL) curve using Standard Concentration [RU] (x) against OD – Blank absorbance [OD 450nm – OD 630nm] (y). Sample dilution factor has already been taken into consideration when expressing standards in REAAD™ units.

Example:

Standard	RU	OD	OD – Blank
#1	200000	3.94	3.795
#2	100000	3.9	3.756
#3	50000	3.748	3.604
#4	25000	3.381	3.236
#5	12500	2.388	2.243
#6	6250	1.64	1.496
#7	3125	0.82	0.675
#8	1562.5	0.591	0.447
#9	781.25	0.345	0.201
#10	390.625	0.263	0.118
#11	195.3125	0.198	0.053
Blank	-	0.145	0

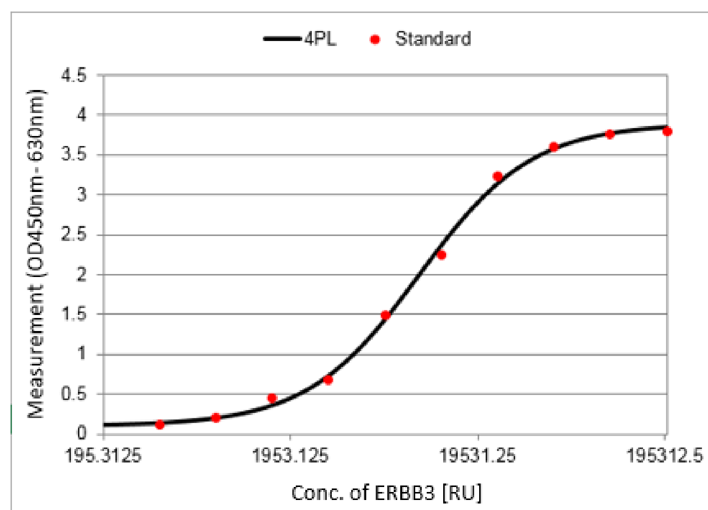

Figure 1: A representative standard curve

Note: The standard curve in Figure 1 is for illustration only. Do not use to derive test result. A new standard curve should be established for each assay.

MEASURING RANGE

195.31 - 20,000.00 RU (defined by the Limit of Quantification and the maximum of the master curve). Values below the Limit of Quantification are reported as < 195.31 RU. Values above the measuring range are reported as > 20,000.00 pmol/L.

The detection limits stated above define the lowest and highest quantifiable analyte concentration using the calibration curve. ERBB3 concentration of 195.31 RU or 20,000.00 RU should be incorporated into the algorithm if results fall below or above the calibration curve, respectively, and could not be quantifiable.

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ALGORITHM

PI = -14.681 + 0.003*[ERBB3] + 0.143*[AFP] + 0.089*[Age]
PP(%) = exp(PI) / [1 + exp(PI)] * 100

RESULTS

Calculate the PP(%) value for each test sample as described above (section "ALGORITHM")
Results are interpreted as follows:

PP(%) of sample ≤48 = "Negative" – Low HCC Correlation
PP(%) of sample >48 = "Positive" – High HCC Correlation

METHOD VALIDATION

HCC-REAAD™ ERBB3 ELISA was found to have high sensitivity of 91.1% and specificity of 91.3% at PP(%) of 32.

The test has also been found to not be affected by haemoglobin.

PRECISION

Both intra- and inter-assay precision was determined by testing 2 samples – 1 negative and 1 weak positive sample. For intra-assay, CVs for both samples were 2.2% and 2.9% respectively. For inter-assay, CVs for both samples were 8.1% and 6.4%.

LIMITATIONS

1. Research use only. Not for diagnostic procedures.
2. 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.
3. The performance characteristics have not been established for visual result determination.

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TECHNINCAL PROBLEMS/ COMPLAINTS

Should there be a technical problem/ complaint, please do the following:

1. Note the kit lot number and the expiry date.
2. Retain the kits and the results that were obtained.
3. Contact Restalyst office or your local distributor.

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