ab109717
– Carboxylesterase 1 (CES1)
Specific Activity Assay Kit

Instructions for Use

For the quantitative measurement of Carboxylesterase 1 (CES1) activity in samples from Human and Rat (activity only).

This product is for research use only and is not intended for diagnostic use.

Version 3 Last Updated 3 March 2016
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1. Introduction

Abcam’s enzyme activity assays apply a novel approach, whereby target enzymes are first immunocaptured from tissue or cell samples before subsequent functional analysis. All of our ELISA kits utilize highly validated monoclonal antibodies and proprietary buffers, which are able to capture even very large enzyme complexes in their fully-intact, functionally-active states.

Capture antibodies are pre-coated in the wells of premium Nunc MaxiSorp™ modular microplates, which can be broken into 8-well strips. After the target has been immobilized in the well, substrate is added, and enzyme activity is analyzed by measuring the change in absorbance of either the substrate or the product of the reaction (depending upon which enzyme is being analyzed). By analyzing the enzyme's activity in an isolated context, outside of the cell and free from any other variables, an accurate measurement of the enzyme's functional state can be understood.

Carboxylesterase 1 (CES1, P23141) is a 61 kDa serine hydrolase family enzyme that catalyzes the hydrolysis of an ester into an alcohol and an acid (EC 3.1.1.1) in the liver cell endoplasmic reticulum lumen. Carboxylesterases have broad overlapping specificity, this promiscuity is due to a large conformable active site. Many environmental toxicants, therapeutic and illicit drugs are esterified. Therefore carboxylesterases are involved in the detoxification of a number of different xenobiotics and in the activation of ester and amide prodrugs. Therefore carboxylesterases play a major role as determinants of pharmacokinetic behavior for
most therapeutic agents containing an ester. There is also growing appreciation of the role of carboxylesterases in the processing of endobiotics, including cholesterol esters and triacylglycerol. cholesterol esters and triacylglycerol.

ab109717 (MS789) improves upon conventional CES1 activity assays in two ways. First, this assay immunocaptures in each well only native CES1 from the chosen liver sample or liver derived cell line; this removes all other enzymes. Second, after activity measurement, in the same well/s, the quantity of enzyme is measured by adding a second CES1 specific antibody which is detected by a colorimetric label (HRP). This reaction takes place in a time dependant manner proportional to the amount of enzyme captured in each well. By combining activity and quantity measurements, the relative specific activity can be determined. Specific activity is a useful for measuring up or down regulation of activity by site-specific modification or damage.
2. Assay Summary

Prepare sample (approximately 1 hour)

- Extract the sample pellet
- Incubate on ice for 20 minutes.
- Centrifuge at 12000 x g for 20 minutes and then collect supernatant.
- Determine protein concentration and dilute to within recommendation range

Load plate (2-3 hours)

- Load sample(s) on the plate being sure to include a dilution series of a reference or normal sample and a buffer control as null reference.
- Incubate 2 hrs at room temperature

Activity measurement (1 hour)

- Empty wells and wash each well twice with 300 μl 1X Wash Buffer.
- Prepare activity solution
- Measure activity in a standard microplate reader for up to 10-60 mins \( \text{OD}_{402} \)
Detector antibody incubation (1 hour)

- Empty wells and wash each well twice with 300 μl 1X Wash Buffer.
- Add 100 μl of 1X Detector antibody to every well used.
- Incubate for 30 mins at room temperature.

HRP label incubation (1 hour)

- Empty wells and wash each well twice with 300 μl 1X Wash Buffer.
- Add 100 μl of 1X HRP Label to every well used.
- Incubate for 30 mins at room temperature

Quantity measurement (15 min)

- Empty wells, wash each four times with 300 μl 1X Wash Buffer.
- Add 100 μl of Development Solution.
- Measure OD\textsubscript{600} at 20-second intervals for 15 minutes at room temperature.
3. Kit Contents

Sufficient materials are provided for 96 measurements in a microplate.

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5700X 4-nitrophenol (5.7 M)</td>
<td>20 µL</td>
</tr>
<tr>
<td>Extraction Buffer</td>
<td>15 ml</td>
</tr>
<tr>
<td>Buffer</td>
<td>25 ml</td>
</tr>
<tr>
<td>10X Blocking Solution</td>
<td>10 ml</td>
</tr>
<tr>
<td>1X Development Solution</td>
<td>12 ml</td>
</tr>
<tr>
<td>1X Base Buffer</td>
<td>25 ml</td>
</tr>
<tr>
<td>100X Detector Antibody</td>
<td>125 µL</td>
</tr>
<tr>
<td>100X HRP Label</td>
<td>125 µL</td>
</tr>
<tr>
<td>CES1 Microplate (12 x 8 antibody coated well strips)</td>
<td>96 Wells</td>
</tr>
</tbody>
</table>
4. Storage and Handling

Store all components at 4°C. This kit is stable for 6 months from receipt. Unused microplate strips should be returned to the pouch containing the desiccant and resealed.

5. Additional Materials Required

- Standard absorbance microplate reader, Activity (402 nm), Quantity (450, 600, or 650 nm)
- Multichannel pipette (50 - 300 μl) and tips
- 1.5 mL microtubes
- A fine needle
- Optional 1N HCl
6. Preparation of Buffers and Samples

Note: This protocol contains detailed steps for measuring CES1 activity in human samples. Be completely familiar with the protocol before beginning the assay. Do not deviate from the specified protocol steps or optimal results may not be obtained.

6.1 Preparation of buffers

6.1.1 Prepare 1X Wash Buffer by adding 25 ml Buffer to 475 ml nanopure water.

6.1.2 Prepare 1X Incubation Buffer by adding 10 ml 10X Blocking Solution to 90 mL 1X Wash Buffer.

6.1.3 Before use prepare 1X Activity Solution. For an entire plate add 4.4 µl 5700X 4-nitrophenyl butyrate to 25 ml 1X Base Buffer provided.

Note – the final concentration of substrate is now 1 mM 4-NPB. NPB can be used at greater dilution to achieve lower substrate concentration if desired for example when used in a competitive assay.

6.1.4 Before use prepare 1X Detector Antibody. For an entire plate add 125 µL 100X Detector Antibody to 12.375 ml 1X Incubation Buffer.

6.1.5 Before use prepare 1X HRP Label. For an entire plate add 125 µL 100X HRP Label to 12.375 ml 1X Incubation Buffer.
6.2 Sample Preparation

*Note: Samples must be detergent extracted by the provided Extraction buffer. To do this tissue homogenates, microsomal preparations or cell pellets must be prepared.*

6.2.1 For cultured cells, microsomal preparations or tissue homogenates: Pellet the sample by centrifugation and resuspend the pellet in 9 volumes of Sample Extraction Buffer. A total of 15 ml 1X Extraction buffer is provided. Example - for analyses of multiple samples this is enough to extract 15 samples in 1 ml, 30 in 0.5 ml, 60 in 0.25 ml etc.

6.2.2 Incubate extracts on ice for 20 minutes. Centrifuge 12,000 x g, 4°C, for 20 minutes. Remove the supernatant and discard the pellet.

6.2.3 Determine the protein concentration of the supernatant extract (a protein assay method unaffected by the presence of detergent is critical e.g. BCA assay recommended).

6.2.4 The sample should be diluted to within the working range of the assay as described in Assay Method Step A1 and first consulting Table I. Undiluted extract can be frozen at -80°C.
7 Assay Method

7.1 Activity Measurement

7.1.1 Detergent extracted samples should be diluted in 1X Incubation Buffer. A dilution series of samples is recommended to ensure that samples are within the working range of the activity and the quantity assays. Also include a buffer control (1X Incubation Buffer only) as a null or background reference. Add 100 μl of each diluted sample into individual wells.

7.1.2 Cover/seal the plate and incubate for 2 hours at room temperature with shaking.

7.1.3 Wash the plate

7.1.3.1 Empty the wells by turning the plate over a receptacle and firmly shaking out the well contents in one rapid downward motion. Dispose of biological samples appropriately.

7.1.3.4 Rapidly add 300 μl 1X Wash Buffer to each well. The wells must not become dry during any step. Repeat this wash once more for a total of two washes. After the last wash strike the microplate surface onto paper towels to remove excess liquid.

7.1.4 Gently add 200 μl 1X Activity Solution to each well minimizing the production of bubbles.

7.1.5 Pop any bubbles immediately and record absorbance in the microplate reader prepared as follows:
<table>
<thead>
<tr>
<th>Mode</th>
<th>Kinetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>402 nm</td>
</tr>
<tr>
<td>Time</td>
<td>10 min – 1 hr (as desired)</td>
</tr>
<tr>
<td>Interval</td>
<td>20 sec -1 min</td>
</tr>
<tr>
<td>Shaking</td>
<td>Shake between readings</td>
</tr>
</tbody>
</table>

Alternative— In place of a kinetic reading, at a user defined time/s record the OD 402 nm in all wells. Record the data for analysis and proceed to section B – Quantity Measurement.

### 7.2 Quantity Measurement

#### 7.2.1
Repeat the wash procedure in Assay Method Step A3. Collect and dispose of solutions and any added enzyme inhibitors appropriately.

#### 7.2.2
Add 100 µl of 1X Detector Antibody to each well used. Cover/seal the plate and incubate for 30 minutes at room temperature with shaking.

#### 7.2.3
Repeat the wash procedure in step Assay Method Step A3.

#### 7.2.4
Add 100 µl of 1X HRP Label to each well used. Cover/seal the plate and incubate 30 minutes at room temperature with shaking. Meanwhile, prepare the microplate spectrophotometer using the parameters described below (Step 6).

#### 7.2.5
Repeat the wash procedure in step Assay Method Step A3 however performing a total of four washes.
7.2.6 Add 100 μl HRP Development Solution to each empty well, rapidly pop bubbles, and immediately record the blue color development in the microplate reader prepared as follows:

<table>
<thead>
<tr>
<th>Mode</th>
<th>Kinetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>600 nm</td>
</tr>
<tr>
<td>Time</td>
<td>Up to 15 min</td>
</tr>
<tr>
<td>Interval</td>
<td>20 sec -1 min</td>
</tr>
<tr>
<td>Shaking</td>
<td>Shake between readings</td>
</tr>
</tbody>
</table>

Alternative – In place of a kinetic reading, at a user defined time record the endpoint OD data at 600 nm or 450 nm. The reaction can be stopped by adding 50 μl stop solution (1N HCl) to each well and record the OD at 450 nm - however an intense, sub-saturated, blue color could become saturating by stopping the reaction and measuring at 450nm. Record the data and proceed to Data Analysis

8 Data Analysis

Two example data sets are shown in Figure 1 illustrating data manipulation and analysis of CES1 activity/quantity measurements in an example human cell line – liver derived HepG2 cells.

Determine the relative activity and quantity of an unknown sample by first creating standard curves for the reference sample.
The following two-fold dilution series of HepG2 cell extract was prepared in incubation buffer:

0, 1, 2, 4, 8, 16, 32, 64, 125, 250, 500, 1000 μg/ml of cell extract

Activity and quantity data were sequentially collected as described in this protocol using a microplate reader. Standard curves of these reference sample data were prepared using the microplate reader software which is capable of a 4-parameter data analysis (shown below). Activity and quantity are clearly measurable in the 10-500 μg/ml range when such a fit was applied (alternatively raw data can be exported to Graph Pad Prism or other graphing software).
Unknown samples are interpolated from these reference sample graphs. By doing this for activity, the amount of reference sample required to generate the same amount of activity as the unknown sample is determined. This ratio (or %) of amount of unknown sample: reference sample is the relative activity. Similarly this is performed to determine the relative quantity in an unknown sample. Together these numbers identify any alteration in the activity and or in the quantity of CES1 of an unknown sample.
Figure 2. The ratio of the determined activity and quantity gives the relative specific activity. An increase or decrease in this ratio indicates an increase or decrease in the specific activity of CES1. In this example, the ratio of the raw quantity and activity data measured for each dilution point of reference sample has a clear linear relationship.

8.1 Determination of effect (IC$_{50}$) of Inhibitors on CES1 activity.

CES1 metabolizes many drugs and is also a well known enzyme for off-target inhibition by drugs. Frequently recombinant human CES1 is used as the enzyme for in vitro assessment of drug interaction and inhibition. Here we compare the results found in two studies with data generated using this assay.

Procedure: a normal human HepG2 lysate was applied to each microplate well at 6.25 µg / well i.e. 62.5 µg/ml. Meanwhile using the supplied 12-channel reagent reservoir the following three-fold dilution series of each inhibitor was prepared in Activity solution: 0, 0.02, 0.05, 0.14, 0.4, 1.2, 3.7, 11, 33, 100 µM After two hour sample
incubation, wells were washed according to the protocol and the above inhibitor series, in activity solution, was added to the plate using a 12 channel pipette. Activity rate was measured over a 10 minute period.

Results:

Example 1 - Bencharit et al. Chem Biol 2003; 10(4) 341-9

Tacrine (9-amino-1,2,3,4-tetrahydroacridine) was the first drug approved to treat the symptoms of Alzheimer’s disease. Tacrine is an inhibitor of acetylcholinesterases (serine hydrolase enzymes related to the carboxylesterases). Tacrine was shown in the above study to co-crystalize in the active site of human recombinant CES1 but was not found to inhibit the enzyme due to the large size of the active site. In agreement with this study, tacrine did not significantly inhibit CES1 activity in this assay (Figure 3A).

Example 2 - Fukami et al. Drug Metab Dispos 2010; 38(12):2173-8
Thiazolidinediones (TZD) are a class of antidiabetic drugs which act by binding to peroxisome proliferator activated receptors (specifically PPARγ). The three principle TZDs are troglitazone, pioglitazone and rosiglitazone. In this study, only troglitazone demonstrated a significant inhibition of CES1 at relevant (i.e. bioavailable) concentrations (IC50 5.6 μM).

Using this assay, these TZDs were also shown to inhibit CES1 and in the same order of significance: pioglitazone (IC50 >100 μM), rosiglitazone (IC50 >100 μM) and troglitazone (IC50 3 μM) (Figure 3B).

8.2 Reproducibility

Typical intra-assay (well-well) variations (same sample) <6% (n=8)

Typical inter-assay (plate-plate) variation (same sample) <11% (n=8)
9 Specificity

Species Reactivity: Human and Rat (activity only).

This assay has been demonstrated with human liver tissue samples and HepG2, a liver derived human cultured cell line. Typical ranges for several sample types are described below. It is highly recommended to prepare multiple dilutions for each sample to ensure that each is in the working range of the assay (see Data Analysis section).

Table 1: Typical working ranges for activity and quantity of samples in µg/ml (Note however the assay requires 100 µl sample in each well):

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured Human whole cell extracts (HepG2)</td>
<td>10 µg - 500 µg/ml</td>
</tr>
<tr>
<td>Human liver tissue extract</td>
<td>1 µg - 50 µg/ml</td>
</tr>
<tr>
<td>Rat Liver extract (activity only)</td>
<td>10 µg - 500 µg/ml</td>
</tr>
</tbody>
</table>
## 10 Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor standard curve</td>
<td>Inaccurate Pipetting</td>
<td>Check pipettes</td>
</tr>
<tr>
<td></td>
<td>Improper standard dilution</td>
<td>Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing</td>
</tr>
<tr>
<td>Low Signal</td>
<td>Incubation times too brief</td>
<td>Ensure sufficient incubation times; change to overnight standard/sample incubation</td>
</tr>
<tr>
<td></td>
<td>Inadequate reagent volumes or improper dilution</td>
<td>Check pipettes and ensure correct preparation</td>
</tr>
<tr>
<td>Large CV</td>
<td>Plate is insufficiently washed</td>
<td>Review manual for proper wash technique. If using a plate washer, check all ports for obstructions</td>
</tr>
<tr>
<td></td>
<td>Contaminated wash buffer</td>
<td>Prepare fresh wash buffer</td>
</tr>
<tr>
<td>Low sensitivity</td>
<td>Improper storage of the ELISA kit</td>
<td>Store the reconstituted protein at -80°C, all other assay components at 4°C. Keep substrate solution protected from light.</td>
</tr>
</tbody>
</table>
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