DRG® Oxidized LDL ELISA (Competitive) (EIA-4261)

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1 NAME AND INTENDED USE

The DRG Oxidized LDL competitive Enzyme-Linked Immunosorbent Assay (ELISA) kit is intended to be used for the in vitro quantitative measurement of oxidized low density lipoproteins (oxidized LDL) in human blood plasma.

Lipoprotein measurements are used in the diagnosis and treatment of lipid disorders (such as diabetes mellitus), atherosclerosis, and various liver and renal diseases.

For Research Use only. Not for Use In Diagnostic Procedures

2 SUMMARY AND EXPLANATION OF THE TEST

The oxidative conversion of low density lipoproteins (LDL) to oxidized low density lipoproteins (oxidized LDL) is now considered to be a key event in the biological process that initiates and accelerates the development of the early atherosclerotic lesion, the fatty streak [1-5].

Experimental studies have shown that native LDL becomes atherogenic when it is converted to oxidized LDL, and that oxidized LDL is more atherogenic than native LDL [1-5]. Oxidized LDL is found in monocyte-derived macrophages in atherosclerotic lesions, but not in normal arteries [6]. The uptake of LDL into macrophages does not occur by way of the classic Brown/Goldstein LDL receptor [7]. Numerous studies [1-5, 8] have established that LDL, the major carrier of blood cholesterol, must first be converted to oxidized LDL so that it can be recognized by "Scavenger" or "oxidized LDL" receptors on monocyte-derived macrophages. The binding of oxidized to macrophages is a necessary step by which oxidized LDL induces cholesterol accumulation in macrophages, and thus transforms the macrophages into lipid-laden foam cells [8].

Holvoet and his colleague's [9] were the first to clearly demonstrate that patients with coronary artery disease had significantly elevated plasma levels of oxidized LDL, and that these circulating levels of oxidized LDL were very similar in patients with stable coronary artery disease and in patients with acute coronary syndromes. They found plasma oxidized LDL results to be significantly higher in patients with stable angina, unstable angina and acute myocardial infarction when compared to age matched, presumably healthy, control subjects.

In the publication of Holvoet [9], plasma oxidized LDL levels were measured by a competitive ELISA utilizing a specific murine monoclonal antibody, mAb-4E6. The DRG Oxidized LDL Competitive ELISA kit utilize the same assay technology and the same specific murine monoclonal antibody, mAb-4E6, that Holvoet used in his assay [9,10,11,12,13].

Several noteworthy studies have been reported by clinical researchers who have used the Oxidized LDL ELISA kits. Hulthe and Fagerberg [14] demonstrated the relationship between subclinical atherosclerosis and circulating oxidized LDL levels by showing that oxidized LDL levels were related to intima-media thickness and plaque occurrence in the carotid and femoral arteries. Sigurdardottir, Fagerberg and Hulthe [15] found elevated levels of oxidized LDL in patients with metabolic syndrome. In addition, they found that elevated oxidized LDL levels in metabolic syndrome patients were associated with small LDL-particle size. Kopprasch et al [16] found elevated levels of circulating oxidized LDL in subjects with impaired glucose tolerance (IGT). And Duntas, Mantzou, and Koutras [17] found significantly elevated plasma oxidized LDL levels in untreated patients with overt hypothyroidism.

At the American Heart Association Scientific Sessions 2002, Johnston et al [18] reported that plasma levels of oxidized LDL were substantially higher in patients with unstable coronary artery disease compared to healthy controls. Most important, there was no significant difference between the cholesterol levels of the unstable coronary artery disease patients and the healthy controls.

3 PRINCIPLE OF THE ASSAY

The DRG Oxidized LDL competitive ELISA is based on the monoclonal antibody 4E6. Oxidized LDL in the sample competes with a fixed amount of oxidized LDL bound to the microtiter well for the binding of the biotin- labelled specific antibodies. After a washing step that removes unreactive sample components, the biotin-labelled antibody bound to the well is detected by HRP-conjugated streptavidin. After a second incubation and an additional washing step, the bound conjugate is detected by reaction with 3,3´,5,5´-tetramethylbenzidine TMB). The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

4 PRECAUTIONS

- For Research Use only. Not for Use in Diagnostic Procedures. Not for internal or external use in humans or animals.
- The content of this kit and their residues must not be allowed to come into contact with ruminating animals or swine.
- The Stop Solution in this kit contains 0.5 M H_2SO_4. Follow routine precautions for handling hazardous chemicals.
- All patient samples should be handled as capable of transmitting infections.

Warning! This kit contains reagents that may be infectious!

This kit contains reagents manufactured from human blood components. The source of material have been tested by immunoassay for hepatitis B surface antigen and for antibodies for HIV virus and found to be negative. Nevertheless, all recommended precautions for the handling of blood derivates should be observed. Please refer to HHS Publication no. (CDC) 88-8395 or corresponding local/national guidelines on laboratory safety procedures.

5 SPECIMEN COLLECTION AND HANDLING

The recommended use of specimen in the Oxidized LDL Competitive ELISA is fresh EDTA-plasma.

Collect blood by venipuncture into tubes containing EDTA as anticoagulant and separate the plasma fraction. Samples can be stored at 2–8°C up to one week or at –80°C for at least six months. Avoid repeated freezing and thawing.

Serum and heparin-plasma may also be used.

6 MATERIALS REQUIRED BUT NOT PROVIDED

- 25 µL micropipette with disposable tips.
- 50 µL, 100µL, 200µL and 1000 µL repeating pipettes
- 1000 ml beaker
- Redistilled water
- Test tubes, 3.5 ml
- Micro plate reader with 450 nm filter
- Plate shaker
- Wash device for microtitration plates
- “Vortex”-mixer

7 REAGENTS

Each kit contains reagents for 96 wells, sufficient for 41 samples, two controls and one standard curve in duplicate. For larger series of assays, use pooled reagents from packages bearing identical Lot numbers. The expiry date for the complete kit is stated on the outer label. The recommended storage temperature is +2-8°C.

Reagents	Quantity	Preparation
Coated Plate (Human oxidized LDL)	1 plate, 96 wells 8-wells strips	Ready for use
Calibrators (Human oxidized LDL) Concentrations stated at vial label. Color coded yellow	5 vials 500 µl	Lyophilized Add 500µl redistilled water per vial
Calibrator 0 Color coded yellow	1 vial 2.0 ml	Ready for use
Enzyme Conjugate 11X (Streptavidin-HRP)	1 vial 1.2 ml	Concentrate; Preparation, see below
Enzyme Conjugate Buffer Color coded blue	1 vial 12 ml	Ready for use
Antibody 6X (mouse monoclonal anti-oxidized LDL)	1 vial 1000 µl	Lyophilized Add 6ml Antibody Buffer to make Antibody
Antibody Buffer Color coded red	1 vial 6 ml	Ready for use
Sample Buffer Color coded yellow	1 bottles 50 ml	Ready for use
Wash Buffer 21X	1 bottle 40 ml	Concentrate; Dilute 1:21; add 1 part concentrate to 20 parts of redistilled water to make Wash Buffer
Substrate TMB Note! Light sensitive!	1 vial 22 ml	Ready for use
Stop Solution 0.5M H_2SO_4	1 vial 7 ml	Ready for use

7.1 Preparation of Enzyme Conjugate

Dilute the Enzyme Conjugate 11X (1.2 ml) by adding the Enzyme Conjugate Buffer (12 ml).

If less than 12 strips are used, prepare the needed volume of Conjugate solution by mixing 100 µl Enzyme Conjugate 11X with 1000 µl Enzyme Conjugate Buffer (1+10) for each strip, as indicated table below:

Number of strips	Enzyme Conjugate 11X	Enzyme Conjugate Buffer
2 strips	200 µl	2.0 ml
4 strips	350 µl	3.5 ml
6 strips	600 µl	6.0 ml
8 strips	700 µl	7.0 ml
10 strips	900 µl	9.0 ml

Enzyme Conjugate preparation is stable for 4 weeks at +2–8°C following preparation.

7.2 Dilution of samples

Samples must be diluted the same day as the assay performance. Prepare a tubes for each patient sample. Each sample is diluted in one step to a final dilution of 41 times as follows:

Patient sample 25 µl
Sample buffer 1000 µl

*It is IMPORTANT to ensure that each dilution step is properly mixed before further use.

Sample diluted 41 times in Sample Buffer is stable for 1 day at +4°C.

7.3 Stability of reconstituted and opened reagents

Calibrators	2 weeks at 2–8°C
Antibody Solution	2 weeks at 2–8°C
Enzyme Conjugate	2 weeks at 2–8°C
Sample Buffer	2 weeks at 2–8°C
Wash Buffer	4 weeks at 2–8°C
Coated plate, unused strips, resealed bag	2 weeks at 2–8°C

8 TEST PROCEDURE

All reagents and samples must be brought to room temperature before use.

Perform each determination in duplicate for B_0, Blank, Calibrators, Controls and Unknowns.

Prepare a Calibration curve for each assay run.

	Add to wells:	B_0	Blank	Calibrators	Unknown
1.	Calibrators 0	50 µl	100µl	–	–
2.	Calibrators	–	–	50 µl	–
3.	Unknown	–	–	–	50 µl
4.	Antibody	50 µl	–	50 µl	50 µl

Please observe that mAb solution is added to all wells except to the Blank!

5. Incubate on a shaker for 2 hours at room temperature (18–25°C)
6. Wash 6 times with automatic washer, or manually by hand.
   Wash 6 times with 350 µl Wash Buffer. Aspirate completely between each washing step.
   After final wash, invert and tap the plate firmly against absorbent paper.
7. Add Enzyme Conjugate 100 µl to all wells
8. Incubate on a shaker for 1 hour at room temperature (18–25°C)
9. Wash 6 times with automatic washer, or manually by hand.
   Wash 6 times with 350 µl Wash Buffer. Aspirate completely between each washing step.
   After final wash, invert and tap the plate firmly against absorbent paper.
10. Add substrate TMB 200 µl to all wells
11. Incubate for 15 minutes at room temperature, no shaking.
12. Add Stop Solution 50 µl to all wells
    Place the plate on a shaker for 5 seconds to ensure mixing.
13. Measure the absorbance at 450 nm and calculate the results.

9 INTERNAL QUALITY CONTROL

Commercial controls and/or internal plasma/serum pools with low, intermediate and high oxidized LDL concentrations should routinely be assayed as unknowns, and results charted from day to day.

It is good laboratory practice to record the following data for each assay: kit lot number; reconstitution dates of kit components; OD values for the B_0, Blank, Calibrators and Controls.

* *Commercial controls (e.g. Ox-LDL Control) are available at request. Please contact DRG.*

10 CALCULATION OF RESULTS

10.1 Manual calculation

1. Express the absorbance (B) for Calibrators, Controls and Unknowns as a percentage inhibition of the mean absorbance of the maximum binding at zero inhibition (B_0)

$$\%B_0 = \frac{\text{B (of Calibrators, Controls or Unknowns)}}{B_0 \text{ (mean abs at zero inhibition)}} \times 100$$

2. Plot the calculated percentage values ($\%B_0$) obtained for the Calibrators against the log concentration on lin-log paper and construct a calibration curve.
3. Read the concentration of the Controls and Unknown samples from the calibration curve.
4. Multiply the concentration of the Controls and the Unknown samples with the dilution factor (eg. × 41).

10.2 Computerized calculation

Computerized data reduction of "$\%B_0$" for the calibrators versus the log (concentration) using a cubic spline regression algorithm may be performed to obtain the concentration of oxidized LDL. Multiply the concentration of the Controls and Unknown samples with the dilution factor (eg. × 41).

10.3 Example of worksheet

Wells	Identity	Mean A 450 nm	Mean $\%B/B_0$	Mean Conc. mU/L	x 41 U/L
1 A-B	B_O	2.104			
1 C-D	Blank	0.121	5.7		
	Calibrator				
1 E-F	0.5 U/l	1.433	68.1		
1 G-H	1.2 U/l	1.074	51.0		
2 A-B	2.6 U/l	0.740	35.2		
2 C-D	5.0 U/l	0.488	23.2		
2 E-F	9.7 U/l	0.367	17.4		
	Controls				
2 G-H	Control A	1.185	56.3	0.9	37
3 A-B	Control B	0.844	40.1	2.0	82
3 C-D	Control C	0.670	31.7	3.1	127
	Unknown				
3 E-F	Sample 1	1.211	57.6	0.9	37
3 G-H	Sample 2	0.914	43.4	1.7	70
4 A-B	Sample 3	0.758	36.0	2.5	103

11 PERFORMANCE CHARACTERISTICS

11.1 Detection limit

The detection limit is ≤ 0.3 U/l

11.2 Recovery.

Recovery upon addition is 93 – 115% (mean value is 101%).

11.3 Precision

Precision was calculated from three samples assayed in 2-4 replicates on 21 different occasions.

Sample	Obtained value	Coefficient of variation %		
	mU/L	within	between	total
1	39	6.7	6.9	9.6
2	82	4.8	4.5	6.5
3	128	6.1	7.0	9.3

11.4 Dilutions

Recovery upon dilution for samples 1:21–1:42–1:84 is 1.01, range 0.85–1.16.

11.5 Interference

Grossly lipemic, icteric or hemolyzed samples do not interfere in the assay.

11.6 Calibration

No international reference is at date available. The Oxidized LDL competitive ELISA is calibrated in relative arbitrary units against an in house reference standard.

12 WARRANTY

The performance data presented here was obtained using the procedure indicated. Any change or modification in the procedure not recommended by DRG may affect the results, in which event DRG disclaims all warranties expressed, implied or statutory, including the implied warranty of merchantability and fitness for use. DRG in such event, shall not be liable for damages indirect or consequential.

13 REFERENCES

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14 SUMMARY PROTOCOL SHEET

Add Calibrator 0 for B_0	50 µl
Add Calibrator 0 for Blank	100 µl
Add Calibrators, Controls and Samples	50 µl
Add Antibody to all wells except the Blank	50 µl
Incubate 2 hours at 18–25°C on a plate shaker	
Wash plate with Washing Buffer 6 times	
Add Enzyme Conjugate to all wells	100 µl
Incubate 1 hour at 18–25°C on a plate shaker	
Wash plate with Washing Buffer 6 times	
Add Substrate TMB	200 µl
Incubate 15 minutes	
Add Stop Solution	50 µl
Shake for 5 seconds to ensure mixing	
Measure A450 Evaluate results	