Adenosine Deaminase Activity Assay Kit (Fluorometric) (#BN00595)

(Catalog # BN00595; 100 assays; Store at -20°C)

I. Introduction:

Adenosine Deaminase (ADA) (E.C. 3.5.4.4.) is an enzyme that catalyzes the conversion of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine. Adenosine Deaminase is widely distributed in various tissues and cells. There are two isoforms, ADA1 and ADA2. ADA1 is widely expressed in most cells in the body, particularly in lymphocytes and macrophages. It is present in the cytosol, nucleus and found associated with dipeptidyl peptidase-4 on the cell membrane. ADA2 was first found in the spleen but is predominantly found in the plasma and serum. Increased serum ADA levels are found in certain infectious diseases such as tuberculosis and various liver diseases such as acute hepatitis, alcoholic hepatic fibrosis, chronic active hepatitis to name a few. Adenosine Deaminase is also a marker for T-lymphocyte proliferation. In Assay Genie's ADA Activity Assay, inosine formed from the breakdown of adenosine is detected via a multi-step reaction, resulting in the formation of an intermediate that reacts with the ADA Probe to generate a fluorescent product. The kit measures total Activity of Adenosine Deaminase with limit of quantification of 10 µU recombinant Adenosine Deaminase. The range of the assay is from 10-100 µU.

II. Application:

- Detection of Adenosine Deaminase activity

III. Sample Type:

- Cell and tissue lysate
- Nuclear Extract
- Cell Medium
- Purified recombinant protein

IV. Kit Contents:

Components	BN00595	Cap Code	Part Number
ADA Assay Buffer (10x)	25 ml	WM	BN00595-1
ADA Convertor	1 Vial	Blue	BN00595-2
ADA Developer	1 Vial	Clear	BN00595-3
ADA Substrate	500 µl	Brown	BN00595-4
ADA Probe	200 µl	Red	BN00595-5
ADA Positive Control	1 Vial	Green	BN00595-6
Inosine Standard (10 mM)	100 µl	Yellow	BN00595-7

V. User Supplied Reagents and Equipment:

- 96-well plate with flat bottom. White plate is preferred for this assay.
- Fluorescence microplate reader
- Protease Inhibitor Cocktail
- Dounce homogenizer

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protected from light. Briefly centrifuge small vials prior to opening. Read entire protocol before performing the assay.

- **ADA Assay Buffer (10x):** Make 1x Assay Buffer by adding one part 10x Assay Buffer to nine parts deionized water. Store at -20°C or 4°C. Bring to 37°C before use.
- **ADA Convertor and ADA Developer:** Reconstitute each with 210 µl ADA Assay Buffer and mix gently by pipetting. Briefly centrifuge to collect the contents at the bottom of the tube. Aliquot and store at -20°C. Avoid repeated freeze/thaw.
- **ADA Substrate:** Aliquot and store at -20°C. Avoid repeated freeze/thaw.
- **ADA Positive Control:** Reconstitute with 22 µl ADA Assay Buffer and mix gently by pipetting. Briefly centrifuge to collect the contents at the bottom of the tube. Aliquot and store at -20°C. Avoid repeated freeze/thaw.

VII. Adenosine Deaminase Activity Assay Protocol:

1. **Sample Preparation:** Rinse tissue and transfer ~100 mg of fresh or frozen tissue (stored at -80°C) to a pre-chilled homogenizer. Add 300 µl cold ADA Assay Buffer containing protease inhibitor cocktail (not provided) and thoroughly homogenize tissue on ice. Transfer the tissue homogenate to a cold microfuge tube.

   To prepare cell extract, add 150-300 µl cold ADA Assay Buffer containing protease inhibitor cocktail (not provided) to 1-5 x 10^6 fresh or frozen cells and pipette several times to disrupt the cells. Transfer cell homogenate including cell debris to a cold microfuge tube and agitate on a rotary shaker at 4°C for at least 15 min.

   Centrifuge the tissue or cell homogenate at 16,000 X g, 4°C for 10 min. Transfer the clarified supernatant to a fresh pre-chilled tube & store on ice. Use lysates immediately to assay ADA activity.

   Note: Lysates can be aliquoted and snap frozen in liquid nitrogen before storing at -80°C. Avoid freeze/thaw.

2. **Inosine Standard:** Dilute Inosine Standard to 1 mM by adding 10 µl of 10 mM Inosine Standard to 90 µl ADA Assay Buffer. Further dilute the Inosine Standard to 10 µM by adding 10 µl of 1 mM Inosine to 990 µl ADA Assay Buffer. Add 0, 2, 4, 6, 8 and 10 µl of diluted 10 µM Inosine Standard into a series of wells in a 96-well plate to generate 0, 20, 40, 60, 80 and 100 pmol/well Inosine Standard. Adjust the volume to 50 µl/well with ADA Assay Buffer.

3. **Adenosine Deaminase Activity Assay:** Add 2-50 µl of sample into desired well(s) in 96-well plate. For Positive Control, dilute the Positive Control 1:10 into ADA Assay Buffer and add 1-2 µl into desired well(s). Adjust the volume of sample and Positive Control to 50 µl/well with ADA Assay Buffer. Add 50 µl ADA Assay Buffer to one well as reagent Background Control.

Notes:

a. For unknown samples, we suggest doing pilot experiment and testing several doses to ensure the readings are within the Standard Curve range.

b. Small molecules such as adenosine, inosine, xanthine, and hypoxanthine in the samples will contribute to the background. Remove these molecules by passing through a desalting column or by buffer exchange using a 10 kDa spin column. Use this modified sample for the assay. **Optional:** Prepare a parallel sample well as sample background control to ensure that these small molecules are removed by either using a desalting column or spin column.

4. **Reaction Mix:** Prepare enough reagents for the number of assays to be performed. Make 50 µl of Reaction Mix and Background Control Mix containing:

	Reaction Mix	Background Control Mix
ADA Assay Buffer	40 µl	45 µl
ADA Convertor	2 µl	2 µl
ADA Developer	2 µl	2 µl
ADA Probe	1 µl	1 µl
ADA Substrate	5 µl	-----

Add 50 µl of Reaction Mix into each sample, reagent background control and Positive Control wells and 50 µl of Background Control mix to Standards and sample background control well(s). Mix well.

5. **Measurement:** Measure fluorescence (Ex/Em = 535/587 nm) in kinetic mode for at least 30 min. at 37°C. Choose two time points (T_1 & T_2) in linear range (can be as short as 2 min.) of plot and obtain corresponding RFU for sample (RFU_{S1} and RFU_{S2}) and reagent background control (RFU_{BG1} and RFU_{BG2}). Read the Inosine Standard Curve along with the samples.

6. **Calculations:** Subtract 0 Standard reading from all Standard Readings. Plot the Inosine Standard Curve. Subtract reagent background control reading from sample reading. Apply the ΔRFU [(RFU_{S2}- RFU_{BG2}) – (RFU_{S1}- RFU_{BG1})] to the Standard Curve to get B pmol of Inosine generated by the sample during the reaction time ($\Delta T = T_2 - T_1$).

Note: Sample background control reading should be less than reagent background control reading. We recommend removing the small molecules again using desalting column or a 10 kDa spin column if sample background control reading is higher than reagent background control

$$\textbf{Sample's ADA Activity} = \frac{\mathbf{B}}{\Delta T \times \mu g\ of\ protein} \textbf{ x DF = pmol/min/µg = µU/µg}$$

Where: **B** is Inosine amount from the Standard Curve (pmol).
ΔT is reaction time (min.)
µg of protein is amount of protein/well (µg)
DF is dilution factor of the sample

Sample ADA Activity can also be expressed as mU/mg (nmoles/min. inosine generated per mg) of protein.

Unit Definition: One unit of Adenosine Deaminase Activity is the amount of enzyme that hydrolyzes adenosine to yield 1.0 µmol of Inosine/min. at 37°C.

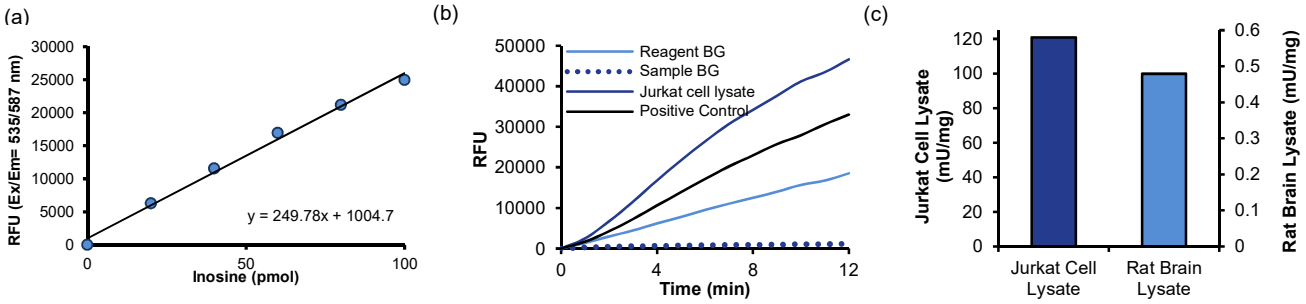

Figure: (a) Inosine Standard Curve, (b) Adenosine Deaminase Activity in Jurkat Cell (T-lymphocyte) lysate (105 ng) and Positive Control; BG: Background (c) Adenosine Deaminase specific activity in Jurkat cell lysate (105 ng) and rat brain lysate (2 µg). Assays were performed following the kit protocol.

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