CREATIVE BIOMART®
Assay Kit

AGE-RAGE in vitro Binding Assay Kit

Product Information

Cat

Kit-0060

Cat.No.

Kit-0060

Product Overview

The AGE-RAGE in vitro Binding Assay Kit is primarily designed to screening inhibitors of AGE2 (glyceraldehyde-modified AGE)-sRAGE interaction (soluble RAGE) in vitro. In addition, this kit can be used for the rapid and sensitive evaluation of inhibitor candidates as well as characterization of AGE2-sRAGE interaction in vitro. The recombinant sRAGE used in this assay kit was specially designed for keeping the correct conformation to bind efficiently to AGE2-BSA but not to BSA, which are immobilized on the microplate surface. This technique allows us measuring the interaction of recombinant sRAGE to AGE2-BSA in a solid-phase assay system such as a conventional ELISA system.

Description

Advanced glycation end products (AGEs) are important biochemical compounds found in diabetes and may be associated with inflammatory processes. In the vessel wall, AGEs are bound to specific receptors to modulate many cellular properties by activating several signaling pathways. One of these receptors is called "Receptor for Advanced Glycation End product" (RAGE). RAGE is a multi-ligand member of the immunoglobulin superfamily of cell surface molecules that is expressed in a variety of cell lines, including endothelial cells, smooth muscle cells, mononuclear phagocytes, pericytes, neurons, cardiac myocytes, mesangial cells and hepatocytes. RAGE interacts with different structures to transmit a signal into the cell and recognizes three-dimensional structures rather than specific amino acid sequences. Therefore, RAGE seems to fulfill the requirements of a pattern-recognition receptor. As a member of the immunoglobulin superfamily, it interacts with a diverse class of ligands, including AGEs, HMGB1 (also known as Amphoterin), amyloid β-peptide, amyloid A, leukocyte adhesion receptors, prions, Escherichia coli curli operons, β-sheet fibrils and several members of the S100 protein superfamily including S100/calgranulins. Thus RAGE may have

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potential involvement in several pathological processes
including inflammation, diabetes, Alzheimer's disease (AD), systemic amyloidosis and tumor growth. AGE-RAGE interaction enhances the expression of genes encoding cytokines, growth factors and adhesion molecules, and increases the classical acute phase proteins. Potential approaches to prevention and treatment of diabetes and its complications include inhibition of AGE formation, breakage of preformed AGE-proteins crosslink, blockade of AGE-RAGE interactions with RAGE competitors, antibody antagonists and RAGE specific metabolic inhibition. Inhibition of AGE-RAGE complex formation is able to suppress the levels of pro-inflammatory cytokines and growth factors and may be considered as a target for overcoming diabetic complications.

Characteristic

The Research Products AGE-RAGE in vitro Binding Assay Kit is a semi-quantitative solid phase binding assay between recombinant His-tagged soluble RAGE (sRAGE) and glyceraldehyde-modified BSA (AGE2-BSA). Plates are pre-coated with AGE2-BSA, which is able to bind to recombinant His-tagged sRAGE. Horse radish peroxidase (HRP)-conjugated anti-His-tag monoclonal antibody specifically reacts with recombinant His-tagged sRAGE that is trapped with AGE2-BSA immobilized on the microplate well surface. The AGE-RAGE in vitro Binding Assay Kit may be used to characterize AGE-RAGE interaction as well as to screen inhibitors of AGE-RAGE interaction in vitro.
To perform the test, the recombinant His-tagged sRAGE is diluted in Reaction Buffer, pipetted into the wells and allowed to bind to AGE2-BSA immobilized on the wells. After wash the wells, the amount of bound recombinant His-tagged sRAGE is measured by binding it with HRP-conjugated anti-His-tag monoclonal antibody, which then catalyzes the conversion of the chromogenic substrate tetra methylbenzidine (TMB) from a colorless solution to a blue solution (or yellow after the addition of stopping reagent). The color is quantitated by spectrophotometry and reflects the relative amount of recombinant His-tagged sRAGE that binds to AGE2-BSA on the wells.
For screening inhibitors of AGE-RAGE interaction in vitro, test compounds or the inhibitor candidates including a monoclonal antibody are added to appropriate amount of His-tagged sRAGE in the wells pre-coated with AGE2-BSA in a similar fashion as described above, followed by evaluation of inhibitory effect on AGE-RAGE interaction by measuring the amount of His-tagged sRAGE2 on the wells.

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Applications

1) Screening inhibitors of AGE-sRAGE interaction in vitro.
2) Characterization of inhibitor candidates of AGE2-sRAGE interaction in vitro.
3) Screening monoclonal antibodies that inhibit AGE2-sRAGE interaction in vitro.
4) Characterization of AGE2-sRAGE interaction in vitro.

Notes

Stop Solution is a strong acid. Wear disposable gloves and eye protection when handling the solution.

Storage

Upon receipt, store all other components at 4°C;
Don't expose reagents to excessive light.
All of the reagents included in the Research Product AGE-RAGE in vitro Binding Assay Kit have been tested for stability. Reagents should not be used beyond the stated expiration date.

Size

96 assays

Kit Components

All samples should be assayed in duplicate. The following components are supplied and are sufficien for the one 96-well microtiter plate kit.
AGE2-BSA coated Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a foil, zip-lock bag with a desiccant pack. Wells are coated with AGE2-BSA (glyceraldehyde-modified BSA).
BSA coated Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in foil, zip-lock bag with a desiccant pack for non-specific binding control. Wells are coated with BSA.
Recombinant His-tagged sRAGE: One vial containing 1600AU of lyophilized recombinant His-tagged human sRAGE.
Reaction Buffer: One bottle containing 50 mL of 1X buffer; used for for reconstitution and dilution of Recombinant His-tagged sRAGE, and binding reaction. Ready to use.
20X Inhibitor Control Compound: One vial containing 200uL of compound DP, which was

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discovered in commercial chemical library using this AGE-RAGE in vitro Binding Assay Kit. This compound DP may be irritating to the skin, eyes and upper respiratory tract and hazardous in case of ingestion.
HRP conjugated anti-His-tag monoclonal antibody: One bottle containing 30 mL of HRP (horseradish peroxidase) conjugated anti-His-tag monoclonal antibody. Ready to use.
10X Wash Buffer: One bottle containing 100 mL of 10X buffer containing Tween-20.
Substrate Reagent: One bottle containing 20 mL of the chromogenic substrate, tetra methylbenzidine (TMB). Ready to use.
Stop Solution: One bottle containing 20 mL of 1 N H2SO4. Ready to use.

Materials Required but Not Supplied

Pipettors: 2-20 µL, 20-200 µL and 200-1000 µL precision pipettors with disposable tips.
Precision repeating pipettor
Orbital microplate shaker
Wash bottle or multichannel dispenser for plate washing.
Microcentrifuge and tubes for sample preparation.
Vortex mixer
Microplate washer: optional (Manual washing is possible but not preferable)
Plate reader capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.
Software package facilitating data generation and analysis :optional
500 or 1000 mL graduated cylinder
Reagent reservoirs
Deionized water of the highest quality
Disposable paper towels

Preparation

I. Preparation of Working Solutions
1. Prepare a working solution of Wash Buffer by adding 100 mL of the 10X Wash Buffer to 900 mL of deionized (distilled) water (ddH2O). Mix well. Store at 4°C for two weeks or -20°C for long-term

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storage.
2. Reconstitute Recombinant His-tagged sRAGE with X* mL of Reaction Buffer by gently mixing. After reconstitution, immediately dispense it in small aliquots (e.g. 200 µL) to plastic micro-centrifuge tubes and store below -70°C to avoid non-specific adsorption to glass surface and multiple freeze-thaw cycles. The concentration of the recombinant His-tagged sRAGE in vial should be 2,000 AU/mL, which is referred to as a Master Solution of recombinant His-tagged sRAGE.
3. Prepare His-tagged sRAGE Solutions by 2-fold serially diluting the Master Solution (2,000 AU/mL) to an appropriate concentration (usually 100-1.56 AU/mL only for drawing dose-response curve) with Reaction Buffer at the time of assay (See "II. Standard Assay for Drawing Dose-Response Curve" below.).
Note-1: For an inhibitor screening assay, the Master Solution should be diluted with Reaction Buffer to appropriate concentration, which shows OD value does not exceed plateau range in dose-response curve.
Note-2: Prepare appropriate volume for your assay. Discard any unused His-tagged sRAGE Solutions after diluted.

Assay Protocol

II. Standard Assay for Drawing Dose-Response Curve
1. Remove the appropriate number of microtiter wells of AGE2-BSA coated Microplate from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.
2. Prepare 2-fold serially diluted His-tagged sRAGE Solutions (100-1.56 AU/mL and Reaction Buffer only).
3. Pipette 100 µL of the His-tagged sRAGE Solutions and Reaction Buffer in duplicates, into the wells. Cover with plate sealer or lid, and incubate at room temperature (ca.25°C) for 60 minutes, shaking at ca. 300 rpm on an orbital microplate shaker.
4. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.
5. Add 100 µL of HRP conjugated Anti-His-tag Antibody into each well, cover with plate sealer or lid, and incubate at room temperature (ca.25°C) for 60 minutes, shaking at ca. 300 rpm on an orbital microplate shaker.

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6. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.
7. Add 100 µL of Substrate Reagent into each well. Avoid exposing the microtiter plate to direct sunlight. Covering the wells with e.g. aluminum foil is recommended. Return Substrate Reagent to 4°C immediately after the necessary volume is removed.
8. Incubate the wells at room temperature (ca.25°C) for 5-15 minutes, shaking at ca. 300 rpm on an orbital microplate shaker. (Appropriate incubation time may vary, and incubation time can be extended up to 30 minutes if the reaction temperature is below than 20°C).
9. Add 100 µL of Stop Solution to each well in the same order as the previously added Substrate Reagent.
10. Measure absorbance in each well using a spectrophotometric plate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the plate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.
Note: Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspiration or decantation. Invert the plate and blot it against clean paper towels.

III. Standard Assay for Inhibitor Screening
Special considerations when screening inhibitors of AGE-RAGE interaction
In order to estimate the inhibitory effect on AGE-RAGE interaction in the test compounds correctly, it is necessary to conduct the control experiment of "Vehicle Control" for every experiment, and "Inhibitor Control" at least once for the first experiment, in addition to "Test Compound", as indicated in the following table. When test compounds cause an inhibitory effect on AGE-RAGE interaction, the level of A450 is weakened as compared with "Vehicle Control".

For inhibitor screening
Assay Reagents Inhibitor Control Test Compound Vehicle Control
Reaction Buffer 85 µL 85 µL 85 µL
20X Inhibitor Control Compound * 5 µL -

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20X Test Compound(s) - 5 µL -
Vehicle for 20X Test Compound(s) - - 5 µL
10X His-tagged sRAGE Solution ** 10 µL 10 µL 10 µL
* 20X Inhibitor Control Compound: See the section "Materials Provided" above.
** 10x concentrated solution of the His-tagged sRAGE Solution, which shows OD value does not exceed plateau range in dose-response curve. Usually, it will be 200-400 AU/mL, of which final concentration is 20-40 AU/mL. See "I. Preparation of Working Solutions" above.

1. Following the above table, add the "Reaction buffer" and "20X Test Compound" or "Vehicle for Inhibitor" or 20X Inhibitor Control compound to each well of AGE2-BSA coated Microplate and BSA coated Microplate.
2. Initiate reaction by adding 10 µL of "10X His-tagged sRAGE solution" to each well and mixing thoroughly. Cover with plate sealer or lid, and incubate at room temperature for 60 minutes, shaking at ca. 300 rpm on an orbital microplate shaker.
3. Follow the steps 4-10 of "II. Standard Assay for Drawing Dose-Response Curve" above.
Note-1: The optimal experimental conditions must be determined by the individual user. Especially, appropriate concentration of 10X His-tagged sRAGE Solution must be determined by drawing dose-response curve, which shows OD value does not exceed plateau range in the dose-response curve.
Note-2: To obtain an accurate inhibition percentage of test compounds, draw and calculate form a dose-response curve with appropriate serial dilutions of 10X His-tagged sRAGE Solution in parallel with the inhibition assay of the test compounds.
Note-3: Inhibition percentage of "Inhibitor Control Compound" provided in "Inhibitor Control" of the above table will be approximately 50% or more.

Analysis

Evaluation of Results
To obtain an accurate inhibition percentage of test compounds, it's required to draw and calculate form a dose-response curve with serial dilutions of 10X His-tagged sRAGE Solution in parallel with the inhibition assay of the test compounds.
1. Average the duplicate readings for each "Inhibitor Control", "Test Compound", "Vehicle Control" and "serial dilutions of 10X His-tagged sRAGE Solution" in AGE2-BSA coated Microplate and BSA

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coated Microplate. Subtract the average readings in BSA coated Microplate from those in AGE2-BSA coated Microplate.
2. Inhibition percentage of test compounds should be calculated from the dose-response curve. It is important to make an appropriate mathematical adjustment to accommodate for inhibition percentage.
3. The dose-response curve of this assay may fit to a sigmoidal four-parameter logistic equation. Most microtiter plate readers perform automatic calculations of analyte concentration. The calibration curve is constructed by plotting the absorbance (Y) of calibrators versus log of the known concentration (X) of calibrators, using the four-parameter function. Alternatively, the logit log function can be used to linearize the calibration curve (i.e. logit of absorbance (Y) is plotted versus log of the known concentration (X) of calibrators).
Note: The AGE-RAGE in vitro Binding Assay Kit has been shown to measure AGE-RAGE interaction in vitro. The assay may be used to screen and characterize inhibitors of AGE-RAGE interaction in vitro.