ab204695 – Adenosine Deaminase (ADA) Activity Assay Kit (Fluorometric)

For the detection of ADA activity.
For research use only - not intended for diagnostic use.

Adenosine Deaminase (ADA) Activity Assay Kit (Fluorometric) (ab204695) is an assay where 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 that can be easily quantified at Ex/Em = 535/587 nm. The kit measures total Activity of Adenosine Deaminase with limit of quantification of 5 µU recombinant Adenosine Deaminase.

Storage and Stability

On receipt entire assay kit should be stored at -20°C, protected from light. Upon opening, use kit within 6 months.

Materials Supplied

Item	Quantity	Storage Condition
10X ADA Assay Buffer	25 mL	-20°C or 4 °C
Developer Solution K	1 vial	-20°C
Converter Mix F	1 vial	-20°C
ADA Positive Control	1 vial	-20°C
OxiRed™ Probe	0.2 mL	-20°C
ADA Substrate	500 µL	-20°C
Inosine Standard	100 µL	-20°C

PLEASE NOTE: Converter Mix F was previously labelled as Converter Enzyme VIII and ADA Developer, Developer Solution K as ADA Converter, and OxiRed™ Probe as ADA Probe. The mechanism of detection has not changed.

Materials Required, Not Supplied

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

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

Reagent Preparation

- Before using the kit, spin the tubes prior to opening.

10X ADA Assay Buffer: Thaw completely, and ensure all particulates return to solution. Make 1x Assay Buffer by adding one part 10X ADA Assay Buffer to nine parts deionized water. Store at -20°C or 4°C. Bring to 37°C before use.

Developer Solution K: Reconstitute each with 220 µl ADA Assay Buffer (1X) 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.

Converter Mix F: Reconstitute each with 440 µl ADA Assay Buffer (1X) 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 Positive Control: Reconstitute with 22 µl ADA Assay Buffer (1X) 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. Dilute directly before use as directed in assay.

OxiRed™ Probe: Ready to use as supplied. Warm by placing in a 37°C bath for 1 – 5 minutes to thaw the DMSO solution before use. Keep at room temperature during the assay. Store at -20°C and protect from light and moisture. Once the probe is opened and thawed, it is stable for at least 3 additional freeze/thaw cycles but should be used within two months. After use, promptly retighten the cap to minimize adsorption of airborne moisture

ADA Substrate and Inosine standard: Ready to use when thawed. Aliquot and store at -20°C. Avoid repeated freeze/thaw.

Assay Protocol

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- **Δ Note:** Only use 1X ADA Assay Buffer diluted from 10X stock.

Sample preparation:

1. Rinse tissue and transfer ~100 mg of fresh or frozen tissue (stored at -80°C) to a pre-chilled homogenizer.
2. Add 300 µl cold ADA Assay Buffer containing protease inhibitor cocktail (not provided) and thoroughly homogenize tissue on ice.
3. Transfer the tissue homogenate to a cold microfuge tube.
4. 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.
5. 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.
6. Centrifuge the tissue or cell homogenate at 16,000 x *g*, 4°C for 10 min.
7. Transfer the clarified supernatant to a fresh pre-chilled tube & store on ice. Use lysates immediately to assay ADA activity.

Δ Note: Small molecules such as adenosine, inosine, xanthine, and hypoxanthine present in the samples will contribute to the background. The molecules can be removed by passing the sample through a desalting column (and using the retentate) or by buffer exchange using a 10 kDa spin column (ab93349). Use this modified processed sample for the assay.
Δ Note: Lysates can be aliquoted and snap frozen in liquid nitrogen before storing at -80°C. Avoid freeze/thaw.

Inosine Standard:

1. Dilute Inosine Standard to 1 mM by adding 10 µl of Inosine Standard to 90 µl ADA Assay Buffer.
2. Further dilute the Inosine Standard to 10 µM by adding 10 µl of 1 mM Inosine to 990 µl ADA Assay Buffer.
3. 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 and adjust the volume to 50 µl/well with ADA Assay Buffer. **OR** build replicate standard curves using the table below:

Standard #	Volume of 10 µM Standard (µL)*	Assay Buffer X (µL)	Final volume standard in well (µL)	End Inosine Amount (pmoles/well)
1	0	125	50	0
2	5	120	50	20
3	10	115	50	40
4	15	110	50	60
5	20	105	50	80
6	25	100	50	100

Each dilution has enough volume to set up duplicate readings (2 x 50 µL).

Adenosine Deaminase Activity Assay:

1. Add 2-50 µl of sample into desired well(s) in 96-well plate.
2. For Positive Control, Dilute stock by a factor of 100 (eg 2 µL of ADA Positive control with 198 µL of ADA Assay Buffer). and add 2-5 µl into desired well(s).
3. Adjust the volume of sample and Positive Control to 50 µl/well with ADA Assay Buffer.
4. Add 50 µl ADA Assay Buffer to one well as reagent Background Control.

Δ 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

Reaction Mix:

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

Item	Reaction Mix	Background Control Mix
ADA Assay Buffer	41 µL	46 µL
Developer Solution K	2 µL	2 µL
Converter Mix F	1 µL	1 µL
OxiRed™	1 µL	1 µL
ADA Substrate	5 µL	-

2. 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.

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.

Calculations

1. Average the duplicate reading for each standard and sample.

2. Subtract 0 Standard reading from all Standard Readings. Plot the Inosine Standard Curve. Plot the corrected absorbance values for each standard as a function of the final amount of inosine in pmoles.
3. Subtract reagent background control reading from sample reading.
4. Apply the ΔRFU [(RFU_{S2}- $RFUB_{G2}$) – (RFU_{S1}- RFU_{BG1})] to the Standard Curve to get B pmol of Inosine generated by the sample during the reaction time ($\Delta T = T_2 - T_1$).

Δ 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**.**

$$ADA\ activity = \left(\frac{B}{\Delta T * V}\right) * D = pmol/(min * \mu L) = \mu U/\mu L$$

Where: **B** = Inosine amount from the Standard Curve (pmol)
ΔT = Reaction time (min)
V = Volume of sample added to well (µL).
D = Dilution factor of the sample

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Unit Definition:
1 Unit ADA activity = amount of Adenosine Deaminase which generates 1.0 µmol of inosine per minute at 37°C under the assay conditions.

Δ Note: Once the activity per /µL of sample is calculated, this can easily be converted to activity / cell or µg of tissue lysed. It may also be expressed in terms of units/µg of protein by dividing by the sample protein concentration (obtained with a BCA or similar protein quantification assay).

Technical Support

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