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ab204694 Adenosylhomocysteinase (AHCY) Inhibitor Screening Assay Kit (Fluorometric)

Instructions for Use

For rapid, sensitive and accurate screening of potential Adenosylhomocysteinase (AHCY) inhibitors.

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

Version 1 Last Updated 23 July 2015

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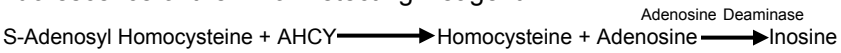
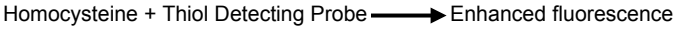
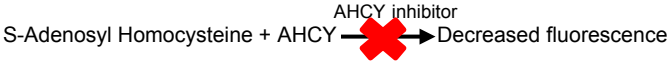
1. BACKGROUND

Adenosylhomocysteinase (AHCY) Inhibitor Screning Kit (Fluorometric) (ab204694) is a simple assay to study, screen or characterize potential inhibitors of AHCY. In this assay, the homocysteine generated from the breakdown of S-Adenosyl Homocysteine (SAH) is measured using a Thiol Detecting Reagent which results in enhanced fluorescence that can be measured at Ex/Em = 392/482 nm. The AHCY reaction is reversible, and therefore Adenosine Deaminase is included in the reaction to ensure that the reaction proceeds towards hydrolysis of SAH. In the presence of AHCY inhibitor, there is a decrease in fluorescence of the Thiol Detecting Reagent.

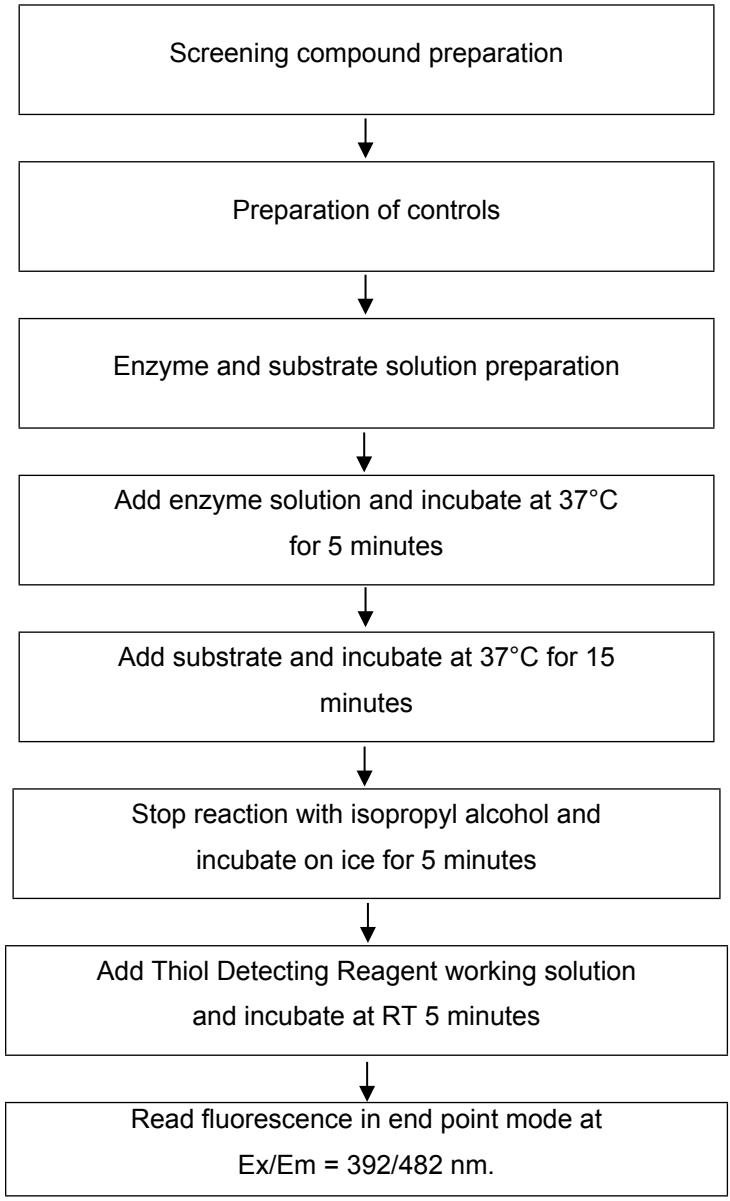






Adenosylhomocysteinase (AHCY) (EC 3.3.1.1) or S-adenosylhomocysteine hydrolase (SAHH); is an enzyme that catalyzes the reversible hydrolysis of S-Adenosyl Homocysteine (SAH) to adenosine and homocysteine. Inhibition of AHCY results in the accumulation of SAH, a product inhibitor of S-adenosyl methionine (SAM)-dependent methyltransferases. AHCY is an important target as an antiviral and anticancer drug. Several characterized SAH inhibitors inhibit some DNA viruses (e.g. Pox viruses) and some negative stranded RNA viruses (e.g.: Marburg virus, Ebola virus & rabies).

2. ASSAY SUMMARY



3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at -20ºC in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
AHCY Assay Buffer	25 mL	-20°C	-20°C / +4C
AHCY Reconstitution Buffer	250 µL	-20°C	-20°C
AHCY Enzyme	1 vial	-20°C	-80°C
Adenosine Deaminase	1 vial	-20°C	-80°C
3-Deazaneplanocin A (10 µM) (in DMSO)	10 µL	-20°C	-20°C
AHCY Substrate (in DMSO)	100 µL	-20°C	-20°C
Thiol Detecting Reagent (in DMSO)	200 µL	-20°C	-20°C

7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- MilliQ water or other type of double distilled water (ddH_2O)
- DMSO
- Isopropyl alcohol (chilled at -20°C)
- Pipettes and pipette tips
- Microcentrifuge
- Fluorescence microplate reader – equipped with filter for Ex/Em = 392/482 nm
- 96 well plate: white plate with flat bottom
- Heat block or water bath

8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required.
- Keep enzymes and heat labile components and samples on ice during the assay.
- Make sure all buffers and developing solutions are at room temperature before starting the experiment.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the appropriate type of plate for the detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **AHCY Assay Buffer:**

Ready to use as supplied. Equilibrate to 37°C before use. Store at 4°C or -20°C.

9.2 **AHCY Reconstitution Buffer:**

Ready to use as supplied. Store at -20°C.

9.3 **AHCY Enzyme:**

Reconstitute in 220 µL AHCY Reconstitution Buffer. Mix gently by pipetting up and down. Aliquot so that you have enough volume to perform the desired number of assays. Store at -80°C. Avoid repeated freeze/thaw. After reconstitution, use within two months. Keep on ice while in use.

9.4 **Adenosine Deaminase:**

Reconstitute with 110 µL ddH_2O. Mix gently by pipetting up and down. Aliquot so that you have enough volume to perform the desired number of assays. Store at -80°C. Avoid repeated freeze/thaw. After reconstitution, use within two months. Keep on ice while in use.

9.5 **3-Deazaneplanocin A (10µM) (in DMSO):**

Ready to use as supplied. Warm by placing in a 37°C bath for 1 – 5 minutes to thaw the DMSO solution before use. ***NOTE: DMSO tends to be solid when stored at -20°C, even when left at room temperature, so it needs to melt for few minutes at 37°C.*** Aliquot inhibitor so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light and moisture. Once the inhibitor is thawed, use with two months.

9.6 **AHCY Substrate (in DMSO):**

Ready to use as supplied. Warm by placing in a 37°C bath for 1 – 5 minutes to thaw the DMSO solution before use. ***NOTE: DMSO tends to be solid when stored at -20°C,***

even when left at room temperature, so it needs to melt for few minutes at 37°C. Aliquot substrate so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light and moisture. Once the substrate is thawed, use with two months.

9.7 **Thiol Detecting Reagent (in DMSO):**

Ready to use as supplied. Warm by placing in a 37°C bath for 1 – 5 minutes to thaw the DMSO solution before use. ***NOTE: DMSO tends to be solid when stored at -20°C, even when left at room temperature, so it needs to melt for few minutes at 37°C.*** Aliquot reagent so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light and moisture. Once the reagent is thawed, use with two months.

10. SAMPLE PREPARATION

- Always prepare a fresh set of samples and controls for every use.

10.1 Screening Compounds:

10.1.1 Dissolve candidate inhibitors at 1000X highest final test concentration into an appropriate solvent.

10.1.2 Dilute to 4X the desired test concentration with AHCY Assay Buffer.

NOTE: *We suggest using different volumes of testing compounds if effective concentration is unknown.*

11. ASSAY PROCEDURE and DETECTION

- **Equilibrate all materials and prepared reagents to room temperature / 37°C prior to use.**
- **It is recommended to assay all controls and samples in duplicate.**

11.1 **Set up reaction wells:**

- Sample wells (S) = 25 µL test inhibitors.
- Inhibitor Control wells (IC) = 1 µL 3-Deazaneplanocin A + 24 µL AHCY Assay Buffer.
- Enzyme Control wells (EC) = 25 µL AHCY Assay Buffer.
- Background Control wells (BC) = 25 µL AHCY Assay Buffer. ***NOTE:*** *Thiol Detecting Reagent reacts with the thiol groups in the enzymes and in the homocysteine. Hence a Background Control (BC) containing AHCY and Adenosine Deaminase should be used.*
- OPTIONAL: Solvent control (SC) = 25 µL solvent. ***NOTE:*** *preferred final solvent concentration should not be more than 2% by volume. If solvent exceeds 2%, include solvent control to test the effect on the solvent on enzyme activity.*

11.2 **Prepare AHCY Enzyme Solution:**

Prepare 25 µL of AHCY Enzyme Solution for each reaction:

Component	Enzyme Solution (µL)
AHCY Assay Buffer	**23**
AHCY Enzyme	**2**

Mix enough reagents for the number of assays (samples and controls) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation: X µL component x (Number reactions +1)

11.3 Add 25 µL of AHCY Enzyme Solution to each well. Mix gently.

11.4 Incubate plate at 37°C for 5 minutes.

11.5 **AHCY Reaction Mix:**

Prepare 50 µL of AHCY Reaction mix for each reaction.

Component	Reaction Mix (µL)	Background Control Mix (µL)
AHCY Assay Buffer	**48**	**49**
AHCY Substrate	**1**	**0**
Adenosine Deaminase	**1**	**1**

Mix enough reagents for the number of assays (samples background control) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation: X µL component x (Number reactions +1)

11.6 Add 50 µL of Reaction Mix Solution to each of S, EC, IC and SC wells.

11.7 Add 50 µL of Background Control Mix to each BC well.

The table below shows the experimental set up:

Component	Sample Well (S) (µL)	Inhibitor Control (IC) (µL)	Enzyme control (EC) (µL)	Background control (BC) (µL)
Test inhibitor compound	**25**	**0**	**0**	
AHCY Assay Buffer	**0**	**24**	**25**	**25**
AHCY Inhibitor	**0**	**1**	**0**	**0**
AHCY Enzyme Solution	**25**	**25**	**25**	**25**
AHCY Enzyme Reaction Mix	**50**	**50**	**50**	**0**
AHCY Background Control Mix	**0**	**0**	**0**	**50**

11.8 Incubate at 37°C for 15 minutes.

11.9 Stop the reaction by adding 50 µL of pre-chilled isopropyl alcohol into each well.

11.10 Mix and keep on ice for 5 min.

11.11 **Thiol Detection Probe:**

Prepare 50 µL of Thiol Detecting Probe working solution for wells just prior to use:

Component	Reaction Mix (µL)
DMSO	**48**
Thiol Detecting Probe	**2**

Mix enough reagents for the number of assays (samples background control) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation: X µL component x (Number reactions +1)

11.12 Add 50 µL of Thiol Detecting Reagent working solution into each well, mix and incubate at room temperature for 5 minutes. (Do not incubate more than 5 minutes.).

11.13 Measure fluorescence in a microplate reader in end point mode at Ex/Em = 392/482 nm.

12. CALCULATIONS

- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

12.1 Average the duplicate reading for each test sample compound, Inhibitor Control and Enzyme control.

12.2 Subtract the mean fluorescence value of the Blank Control (BC) from all controls and sample readings. This is the ΔRFU.

12.3 Set the ΔRFU of Enzyme Control (EC) as 100%.

12.4 Calculate the % inhibition or % Relative Activity as follows:

$$\%\ Inhibition = \frac{\Delta RFU\ of\ EC\ - \Delta RFU\ of\ S}{\Delta RFU\ of\ EC} \times 100$$

$$\%\ Relative\ Activity = \frac{\Delta RFU\ of\ S}{\Delta RFU\ of\ EC} \times 100$$

NOTE:

If RFU of SC < RFU of EC = make a higher stock of test inhibitor, or dissolve the inhibitor in lower concentration of the solvent; or use a different solvent.

If RFU of S < RFU of BC = treat as 100% inhibition and further dilute the test inhibitor and repeat the assay.

13. TYPICAL DATA

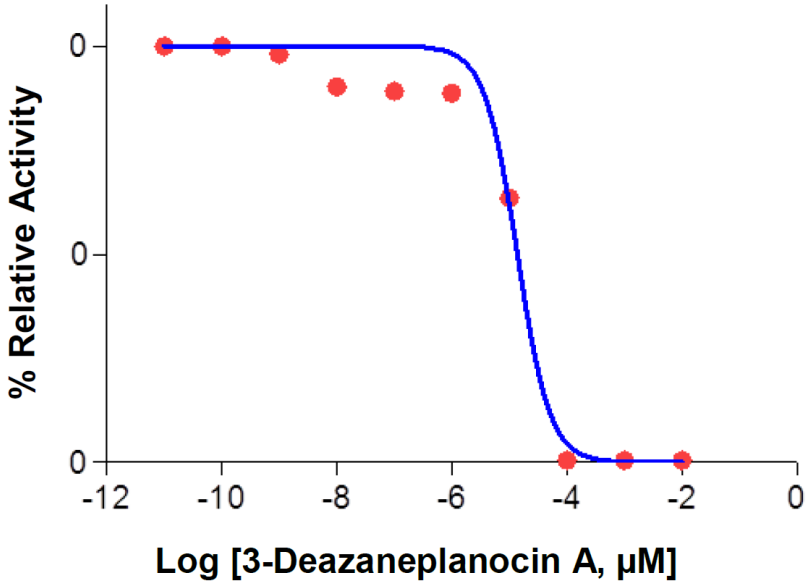


Figure 1. Inhibition of AHCY Enzyme activity by 3-Deazaneplanocin A. Assay was performed following kit protocol. IC_{50} (3-Deazaneplanocin A) = 0.137 nM.

14. QUICK ASSAY PROCEDURE

***NOTE**: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.*

- Prepare enzyme mix, substrate mix, inhibitor (aliquot if necessary) and get equipment ready.
- Prepare samples and dissolve test inhibitors in suitable solvent.
- Prepare enzyme solution for all wells to be set up (25 µL/well)

Component	Enzyme Solution (µL)
AHCY Assay Buffer	23
AHCY Enzyme	2

- Set up plate as follows:

Component	S (µL)	SC (µL)	EC (µL)	IC (µL)	BC (µL)
Enzyme Mix	50	50	50	50	50
Solvent test compound	0	10	0	0	0
Test Inhibitor Compound	10	0	0	0	0
Assay Buffer	0	0	10	9	10
Inhibitor control	0	0	0	1	0

- Incubate plate at 37°C for 5 minutes..
- Prior to use, prepare 50 µL AHCY Reaction Mix for each well (Number wells + 1).

Component	Substrate Mix (µL)	Background Control Mix (µL)
AHCY Assay Buffer	48	49
AHCY Substrate	1	0
Adenosine Deaminase	1	1

- Add 50 µL of Reaction Mix Solution to each of S, EC, IC and SC wells.

- Add 50 µL of Background Control Mix to each BC well.
- Incubate at 37°C for 15 minutes.
- Stop the reaction by adding 50 µL of pre-chilled isopropyl alcohol (not provided) into each well. Mix and keep on ice for five minutes.
- Prepare 50 µL of Thiol Detecting Probe working solution for each well (Number wells + 1):

Component	Reaction Mix (µL)
DMSO	**48**
Thiol Detecting Probe	**2**

- Add 50 µL of Thiol Detecting Reagent working solution into each well
- Mix and incubate at room temperature for 5 minutes. (Do not incubate more than 5 minutes.).
- Measure fluorescence in end point mode at Ex/Em = 392/482 nm.

15. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions on protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

16. **FAQ**

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