コスモ・バイオ株式会社

Roar

Product Information

Revised January 2006

Roar Ex Vivo CETP Activity Assay, 96 assays

Product No. RB-EVAK

Assay Method: Fluorometric

Number of Assays: 96 assays in 54 µl total assay volume

Assay Components:
Reagent A: 73 µl
Reagent B: 311 µl
Caution: Human blood product. HbsAg; HIV, HCV antibodies: Negative; HBc: Negative. Treat all human blood products as hazardous.
Assay Buffer Blank for Negative Control: 500 µl supplied. Sufficient for 10 blank wells at 50 µl per well.

Storage and Handling: Store assay components at 4°C. If stored properly, components are stable for up to 1 year. DO NOT FREEZE.

Instrumentation: Fluorescence microplate reader (excitation: 465 nm; emission: 535 nm)

Introduction

Cholesteryl ester transfer protein (CETP) is present in normal human plasma and transfers neutral lipids from high density lipoproteins (HDL) to very low density lipoprotein (VLDL) and low density lipoprotein (LDL). CETP plays an important role in lipoprotein metabolism and influences the reverse cholesterol transport pathway. The **Roar Ex Vivo CETP Activity Assay** eliminates the assay dilution factor associated with conventional CETP activity determination methods. The method is useful for measuring the efficacy of a CETP inhibitor in plasma without diluting the plasma sample.

The **Roar Ex Vivo CETP Activity Assay** includes proprietary substrates that enable the measurement of plasma CETP activity through CETP-mediated transfer of fluorescent substrates. The CETP transfer activity results in an increase in fluorescence intensity. The assay substrate volume is 7.4% of the total assay volume and the plasma volume is 92.6% of the assay volume. The assay is incubated for 90 minutes at 37ºC.

Materials Required, But Not Supplied

- Microplate reader with wavelength capabilities (excitation: 465 nm; emission: 535 nm)
- 37°C water bath / incubator
- Plasma source
- Adhesive microplate covers
- 100% isopropanol for optional standardization to convert units of fluorescence intensity transferred to pmoles of substrate transferred

Assay Method

1. Pipette 50 µl of plasma into the wells of a black microtiter plate with a gentle action to minimize frothing of the plasma.

2. Pipette 50 µl of assay buffer blank into empty microplate wells to serve as the negative control for the assay.

3. Premix 73 µl Reagent A with 311 µl Reagent B to make Reagent C. Please be sure to maintain an equivalent ratio of Reagent A : Reagent B when running fewer than 96 assays. For example, you may mix 7.3 µl Reagent A to 31.1 µl Reagent B.

4. Add 4 µl Reagent C to each plasma sample and the buffer blanks. Seal the microplate with an adhesive microplate cover and float the plate in a water bath. Incubate the samples at 37ºC for 90 minutes.

5. Following the incubation, take the microplate out of the water bath and carefully remove the adhesive plate cover. Measure the fluorescence intensity in a fluorescence microplate reader equipped with filters to read an emission wavelength of 535 nm with an excitation wavelength of 465 nm.

Standardization

The concentration of the fluorescent substrate in Reagent A is reported on the vial label. A standard curve is generated by dispersing a sample of Reagent A in isopropanol to derive a fluorescence intensity-to-nmoles of substrate relationship. This will allow you to calculate pmoles transferred in the assay.

Spectrally pure (HPLC grade or better) isopropanol is utilized as the solvent. Note: there should be no background fluorescence when isopropanol alone is read at EX 465 nm / EM 535 nm.

Pipette 5 µl Reagent A into 2 ml isopropanol and serially dilute the mix 1:2 to create a standard curve. Pipette 50 µl of each dilution into the wells of a black microplate and read the plate at EX 465 nm / EM 535 nm.

Perform a linear regression utilizing the substrate concentration as the independent variable. Reagent A with a substrate concentration of 2080 nmoles/ml is entered into the standard as (2080 nmoles) x (0.005 ml) = 10.4 nmoles / (2 ml IPA) x (0.05 ml) = 0.26 nmoles.

Determine the fluorescence intensity transferred in the plasma samples by subtracting the fluorescence intensity of the assay buffer blanks from the fluorescence intensity of the plasma samples. The transferred fluorescence intensity may then be converted to the amount of substrate transferred in pmoles from the regression equation. An example is provided below.

Notes

- Microplate incubations must be placed in a sealed container with standing water to prevent evaporation. Microplates should be sealed as tightly as possible with adhesive plate covers.

- The incubator must be able to rapidly raise the microplate temperature to 37°C. Humidified air incubators are not recommended for any CETP assay. Heat transfer is most efficient when the microplate is allowed to float in a 37°C water bath.

- Microplates must be compatible with fluorescent assays. Some clear plates contain fluorescent plastic. We recommend black microplates and top-reading instruments.

- The filter must be within specifications. An excitation filter of 485 nm with a 20 nm bandwidth may **NOT** be used. This filter will incompletely excite the label and the standard curve will appear to work, but protein activity results will be low.

Performance Characteristics of the Roar Ex Vivo CETP Activity Assay

A. Plasma CETP activity inhibition with neutralizing antibody

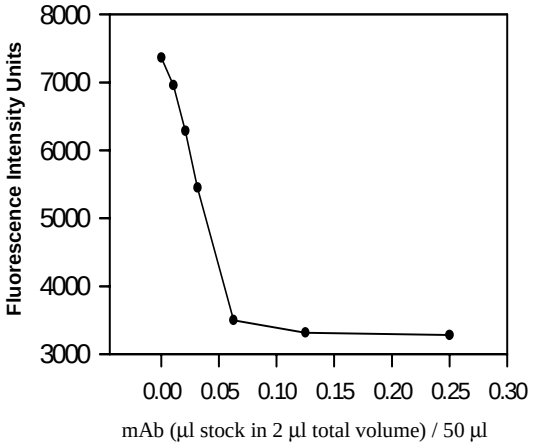

Figure 1. 50 µl samples of human plasma were pre-treated with CETP inhibitory monoclonal antibody. The antibody was diluted from stock mAb in 2 µl total volume.

4 µl Reagent C was added to the plasma samples in a microplate and the samples were incubated for 90 minutes at 37° C.

The microplate was read at an excitation wavelength 465 nm and emission wavelength 535 nm. Total assay volume was 56 µl.

B. Human plasma CETP activity assay results

Plasma samples were assayed in triplicate by pipetting 50 µl of each sample into empty wells of a black 96-well microplate (U-bottom, black Thermo Electron cat. #7205 or VWR cat. #25227-304). Assay buffer blanks (50 µl) were also added to the microplate. Reagent C was prepared by combining 7 µl Reagent A with 30 µl Reagent B in a disposable microfuge tube. Reagent C (4 µl) was added to each of the plasma samples and the buffer blanks. The plate was sealed with an adhesive microplate sealer and placed floating in a preheated 37° C water bath without agitation. After 90 minutes, the plate was read in a fluorescence microplate reader at 465 nm excitation and 535 nm emission.

The fluorescence intensity units transferred during the incubation of Sample 1and Sample 2 was determined by subtracting out the fluorescence intensity in the Buffer Blank:

6077 – 1769 = 4308 FIU transferred for Sample 1
4487 – 1769 = 2718 FIU transferred for Sample 2

Sample	Raw FIU			Average	FIU Transferred
Sample 1	6180	6109	5943	6077	4308
Sample 2	4426	4335	4699	4487	2718
Buffer Blank	1858	1667	1782	1769	

Standard Curve: 5 µl Reagent A was dispersed in 2 ml isopropanol and serially diluted 1:2 x 4. Then 50 µl of each dilution was added to a microplate and the fluorescence intensity was read. Some plasma samples will have more transferred fluorescence intensity than the highest point of a standard curve. You may include higher standard curve values by adding 100 µl volumes of the isopropanol dispersed Reagent A to the plate (see italicized bold in the table below).

Raw FIU		Average	pmoles
100 ul 4909	***100 ul 4634***	***4772***	***520***
2506	2286	2396	260
1250	1194	1222	130
650	650	650	65
340	315	328	33
176	178	177	16

Donor Concentration = 2080 nmoles/ml.

Linear regression from the standard curve:

Intercept	36.6
X Variable 1	9.1

Solve for X pmoles = (FIU transferred – 36.6) / 9.1

Sample	FIU Transferred	pmoles Transferred
Sample 1	4308	469
Sample 2	2718	295

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Contact Information

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Roar

Product Information

Roar Ex Vivo CETP Activity Assay, 92 assays

Catalog No. RB-EVAK version C100
U.S. Pat. No. 7,279,297 and other patents pending

Assay Method:	Fluorometric
Number of Assays:	92 assays in 105 µl total assay volume
Assay Components:	Reagent A: 87 µl Reagent B: 373 µl Assay Blank: 1.5 ml
Storage and Handling:	Store assay components at 4°C. If stored properly, components are stable for up to 1 year. DO NOT FREEZE.
Instrumentation:	Fluorescence microplate reader (excitation: 465 nm / emission 535 nm)

Introduction

Cholesteryl ester transfer protein (CETP) is present in normal human plasma and transfers neutral lipids from high density lipoproteins (HDL) to very low density lipoprotein (VLDL) and low density lipoprotein (LDL). CETP plays an important role in lipoprotein metabolism and influences the reverse cholesterol transport pathway. The **Roar Ex Vivo CETP Activity Assay** eliminates the assay dilution factor associated with conventional CETP activity determination methods. The method is useful for measuring the efficacy of a CETP inhibitor in plasma without diluting the plasma sample.

The **Roar Ex Vivo CETP Activity Assay** includes proprietary substrates that enable the detection of CETP-mediated transfer of neutral lipid among the substrates. The CETP transfer activity results in an increase in fluorescence intensity. The combined assay substrate volume is 4.8% of the total assay volume and the plasma volume is 95.2% of the assay volume. The assay is incubated for 90 minutes at 37°C.

Materials Required, But Not Supplied

Microplate reader with wavelength capabilities (excitation: 465 nm; emission: 535 nm)
37°C water bath / incubator
Plasma source

Assay Method

1. Pipette 100 µl of frozen-then-thawed plasma samples into the wells of a black microtiter plate with a gentle action to minimize frothing of the plasma.

2. Pipette 100 µl of assay buffer into empty microplate wells to serve as assay blanks.

3. Premix 87 µl of Ex Vivo Kit Reagent A per 373 µl of Ex Vivo Kit Reagent B to make Reagent C.

4. Add 5 µl of Reagent C to each well of the microplate containing plasma samples and each well containing assay buffer blanks. Seal the plate with an adhesive plate sealer and float the plate in a water bath at 37 degrees C for 90 minutes.

5. Following incubation, remove the plate from the water bath and carefully remove the plate sealer.

6. Read the plate at an excitation wavelength of 465 nm and emission wavelength of 535 nm.

Standardization

The concentration of fluorescent substrate in the Ex Vivo CETP Activity Kit Reagent A is printed on the vial label. A standard curve is generated by dispersing a sample of Reagent A in isopropanol to derive a fluorescence intensity-to-nmoles of substrate relationship. This will allow you to calculate pmoles transferred by your samples in the assay.

Spectrally pure (HPLC grade or better) isopropanol is utilized as the solvent. Please note: there should be no background fluorescence when isopropanol alone is read at EX 465 nm / EM 535 nm.

Pipette 5 µl Reagent A into 2 ml isopropanol and serially dilute the mix 1:2 to create a standard curve. Pipette 100 µl of each dilution into the wells of a black micro plate and read the plate at EX 465 nm / EM 535 nm.

Perform a linear regression utilizing the concentration reported on the label as the independent variable. For example, a Reagent A substrate concentration of 1967 nmoles/ml means the highest standard is (1967 nmoles) x (0.005 ml) = 9.84 nmoles / (2 ml IPA) x (0.1ml) = 0.492 nmoles.

Assay Notes

- If DMSO is used, pipette 4 µl of DMSO +/- compound into the wells of black microtiter plates with a gentle aspiration of a portion of the contents of the well to mix plasma and DMSO/compound.

- Microplate incubations must be placed in a sealed container with standing water to prevent evaporation. Microplates should be sealed as tightly as possible with plate sealers.

- The microplate incubator must be able to rapidly raise the assay temperature to 37°C. Test the incubator with a small container of water and a thermometer. Large, humidified air incubators may cause problems by slowly increasing the temperature of the assay.

- Microplates must be compatible with fluorescent assays. Some clear plates contain fluorescent plastic. We recommend black microplates (top-reading plate readers only).

- The filter must be within specifications. An excitation filter of 485 nm with a 20 nm bandwidth may **NOT** be used. This filter will incompletely excite the label and the standard curve will appear to work, but protein activity results will be low.

Human Plasma Assay Results: The following data was generated with three human plasma samples.

Test 100 µl human plasma in the Ex Vivo CETP activity assay using 5 µl of Reagent C. Incubate 90 minutes at 37° C. Total assay volume is 105 µl.

Sample	Raw FIU			Average	FIU Transferred
Human Plasma 1	5943	5534	5598	5692	4045
Human Plasma 2	3565	3327	3177	3356	1709
Human Plasma 3	4519	4603	4188	4497	2850
Blank	1663	1633	1644	1647	

Standard Curve: 5 µl Reagent A dispersed in 2 ml isopropanol is serially diluted 1:2 x 6 and 100 µl of each dilution is read. Donor Concentration = 1967 nmoles/ml

pmoles	Average	Raw FIU	
492	4717	4966	4467
246	2389	2339	2438
123	1292	1219	1365
62	633	552	714
31	379	353	404
15	240	209	270
0	87	96	77

Linear regression from standard curve:

Intercept	90
X Variable	9.4

Solve for X pmoles = (FIU transferred – 90) / 9.4

Sample	FIU transferred	pmoles transferred
Human Plasma 1	4045	421
Human Plasma 2	1709	172
Human Plasma 3	2850	294

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