Roar

Product Information

Roar Ex Vivo CETP Activity Assay, 100 assays

Cat. No. RB-EVAK version C100

U.S. Pat. Nos. 7,279,297; 7,642,065, European Pat. No. 1492880, Japanese Pat. No. 4339702 and Canadian Pat. No. 2480439

Assay Method:	Fluorometric
Number of Assays:	100 assays
Kit Contents:	**Reagent A** 95 µl **Reagent B** 405 µl **Assay Buffer** 1.7 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), present in normal human plasma, transfers neutral lipids among the different classes of lipoproteins (HDL, LDL, VLDL). CETP plays an important role in lipoprotein metabolism and influences the reverse cholesterol transport pathway. The **Roar Ex Vivo CETP Activity Assay** (**RB-EVAK**) is a unique test for CETP activity that eliminates the dilution factor associated with conventional CETP activity assays. Unlike other methods, **RB-EVAK** measures CETP activity with all plasma components near physiological concentration, thus giving an *in vitro* measurement that incorporates effects of known endogenous inhibitors (e.g., apo-C1), which are known to occur *in vivo*. The **RB-EVAK** assay has been validated and used extensively by pharmaceutical companies in human clinical trials of CETP inhibitors.

RB-EVAK includes proprietary substrates that enable the detection of CETP-mediated transfer of neutral lipid, and CETP transfer activity results in an increase in fluorescence intensity. The combined assay substrate volume is just 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.

Advantages

- Non-dilution assay, useful for clinical trials that do not allow for interpretations including a dilution factor
- Effects of endogenous inhibitors are represented in activity measurement because plasma is not diluted
- Assay substrates are stable with addition of DMSO, enabling testing of compounds prepared in DMSO
- Assay results are not affected by endogenous plasma HDL, LDL, or VLDL concentrations
  - The Roar donor particle is the preferred substrate by CETP over HDL, thus eliminating competition from endogenous HDL present in the plasma sample.
  - The addition of excess exogenous acceptor normalizes endogenous acceptor lipoprotein concentration present in the sample.

- Other methods, including radioisotopic methods, are affected by endogenous HDL concentration. An increasing plasma HDL concentration in the sample decreases the specific activity of the labeled HDL substrate due to the equal preference by CETP for either labeled or unlabeled HDL.

Materials Required, But Not Supplied

Microplate reader (excitation: 465 nm / emission: 535 nm)
37°C water bath
Microplates (black with round bottom)
Adhesive plate seals
Plasma source, thawed at time of assay

Assay Method

1. Thaw plasma samples at the time of assay. Gently pipette 100 µl of plasma samples into the wells of a black microplate to minimize frothing of the plasma.

2. Pipette 100 µl of **RB-EVAK Assay Buffer** into empty microplate wells (e.g., in duplicate) to serve as assay blanks (this is necessary to calculate transfer activity).

3. Prepare a master mix tube of "Reagent C" that will allow for distribution of 5 µl per assay well, as follows: for 20 wells, you will need 18.8 µl of **RB-EVAK Reagent A** and 81.2 µl of **RB-EVAK Reagent B**.

4. Add 5 µl of freshly prepared Reagent C per well to plasma samples and assay blanks with gentle mixing. Seal the plate with an adhesive plate seal and float it in a water bath at 37˚C for 90 minutes.

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

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

Assay Notes and Tips

- Microplates must be compatible with fluorescent assays (Note: some clear plates contain fluorescent plastic). We recommend black microplates such as Corning's 96 Well Black Round Bottom Polystyrene plates (e.g., Corning #3792).

- Microplates should be sealed as tightly as possible with adhesive plate seals to prevent evaporation and so that the contents of assay wells are not disturbed during incubation.

- The microplate incubator must be able to rapidly raise the assay temperature to 37°C; for this reason we recommend that microplate incubations always occur in a preheated water bath. Large, humidified air incubators may cause problems by slowly increasing the temperature of the assay. If you must use an incubator, then the incubation should occur in a small container of water and temperature should be monitored using a thermometer.

- DMSO (up to 4 µl) may be used in the assay with or without compounds, i.e., CETP inhibitors. Pipette gently to minimize frothing when mixing DMSO/compound treatments with plasma samples or assay blanks. Additionally, assay blanks containing each of the DMSO/compound treatments (e.g., concentrations titrated from 0) should be run alongside samples and used as the blanks for sample wells containing the corresponding DMSO/compound treatments.

- The microplate reader must have a filter within the stated specifications. An excitation filter of 485 nm with a 20 nm bandwidth may not be used; this filter will incompletely excite the label and although a standard curve run with isopropanol may appear to work, transfer activity observed when the assay is run with plasma samples will be low.

Interpretation Guide

A) Data Output

The following data was generated using triplicate wells in the **Roar Ex Vivo CETP Activity Assay** (**RB-EVAK**). To each well either 100 µl **RB-EVAK Assay Buffer** or 100 µl of human plasma (here we used samples from three different individuals) was mixed with 5 µl of freshly prepared Reagent C, incubated for 90 minutes at 37° C, and read at excitation: 465 nm / emission: 535 nm. Calculation of transfer activity is shown in the last column.

Sample	Raw FIU			Average FIU	FIU Transferred = (Sample - Blank)
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	N/A

B) Data Interpretation / Presenting Transfer Activity as Picomoles Transferred

Reporting the units of CETP transfer activity as picomoles transferred is a preferable way to present the data generated by the **Ex Vivo CETP Activity Assay** (**RB-EVAK**) for many applications because those units are easier to interpret than fluorescence intensity and because FIU vary among microplate readers. In order to derive a fluorescence intensity to picomoles of substrate relationship, you may run a standard curve on your fluorimeter by dispersing a small volume of **RB-EVAK Reagent A** in isopropanol (see procedure below). Using this standard curve, you will be able to calculate picomoles (pmol) transferred by your samples in the assay.

Notes:

- We do not recommend running the standard curve alongside samples in the assay.
- Spectrally pure (HPLC grade or better) isopropanol should be utilized as the solvent. Please note: there should be no background fluorescence when isopropanol alone is read at excitation: 465 nm / emission: 535 nm.
- The concentration of fluorescent substrate in **RB-EVAK Reagent A** is printed on the vial's label.

Procedure (preparing the standard curve):

1. Mix 5 µl **RB-EVAK Reagent A** with 2 ml isopropanol.
2. Perform serial dilutions (1:2) of the mixture to create data points on the standard curve.
3. Pipette 100 µl of each dilution into the wells of a black microplate and make sure to include an isopropanol blank without **RB-EVAK Reagent A**.

4. Read the plate at excitation: 465 nm / emission: 535 nm.
5. Determine the concentration of **RB-EVAK Reagent A** in the initial mixture and the serial dilutions based on the starting concentration printed on the reagent's vial. In the example below, **RB-EVAK Reagent A** substrate concentration of 1967 nmol/ml means the initial mixture is (1967 nmol) x (0.005 ml) = 9.84 nmol / (2 ml IPA) x (0.1ml) = 0.492 nmol or 492 pmol.
6. Plot the data and perform a linear regression utilizing the concentration reported on the vial label as the independent variable (***x***).

Example standard curve data output:

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

Example linear regression from standard curve:

Intercept = 90
Slope = 9.4

Example solving for pmol transferred:

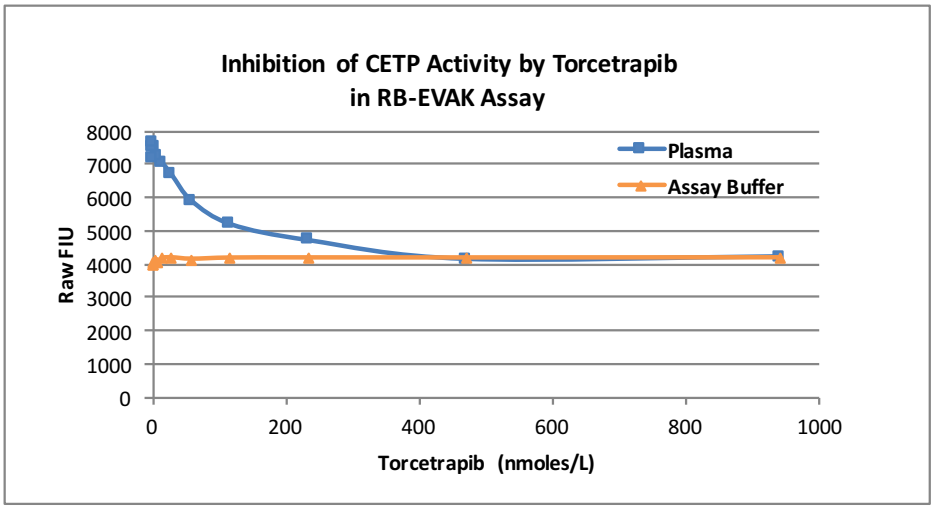
x = pmol transferred = (FIU Transferred - 90) / 9.4

Sample	FIU Transferred	pmol Transferred
Human Plasma 1	4045	421
Human Plasma 2	1709	172
Human Plasma 3	2850	294

Validation of the Assay with a CETP Inhibitor

The following data was generated using the **Roar Ex Vivo CETP Activity Assay** (**RB-EVAK**) with the CETP inhibitor torcetrapib, and demonstrates the assay's measurement of CETP activity near physiological levels, i.e., without a dilution factor.

As shown in the figure below, CETP activity measured in the plasma sample decreases as the concentration of torcetrapib increases. In addition, the figure shows that the fluorescence intensity in the blank (Assay Buffer) does not change as the concentration of torcetrapib increases, which demonstrates that the inhibitor does not quench the fluorescent label in the assay. Details for the procedure are provided following the figure, but it is not necessary for the end-user of the kit to perform this validation.



Procedure used to generate data presented in figure (above):

1. A 100 µM working solution of the CETP inhibitor torcetrapib (Sigma #PZ0170) was made in DMSO.
2. To prepare a range of concentrations of the inhibitor, the torcetrapib working solution was then serially diluted in DMSO.
3. Normal human plasma (100 µl) and **RB-EVAK** Assay Buffer (100 µl) were each added to triplicate wells of a black round bottom microplate (Corning #3792).
4. Reagent C was prepared as directed from **RB-EVAK Reagent A** and **Reagent B**, and 5 µl was added to each well, gently mixing to minimize frothing.
5. Next, 1 µl of each prepared torcetrapib serial dilution was added per well with gentle mixing to triplicate wells of plasma or **RB-EVAK** Assay Buffer. Final % DMSO in assay = 0.94%.
6. The plate was sealed with an adhesive aluminum plate seal and floated in a 37°C water bath for 90 minutes.
7. Following incubation, the microplate was read at excitation: 465 nm / emission: 535 nm, and data points were calculated based on the average of triplicate wells.

For Research Use Only. Not for Diagnostic or Therapeutic Purposes.

Roar Biomedical, Inc., Audubon Biomedical Center, 3960 Broadway, New York, NY 10032 USA
Tel: +1 (212) 280-2983 ▪ Fax: +1 (212) 280-2968 ▪ info@roarbiomedical.com ▪ www.roarbiomedical.com

Roar

Product References

RB-EVAK Citations (2009-2016)

Roar Ex Vivo CETP Activity Assay Kit

1. Deguchi H, Banerjee Y, Elias DJ, Griffin JH. Elevated CETP lipid transfer activity is associated with the risk of venous thromboembolism [published online ahead of print]. J Atheroscler Thromb. 2016;23:000-000.
2. Kawai Y, Iwase N, Kikuchi O, Takata K, Motoyama T, Hagihara M. Acid addition salt of substituted pyridine compound. U.S. Patent 9,321,747. April 26, 2016.
3. Johns DG, Chen Y, Wang S, Castro-Perez J, Previs SF, Roddy TP. Inhibition of cholesteryl ester transfer protein increases cholesteryl ester content of large HDL independently of HDL-to-HDL homotypic transfer: In vitro vs in vivo comparison using anacetrapib and dalcetrapib. Eur J Pharmacol. 2015;762:256-262.
4. Briand F, Thieblemont Q, Muzotte E, et al. Anacetrapib and dalcetrapib differentially alters HDL metabolism and macrophage-to-feces reverse cholesterol transport at similar levels of CETP inhibition in hamsters. Eur J Pharmacol. 2014;740:135-143.
5. Han S, LeVoci L, Fischer P, et al. Inhibition of cholesteryl ester transfer protein by anacetrapib does not impair the anti-inflammatory properties of high density lipoprotein. BBA-Molecular and Cell Biology of Lipids. 2013;1831(4):825-833.
6. Robins SJ, Lyass A, Brocia RW, Massaro JM, Vasan RS. Plasma lipid transfer proteins and cardiovascular disease. the framingham heart study. Atherosclerosis. 2013;228(1):230-236.
7. Siebel AL, Natoli AK, Yap FY, et al. Effects of high-density lipoprotein elevation with cholesteryl ester transfer protein inhibition on insulin secretion. Circ Res. 2013;113(2):167-175.
8. Bentley DD. Evidence of a Drug–Drug interaction linked to inhibition of ester hydrolysis by orlistat. J Cardiovasc Pharmacol. 2012;60(4):390-396.
9. Sarich TC, Connelly MA, Schranz DB, et al. Phase 0 study of the inhibition of cholesteryl ester transfer protein activity by JNJ-28545595 in plasma from normolipidemic and dyslipidemic humans. Int J Clin Pharmacol Ther. 2012;50(8):584-594.
10. Castro-Perez J, Briand F, Gagen K, et al. Anacetrapib promotes reverse cholesterol transport and bulk cholesterol excretion in syrian golden hamsters. J Lipid Res. 2011;52(11):1965-1973.
11. Derks M, Anzures-Cabrera J, Turnbull L, Phelan M. Safety, tolerability and pharmacokinetics of dalcetrapib following single and multiple ascending doses in healthy subjects: A randomized, double-blind, placebo-controlled, phase I study. Clinical Drug Investigation. 2011;31(5):325-335.
12. Connelly MA, Parry TJ, Giardino EC, et al. Torcetrapib produces endothelial dysfunction independent of cholesteryl ester transfer protein inhibition. J Cardiovasc Pharmacol. 2010;55(5):459-468.
13. Niesor EJ, Magg C, Ogawa N, et al. Modulating cholesteryl ester transfer protein activity maintains efficient pre-beta-HDL formation and increases reverse cholesterol transport. J Lipid Res. 2010;51(12):3443-3454.
14. Kuo G, Rano T, Pelton P, et al. Design, synthesis, and biological evaluation of (2R,αS)-3,4-dihydro-2-[3-(1,1,2,2-tetrafluoroethoxy)phenyl]-5-[3-(trifluoromethoxy)-phenyl]-α-(trifluoromethyl)-1(2H)-quinolineethanol as potent and orally active cholesteryl ester transfer protein inhibitor. J Med Chem [supporting information: jm801319d_si_001.pdf]. 2009;52(6):1768-1772.

Roar Biomedical, Inc., Audubon Biomedical Center, 3960 Broadway, New York, NY 10032 USA
Tel: +1 (212) 280-2983 ▪ Fax: +1 (212) 280-2968 ▪ info@roarbiomedical.com ▪ www.roarbiomedical.com