EnBio RCAS for LXRα

Code: LXRA-SRC

STORAGE :
Store at 2 - 8℃

EXPIRATION :
The expiration date is stated on the package and will be a minimum of 4 weeks from the date of dispatch.

WARNING:
For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

FUJIKURA KASEI CO.,LTD.

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1. INTENDED USE

This Receptor Cofactor Assay System (RCAS) kit is sensitive and rapid *in vitro* assay. It is to be used for screening of natural extracts and synthetic chemicals for potential nuclear receptor ligands.

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2. INTRODUCTION

Liver X receptor (LXR) is a member of the nuclear receptor (NR) family. NR is one of the transcription factors that regulate target gene expression. LXR plays an important role in regulation of cholesterol and fatty acid homeostasis, therefore, the study of chemicals bind to LXR is very useful for research of drug screening.

LXR has two subtypes, LXRα and LXRβ. LXRα is expressed in restricted tissues such as liver, kidney, intestine, fat tissue and macrophages, whereas LXRβ is ubiquitously expressed.

NR activation before gene expression consists of 3 steps.

Step1: Ligand binding to NR induces conformational change of NR
Step2: Coactivator is recruited to NR-ligand complex
Step3: Other transcriptional factors are recruited to NR-ligand-coactivator complex

Competitive binding assay detects only the ligand-binding to NR, whereas RCAS detects NR-ligand-coactivator complex. Therefore you can predict the characteristic of your test samples more accurately using this RCAS kit.

3. PRINCIPLE

A peptide containing LXXLL motif of coactivator (SRC1) is immobilized on the microwell plate. The mixture of recombinant human LXRα with potentially agonistic compounds is incubated into the plate. The binding of LXRα-ligand complex to the coactivator peptide on the plate is detected by using HRP conjugated detection antibody.

The HRP activity is determined by the addition of TMB substrate solution. The reaction is stopped by addition of an acid solution and the resultant color read at 450 nm using a microwell plate spectrophotometer. The reactivity of the sample

to the receptor can be determined by calculation of EC50 using the absorbance data.

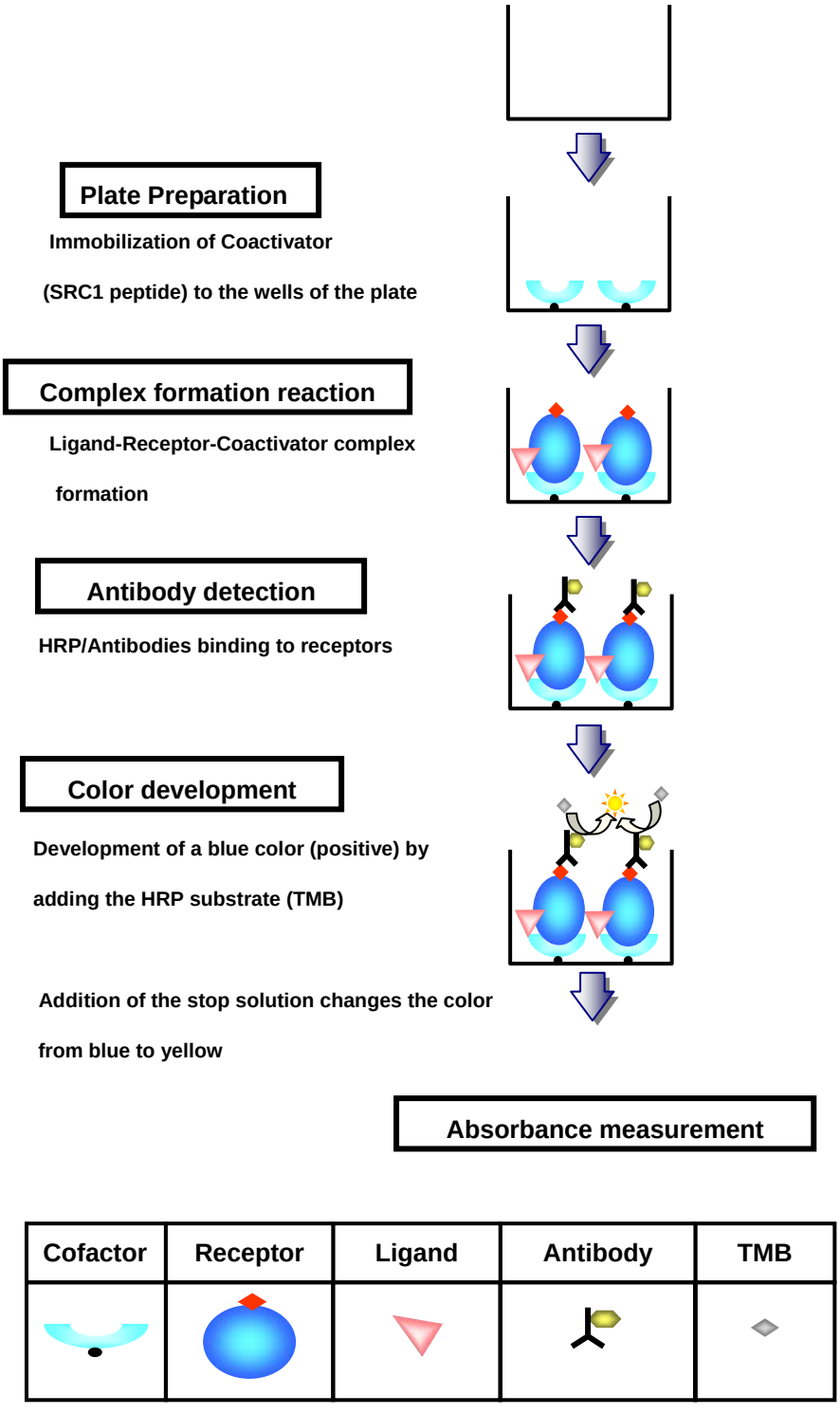
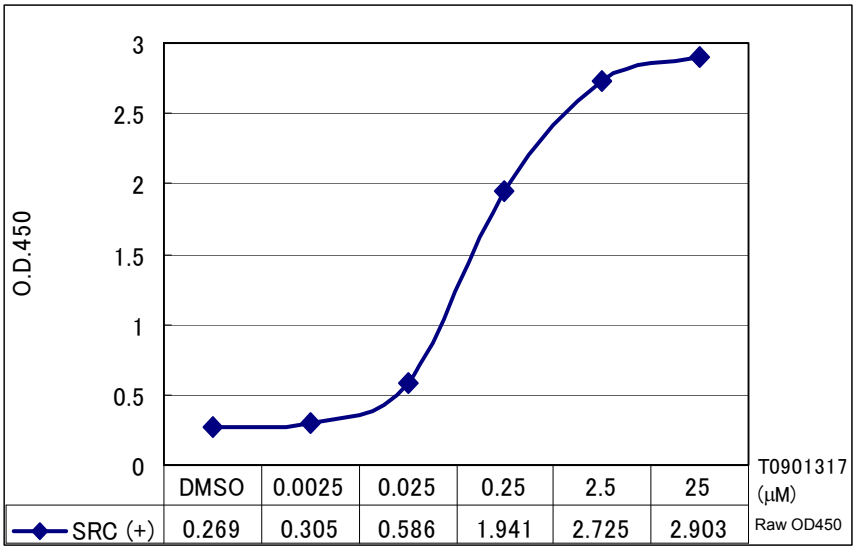


Figure 1. Assay Principle

A.



B.

T0901317(μM)	DMSO	0.0025	0.025	0.25	2.5	25

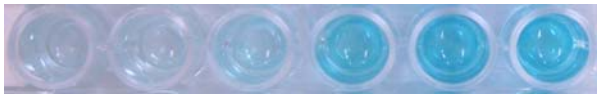


Figure 2. A typical result obtained by using this kit

(A) Dose-response curve of T0901317 (T090) based on raw optical density data obtained from a 10-fold dilution series of T090 from 25 μM.

（B） Actual Photograph of TMB color development in the wells just before adding the stop solution (the photograph was taken when the data described in panel A was measured.)

4. CONTENTS

No	Content	Amount
1	Microtiter plate	1 plate (12 wells x 8 strips) (4 strips in a foil pouch x2)
2	100 x SRC1 peptide	1 tube (0.2 mL in 1.7 mL - plastic tube)
3	LXRα	2 vials (lyophilized in 6 mL - glass vial)
4	100 x Detection antibody <LXRα>	1 tube (0.14 mL in 1.7 mL - plastic tube)
5	Assay buffer	1 bottle (30 mL in 30 mL - PP bottle)
6	10 x Wash buffer, phosphate	1 bottle (50 mL in 60 mL – PP bottle)
7	TMB substrate	1 bottle (25 mL)
8	Stop solution (2.7% sulfuric acid)	1 bottle (14 mL in 15 mL- PP bottle)
9	Dimethyl Sulfoxide (DMSO)	1 vial (4 mL in 5 mL - glass tube)
10	LXRα agonist T0901317, 0.5 mM in DMSO	1 vial (0.3 mL in 1.5 mL - glass tube)
11	Plate seal	2 sheets

5. SAFETY WARNINGS AND PRECAUTION

WARNING: For research use only. Not for clinical diagnostic use. Do not use internally or externally in humans or animals. Avoid contact with skin or eyes.

We recommend that this product and its components be handled only by those who have been trained in laboratory techniques. As all chemicals should be considered as potentially hazardous, it is advisable when handling chemical reagents to wear suitable protective clothing, such as laboratory overalls, safety goggles and gloves.

6. GENERAL NOTES

1. Read the complete procedure before starting the assay.
2. Allow all reagents to reach room temperature prior to performing the assays.
3. Avoid handling the tops and bottoms of the wells both before and after filling.
4. It is recommended that all standards and samples are assayed in duplicate.
5. The total dispensing time for each plate should not exceed 10 minutes.
6. Use only coated wells from the same reagents batch for each assay. Do not mix reagents from different kit lots.
7. New pipette tips should be used for each standard and sample.
8. It is important that the wells are washed thoroughly and uniformly. If using automatic washer, check operation of head and the required amount of Wash buffer before starting. If washing by hand ensure that all wells are completely filled at each wash.

7. ADDITIONAL MATERIALS AND EQUIPMENTS REQUIRED

The following materials and equipment are required:

1. Pipettes or pipetting equipment with disposable tips (1-20 µL, 20-200 µL and 100-1, 000 µL).
2. Multiple pipette (8 channels)
3. Disposable polypropylene test tubes
4. Disposable glass test tubes
5. 500 mL measuring cylinder
6. Distilled or de-ionized water
7. Plate reader capable of reading at 450 nm
8. Microplate shaker （recommend shaking speed : about 800 rpm）
9. DMSO (if you need more for preparation of samples)

8. ASSAY PROCEDURE

(1) Reagent preparation

Note: All reagents must be allowed to equilibrate to room temperature before use. This is a particularly important for the enzyme substrate (TMB).

1. Either distilled or deionized water may be used for reagent preparation.
2. The microtiter plate, substrate and stop reagent are supplied ready to use when equilibrated to room temperature.
3. The three reagents (i.e. 100x SRC1 peptide, 100x Detection antibody and Assay buffer) should be mixed well prior to use.

1x Wash buffer

Stand the 10 x Wash buffer bottle at room temperature, and mix it well by inversion. Transfer 50 mL of the 10 x Wash buffer to a 500 mL measuring cylinder. Adjust the final volume to 500 mL with deionized water and mix thoroughly. Store at 2 - 8℃ for up to one month.

SRC1 (+) solution

Mix 100x SRC1 peptide and Assay buffer with the mixture ratio 1:100. (i.e. preparation of the SRC1 (+) solution for 1 well, 100x SRC1 peptide: Assay buffer= 1.25 µL: 125 µL)

Note: Remaining SRC1 (+) solution can not be stored after the analysis.
If you want to discriminate false positives, preparation of SRC1 (-) solution is required. The assay procedure for discrimination of false positives is described in Section 8 in detail.

Detection antibody solution

Mix 100x Detection antibody and 1x Wash buffer with the mixture ratio 1:100 immediately before use. (i.e. preparation of the detection antibody solution for 1 well, 100x Detection antibody: 1x Wash buffer= 1.25 µL: 125 µL)

Note: Remaining Detection antibody solution can not be stored after the analysis.

(2) Preparation of Coactivator binding plate

The plate consists of 8 strips of 12 microwells each. The strips are supplied as two foil pouches each containing 4 strips. Do not store strips from once-opened foil pouches. Decide the number of strips (4 strips or 8 strips) you wish to run, remove the strips from the foil poach and fit them into the plate frame. Marking each strip is recommended because there is possibility that the strips fall out of the frame during washing. Strips in unopened foil pouches can be stored at 4-8℃ until the expiration date of the kit. If you run assay using 4 strips, retain the plate frame for second partial plate after running the assay.

1. Set up the microtiter plate with sufficient wells for running all blank (DMSO), T090 standard and samples as required. We recommend that all standards and samples are assayed in duplicate.
2. Add 300 µL of 1x Wash buffer (see section 7 (1)) to each well and decant wash buffer by inverting the plate.
3. Repeat step 2 twice more for a total of 3 washes. At the end of 3rd wash remove the residual buffer from all wells by inverting the plate and tapping it briskly on absorbent paper towels several times. Do not let wells dry before proceeding to the next step.
4. Add SRC1 (+) solution (see section 7 (1)) to the each well (100 µL /well).
5. Cover the plate with a plate seal and incubate at room temperature (20-28℃) for 1 hour with shaking (about 800 rpm) on a plate shaker.
6. (During the plate incubation, prepare reaction mixtures (see section 7 (3)-(6).)
7. Aspirate and wash all wells as described in section 7 (2) step 2-3.

(3) Preparation of blank (DMSO) and T090 standard solution

Thaw DMSO and T090 standard solution (0.5 mM) at room temperature and mix well by pipetting.

Note: DMSO and T090 standard solution can be stored at 4℃ after use.

(4) Preparation of sample solution for agonist assay

Dilute samples to be tested in DMSO.

Note: All samples should be prepared in glass tube since chemicals may be absorbed to the surface of plastic tube.

(5) Preparation of LXRα solution

One LXRα bottle is required for running the assay using 4 strips

Add 6 mL of the Assay buffer to a lyophilized LXRα bottle. Mix well by inverting the bottle several times gently. (This LXRα solution should be kept on ice until analysis.)

Note: Remaining LXRα solution can not be stored after the analysis.

(6) Assay protocol

STEP 1: Sample-LXR Reaction

1. Apply the reaction mixtures to the wells of the Coactivator binding plate (see section 7 (2)) as follows:

   Add 95 µL of LXRα solution to each well followed by 5µL of agonist sample. Both positive and negative control should be measured in each assay. As a positive control, add 5 µL of T090 standard (0.5 mM) into the appropriate well after addition of 95 µL of LXRα solution. As a negative control, add 5 µL of DMSO into the appropriate well after addition of 95 µL of LXRα solution.
2. Cover the plate with the plate seal and incubate at room temperature (20-28℃) for 1 hour with shaking (about 800 rpm) on a plate shaker.

STEP 2: Antibody Reaction

1. Aspirate and wash all wells as described in section 7 (2) step 2-3.
2. Pippete 100 µL of Detection antibody solution (see section 7 (1)) to each well.
3. Cover the plate with the plate seal and incubate at room temperature (20-28℃) for 30 min with shaking (about 800 rpm) on a plate shaker.

STEP 3: Detection

1. Dispense the TMB substrate from the bottle only the amount required for

the number of strips you are running, 100 µl per well. Do not use a glass pipette to measure the TMB substrate. Do not combine leftover substrate with that reserved for the second partial plate. Care must be taken to ensure that the remaining TMB substrate is not contaminated. If the substrate reagent is bright blue prior to use, it has been contaminated. DO NOT USE.

2. Aspirate and wash all wells as described in section7 (2) step 2-3.
3. Add 100 µL of TMB substrate to all wells.
4. Incubate at room temperature for 20 min (no shaking!).
5. Add 100 µL of Stop solution to all wells.
6. Read the absorbance at 450 nm.

9. DATA PROCESSING

(1) Discrimination between false and true positive

Some test compounds, particularly at high concentration, may cause non-specific adsorption to the plate and it may yield false positives. To distinguish between true positives and false positives, it is highly recommended to run two tests with or without coactivator (SRC1 (+) or SRC1 (-)).

Assay procedure

a. For preparation of the SRC1 (-) solution, mix DMSO and Assay buffer with the mixture ratio 1:100. (i.e. preparation of the SRC1 (-) solution for 1 well, DMSO: Assay buffer= 1.25 µL: 125 µL)
b. For preparation of the SRC1 (-) wells, SRC1 (-) solution instead of SRC1 (+) solution is added to appropriate wells of the plate. (See Page 9 (2))
c. The two tests, SRC1 (+) and SRC1 (-), are run as described in Assay Procedure on Page 9-11.
d. OD values of blank (DMSO as negative control) and T0901317 (T090) (at 25 µM as positive control) are measured in both SRC1 (+) and SRC1 (-).
e. Determination of true positive/false positive can be done by comparison of the difference in OD values of the test compound between SRC1 (+) and SRC1 (-) with that obtained from the negative control.

Figure 3 shows an example of data analysis using SRC1(+) and SRC1 (-). Panel A shows OD values of the samples observed in SRC1 (+) and SRC1 (-). Panel B indicates the difference in the observed OD values of each sample [SRC1 (+) – SRC1 (-)]. The observed OD values of Sample A and Sample B are almost the same in SRC1 (+), but because of higher OD value of Sample B in SRC1 (-), Sample A turns out to be more active.

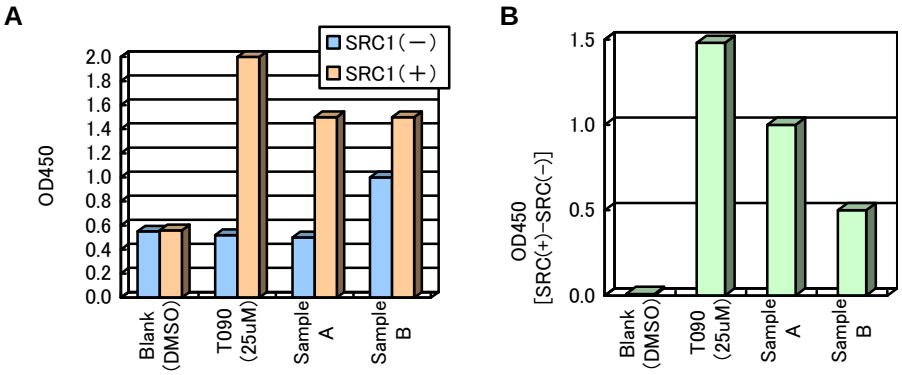


Figure 3. Example of data analysis using SRC1 (+) and SRC1 (-)

(2) Evaluation of analytical precision

When the difference of absorbance value between DMSO and T090 standard (0.5 mM; final concentration is 25 μM) is less than 0.8, the assay values of the samples may not be correct. Retesting is recommended.

(3) Typical assay data

Typical assay data for agonist (T0901317, Paxilline and 24(S),25-Epoxy cholesterol) and antagonist (Geranylgeraniol) are shown in Figure 4. The EC50 or IC50 value of test chemicals will be calculated by logistic 4-parameter curve fit using computer software such as Prism 4 (GraphPad).

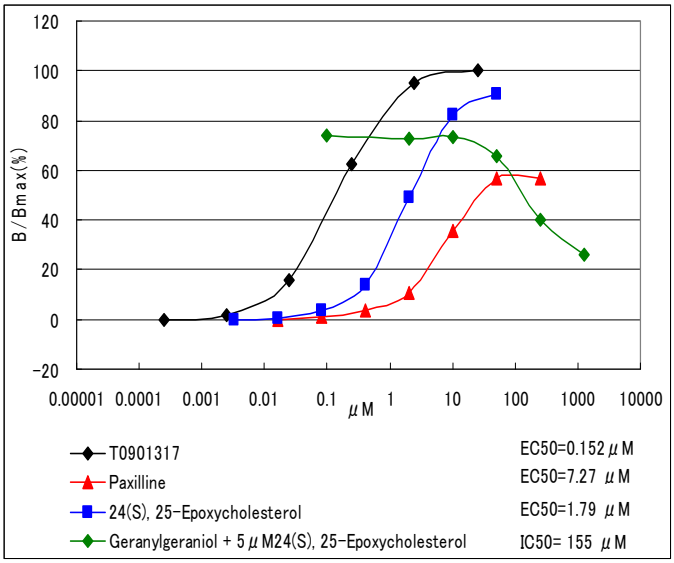


Figure 4. Dose response curve of T0901317, Paxilline, 24(S),25-Epoxycholesterol and Geranylgeraniol (in combination with 5 μM 24(S),25-Epoxycholesterol)

※ B/Bmax : B/Bmax is relative activity and defined as follows.

・The activity of positive control (25 μM T0901317) is defined as 100%

・The activity of negative control (DMSO) is defined as 0%.

B/Bmax values can be calculated according to the following equation:

B/Bmax = (C-B) / (A-B) %

A : (OD_{450} value of the positive control in SRC (+) well)

−(OD_{450} value of the positive control in SRC (-) well)

B : (OD_{450} value of the negative control in SRC (+) well)

−(OD_{450} value of the positive control in SRC (-) well)

C : (OD_{450} value of the test sample in SRC (+) well)

−(OD_{450} value of the test sample in SRC (-) well)

10. TROUBLESHOOTING GUIDE

1. Low optical densities

1.1) Check reader wavelength at 450 nm.

1.2) Check reagents have been correctly reconstituted.

1.3) Check reagents have been stored under the recommended conditions.

1.4) Check incubation time and temperatures.

1.5) Check that TMB substrate has been equilibrated to room temperature before use.

1.6) Read the absorbance of the plate within 30 minutes of adding the stop reagent.

1.7) Check whether the plate is shaked sufficiently by a plate shaker during the reaction.

2. High optical density

2.1) Check the point 1.2.

2.2) Check the point 1.4.

2.3) Ensure the every wash step in the assay procedure is carried out completely.

2.4) Check whether the evaporation of assay solution is occurred in wells during assay incubation due to heat generation from a plate shaker's motor. This induces high background of the assay. To reduce this high background, placing a paper towel between a plate and a shaker is recommended.

3. Poor replication or precision

3.1) Ensure automatic washers are working correctly, and that each well is completely filled and emptied at every wash step when hand washing.

3.2) Check pipette calibration

3.3) Use clean, dedicated reagent trays for dispensing individual reagents.

3.4) Check that plates have been carefully placed into the shaking incubator and the plate reader, to avoid splashing and resultant cross contamination of the wells.

3.5) Check the standard dilution procedure.

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Quick Guide

STEP 1: Plate preparation

1. Pre-wash your plate with 3 x 300 µL/well wash buffer.
2. Add SRC1 solution to the each well (100 µL/well).
3. Incubate the plate with shaking (about 800 rpm) for 1 hr at room temperature.
4. (During the plate incubation) Prepare controls, samples and LXRα solution.

STEP 2: First Reaction (Ligand-Receptor-Coactivator Complex)

1. Wash the plate with 3 x 300 µL/well of wash buffer.
2. Add LXRα solution to the each well (95 µL/well) followed by 5 µL of sample.
3. Incubate the plate with shaking (about 800 rpm) for 1 hr at room temperature.

STEP 3: Second Reaction (HRP/ Antibody-Receptor binding)

1. (After washing the plate with 3 x 300 µL/well of wash buffer), apply 100 µL of Detection antibody solution to each well.
2. Incubate the plate with shaking (about 800 rpm) for 30 min at room temperature.

STEP 4: Detection

1. (After washing the plate with 3 x 300 µL/well of wash buffer), add 100 µL of TMB substrate to each well.
2. Incubate at room temperature for 20 min (no shaking!).
3. Add 100 µL of Stop solution to each well.
4. Read the absorbance at 450 nm.
5. Calculate the results.

Plate layout