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ab273292 Aminopeptidase N (APN/CD13) Activity Assay Kit (Fluorometric)

View Kit datasheet: <https://www.abcam.com/ab273292>
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<https://www.abcam.co.jp/ab273292> for Japan)

For the determination of APN activity in cell and tissue lysates and biological fluids.

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

Table of Contents

1. Overview	1
2. Protocol Summary	2
3. Precautions	3
4. Storage and Stability	3
5. Limitations	4
6. Materials Supplied	4
7. Materials Required, Not Supplied	5
8. Technical Hints	6
9. Reagent Preparation	7
10. Sample Preparation	8
11. Standard Curve	9
12. Assay Procedure	10
13. Calculations	11
14. Typical Data	12
15. FAQ / Troubleshooting	15
16. Notes	16

1. Overview

Aminopeptidase N (APN/CD13) Activity Assay Kit (Fluorometric) (ab273292) enables rapid measurement of APN activity, utilizing a fluorogenic substrate that is converted into a highly fluorescent product (Ex/Em = 384/502 nm). A selective APN inhibitor is provided for verification of specific activity.

The assay is simple, high-throughput adaptable and can detect a minimum of 100 μ U APN activity in a variety of biological samples.

2. Protocol Summary

Prepare samples as directed



Prepare all reagents as directed



Prepare standard curve and measure fluorescence at Ex/Em = 384/502 nm. Calculate slope



Add Positive Control, Samples, Negative Control, Background Control to appropriate wells and adjust volume to 80 μ L



Preincubate plate for 10 minutes at 37°C.



Add Substrate Mix (20 μ L)



Measure fluorescence (Ex/Em = 320/445 nm) in kinetic mode for 45 to 60 minutes at 37°C.

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.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipette by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at -20°C in the dark immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the 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 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	Quantity	Storage temperature (before prep)
Aminopeptidase Assay Buffer	50 mL	-20°C or +4°C
AFC standard (1 mM)	100 µL	-20°C or +4°C
Aminopeptidase N Inhibitor (Lyophilized)	1 vial	-20°C
Aminopeptidase N Substrate (Lyophilized)	1 vial	-20°C
Aminopeptidase N Positive Control (Lyophilized)	1 vial	-20°C

7. Materials Required, Not Supplied

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

- Multiwell fluorescence microplate reader
- Precision multi-channel pipette and reagent reservoir
- 96-well plates with flat bottom (either a clear or a black plate is preferred)

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. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Samples generating values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 **AFC Standard:**

Provided as a 1 mM stock solution in DMSO. Store at -20°C, protected from light. Stable for 5 freeze/thaw cycles.

9.2 **Aminopeptidase N Inhibitor (t-BPAPP):**

Reconstitute with 155 μL of dH_2O and vortex to yield a 14 mM stock solution. To prepare a 3.5 mM working solution (10X final concentration), add 125 μL of the 14 mM stock solution to 375 μL of dH_2O . The 3.5 mM working solution should be stored at -20°C and is stable for at least 3 freeze/thaw cycles

9.3 **Aminopeptidase N Substrate:**

Reconstitute with 110 μL of dH_2O to obtain a 100X stock solution. Store at -20°C, protected from light. Stable for at least 3 freeze/thaw cycles.

9.4 **Aminopeptidase N Positive Control:**

Reconstitute with 110 μL Aminopeptidase Assay Buffer and mix contents thoroughly. Aliquot and store at -20°C, avoid repeated freeze/thaw cycles

10. Sample Preparation

10.1 Tissue/cell lysate preparation:

- 10.1.1 Homogenize tissue (25 mg) or pelleted, pre-washed cells (2.5×10^6) with 250 μ L ice-cold Aminopeptidase Assay Buffer.
- 10.1.2 Centrifuge the homogenate ($10,000 \times g$) at 4°C for 15 min and transfer the supernatant to a new microfuge tube.

11. Standard Curve

- 11.1 Prepare a 200 μM solution of AFC-Standard by diluting 40 μL of 1 mM AFC-Standard with 160 μL of Aminopeptidase Assay Buffer.
- 11.2 Add 0, 2, 4, 6, 8 and 10 μL of 200 μM AFC Standard into a series of wells in a 96-well plate and adjust the final volume to 100 μL /well with APN Assay Buffer. This will generate 0, 0.4, 0.8, 1.2, 1.6 and 2 nmol/well of AFC Standard respectively.

Standard #	200 μM AFC-Standard (μL)	APN Assay Buffer (μL)	AFC (nmol/well)
1	10	90	2
2	8	92	1.6
3	6	94	1.2
4	4	96	0.8
5	2	98	0.4
6	0	100	0

12. Assay Procedure

Thaw all reagents thoroughly and mix gently.

12.1.1 For **Sample**, add 2-20 μL of lysate into desired well(s) in a clear or black flat bottom 96-well plate.

Δ Note: For human serum samples, we recommend adding 10 μL of undiluted serum per reaction, although volumes of 2-20 μL per reaction may be used.

Δ Note: In order to determine specific APN activity in biological samples (which may contain other active ectopeptidase enzymes capable of hydrolyzing the APN Substrate), for each test sample, prepare a parallel sample well to serve as an inhibitor control.

Δ Note: The sample volume and/or dilution factor required can vary based upon the nature of the sample. For unknown samples, we suggest doing a pilot experiment by testing several amounts to ensure the readings are within the range of the standard curve.

12.1.2 Prepare assay reaction wells according to the table below. In addition to the test sample wells, prepare a **background control** (no sample) well to correct for any non-enzymatic substrate hydrolysis. Prepare parallel APN Inhibitor control wells (sample + 350 μM tBPAPP) using the 3.5 mM working solution (10X final concentration). If desired, you may also prepare **positive control** wells using the reconstituted Aminopeptidase N Positive Control. Adjust the volume of all wells to 80 μL /well with Aminopeptidase Assay Buffer.

Component	Test Sample	+APN Inhibitor	Background	Positive Control
Test Sample	2-20 μL	2-20 μL	-	-
Aminopeptidase N Positive Control	-	-	-	10 μL
APN Inhibitor (3.5 mM, 10X)	-	10 μL	-	-

Aminopeptidase Assay Buffer	To 80 μ L	To 80 μ L	80 μ L	70 μ L
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- 12.1.3 Preincubate the plate for 10 min at 37°C to allow the inhibitor to interact with sample APN.
- 12.1.4 During the preincubation, prepare a 5X concentrated APN Substrate solution by diluting the reconstituted 100X Aminopeptidase N Substrate stock solution with Aminopeptidase Assay Buffer at a 1:20 ratio. Prepare 20 μ L of 5X APN Substrate solution for each reaction to be performed (for example, for 10 wells, mix 10 μ L of 100X Aminopeptidase N Substrate stock with 190 μ L Aminopeptidase Assay Buffer).
- 12.1.5 Start the reaction by adding 20 μ L of the 5X APN Substrate solution to each reaction well using a multichannel pipette, yielding a final volume of 100 μ L/well.

Δ Note: Do not add APN Substrate solution to the AFC Standard curve wells.

- 12.1.6 Measure fluorescence (Ex/Em = 384/502 nm) in kinetic mode for 45-60 minutes at 37°C.

Δ Note: Ideal measurement time for the linear range may vary depending upon the sample (we recommend reading sample fluorescence in kinetic mode).

Δ Note: The AFC Standard curve wells may be read in endpoint mode (Ex/Em = 384/502 nm).

13. Calculations

- 13.1 For the AFC Standard curve, subtract the 0 pmol/well reading from all standard readings, plot the background-subtracted values and calculate the slope.
- 13.2 For sample reaction wells (including paired inhibitor control wells), choose two time points (t_1 and t_2) in the linear phase of the reaction progress curves, obtain the corresponding fluorescence values at those points (RFU_1 and RFU_2) and determine the change in fluorescence over the time interval: $\Delta F = RFU_2 - RFU_1$.
- 13.3 Calculate the specific fluorescence generated by aminopeptidase N activity (denoted by CS) by subtracting the APN Inhibitor control fluorescence (ΔFI) from the corresponding test sample reading (ΔFS): $CS = \Delta FS - \Delta FI$.
- 13.4 Aminopeptidase N activity is obtained by applying the CS values to the AFC fluorescence standard curve to get B pmol of substrate metabolized during the reaction time.

$$\text{Sample APN activity} = \frac{B}{(\Delta t \times P)} \times D$$

APN activity is in nmol/min/(mg or mL) = $\mu\text{U}/(\text{mg or mL})$

B = Released AFC in Samples based on the Standard Curve slope (pmol)

Δt = Reaction time ($t_2 - t_1$) in minutes

P = Sample volume used (in mg protein or mL fluid)

D = Sample dilution factor (D = 1 for undiluted Samples)

Unit definition:

One unit of aminopeptidase N activity is the amount of enzyme that generates 1 μmole of AFC per min by hydrolysis of 1 μmole fluorogenic substrate at 37°C and pH 8.

14. Typical Data

Typical standard curve – data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

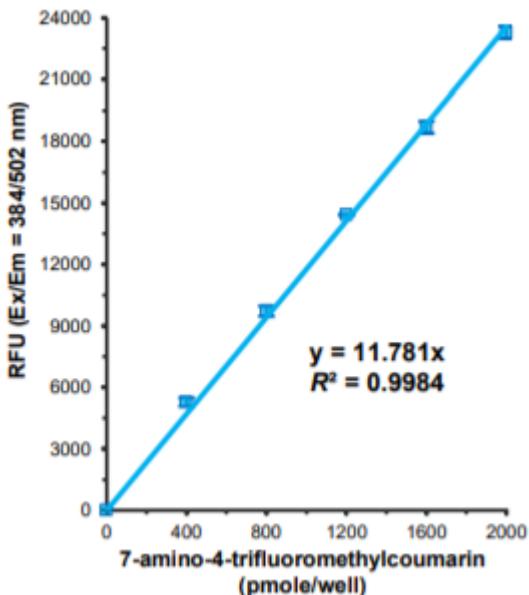


Figure 1. Standard curve of 7-amino-4-trifluoromethylcoumarin (AFC) fluorescence. One mole of AFC corresponds to the metabolism of one mole of Aminopeptidase N Substrate).

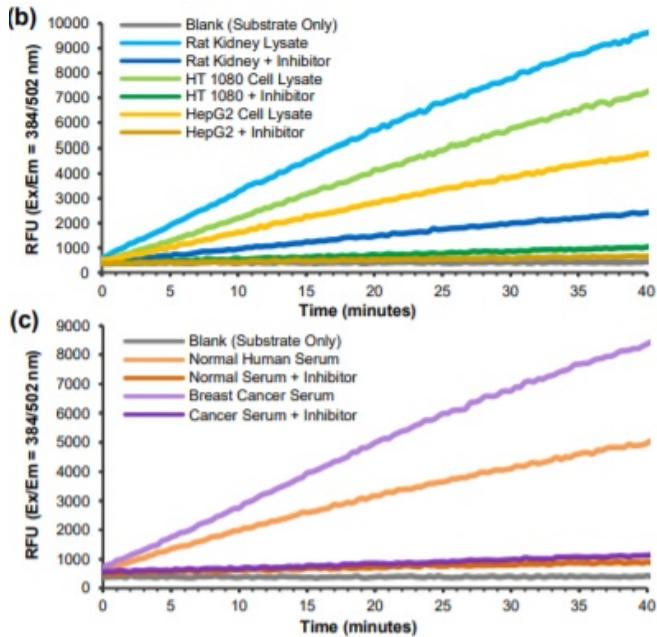


Figure 2. Kinetics of APN Substrate metabolism in rat kidney lysate (5 μ g protein), HT-1080 cell and HepG2 cell lysates (each 10 μ g protein), as well as pooled normal human serum and serum from breast cancer patients (each 10 μ l undiluted serum) in the presence and absence of the included selective APN inhibitor.

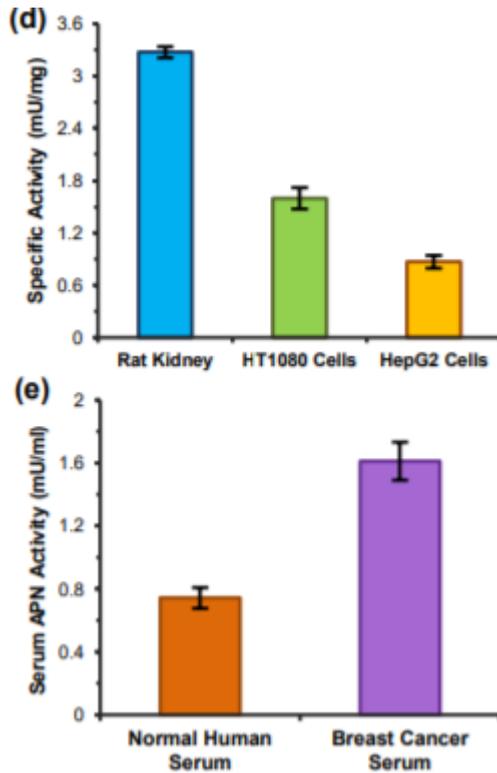


Figure 3. Quantification of APN activity in tissue/cell lysates and human serum samples (mean \pm SEM at least 3 replicates). Assays were performed according to the kit protocol.

15. FAQ / Troubleshooting

General troubleshooting points are found at www.abcam.com/assaykitguidelines.

16. Notes

Technical Support

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