Gentaur Europe BVBA Voortstraat 49, 1910 Kampenhout BELGIUM
Tel 0032 16 58 90 45 info@gentaur.com

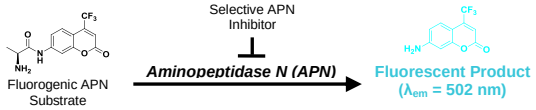
Aminopeptidase N (APN/CD13) Activity Assay Kit (Fluorometric)

06/17

(Catalog # K523-100; 100 Reactions; Store at -20°C)

I. Introduction:

Aminopeptidase N (APN, EC 3.4.11.2) also known as CD13 or membrane alanyl aminopeptidase is a Zn^{2+}-dependent ectopeptidase that cleaves *N*-terminal neutral amino acids (preferentially alanine) from proteins and peptides. APN is a promiscuous, multifunction enzyme consisting of a short cytoplasmic domain, a single transmembrane helix and a large extracellular catalytic domain that can be cleaved to generate a soluble version of the enzyme. Aminopeptidase N expression is upregulated in many different human cancers and the enzyme is involved in metastatic tumor cell proliferation, invasion and angiogenesis. Serum levels of soluble APN are elevated in cancer patients compared with healthy controls and there is a strong inverse correlation between serum APN, tumor load and long-term prognosis in pancreatic, breast and colon cancers. Thus, in addition to being a promising pharmacological target for novel anti-neoplastic drugs, serum APN activity has been proposed as a non-invasive biomarker for the diagnosis and surveillance of breast and pancreatic cancers. BioVision's Aminopeptidase N Activity Assay Kit 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 µU APN activity in a variety of biological samples.



II. Applications:

- Rapid assessment of APN activity in tissues, cell lysates and biological fluids (serum/plasma)

III. Sample Type:

- Lysates of soft tissues (*e.g.* kidney, liver, lung) or cultured cells
- Human or animal biological fluids (*e.g.* serum, plasma)

IV. Kit Contents:

Components	K523-100	Cap Code	Part Number
Aminopeptidase Assay Buffer	50 ml	NM	K523-100-1
AFC Standard (1 mM)	100 µl	Yellow	K523-100-2
Aminopeptidase N Inhibitor (*t*-BPAPP)	1 vial	Clear	K523-100-3
Aminopeptidase N Substrate	1 vial	Red	K523-100-4
Aminopeptidase N Positive Control	1 vial	Violet	K523-100-5

V. User Supplied Reagents and Equipment:

- 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)

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C and protect from light. Briefly centrifuge all small vials prior to opening. Allow the Aminopeptidase Assay Buffer to warm to room temperature prior to use. Read entire protocol before performing the assay procedure.

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

VII. Aminopeptidase N (APN/CD13) Activity Assay Protocol:

1. Sample Preparation:

a. Homogenize mammalian soft tissues (~25 mg) or pelleted, pre-washed cells (~2.5 x 10^6) in 250 µl ice-cold Aminopeptidase Assay Buffer. Centrifuge the homogenate (10,000 x *g*) at 4°C for 15 min and transfer the supernatant to a new microfuge tube. Add 2-20 µl of sample homogenate to desired well(s) in a clear or black flat bottom 96-well plate. 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.

Notes:

- 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.
- 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.

b. 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 *t*-BPAPP) 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:

	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 Solution (10X)	—	10 µl	—	—
Aminopeptidase Assay Buffer	to 80 µl	to 80 µl	80 µl	70 µl

2. **Standard Curve Preparation:** Dilute the AFC Standard by adding 40 µl of the 1 mM stock to 160 µl Aminopeptidase Assay Buffer to obtain a 200 µM AFC Standard solution. Add 0, 2, 4, 6, 8 and 10 µl of the 200 µM solution into a series of wells and adjust the volume of each well to 100 µl with Aminopeptidase Assay Buffer, yielding 0, 0.4, 0.8, 1.2, 1.6 and 2 nmol/well AFC Standard.
3. **Reaction Mix:**
   a. Preincubate the plate for 10 min at 37°C to allow the inhibitor to interact with sample APN. 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 µl of 5X APN Substrate solution for each reaction to be performed (for example, for 10 wells, mix 10 µl of 100X Aminopeptidase N Substrate stock with 190 µl Aminopeptidase Assay Buffer).
   b. Start the reaction by adding 20 µl of the 5X APN Substrate solution to each reaction well using a multichannel pipette, yielding a final volume of 100 µl/well. *Do not add APN Substrate solution to the AFC Standard curve wells.*
4. **Measurement:** Measure the reaction well fluorescence at Ex/Em = 384/502 nm in kinetic mode for 45-60 min at 37°C. Ideal measurement time for the linear range may vary depending upon the sample (we recommend reading sample fluorescence in kinetic mode). *The AFC Standard curve wells may be read in endpoint mode (Ex/Em = 384/502 nm).*
5. **Calculations:** For the AFC Standard curve, subtract the 0 pmol/well reading from all standard readings, plot the background-subtracted values and calculate the slope. 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: $\Delta F = RFU_2 - RFU_1$. Calculate the specific fluorescence generated by aminopeptidase N activity (denoted by C_S) by subtracting the APN Inhibitor control fluorescence (ΔF_I) from the corresponding test sample reading (ΔF_S): $\boldsymbol{C_S = \Delta F_S - \Delta F_I}$. Aminopeptidase N activity is obtained by applying the C_S values to the AFC fluorescence standard curve to get B pmol of substrate metabolized during the reaction time.

$$\textbf{Sample Aminopeptidase N (APN/CD13) Activity} = \frac{\boldsymbol{B}}{\boldsymbol{\Delta T \times P}} \times \boldsymbol{D} = \textbf{pmol/min/(mg or ml) = µU/(mg or ml)}$$

Where: $\boldsymbol{B}$ is the amount of metabolite produced, calculated from the standard curve (in pmol)
$\boldsymbol{\Delta T}$ is the linear phase reaction time $t_2 - t_1$ (in minutes)
$\boldsymbol{P}$ is the amount of sample added to the well (in mg of protein or ml fluid)
$\boldsymbol{D}$ is the sample dilution factor (if applicable, D=1 for undiluted samples)

Aminopeptidase N (APN/CD13) 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.

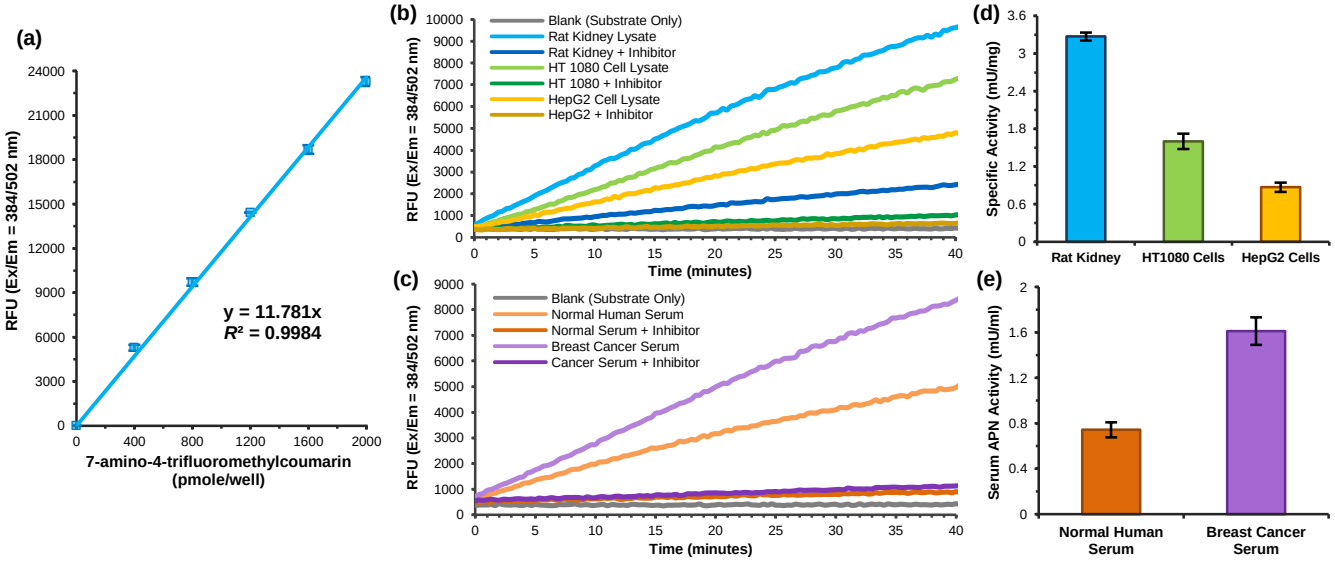


Figure: (a) Standard curve of 7-amino-4-trifluoromethylcoumarin (AFC) fluorescence. One mole of AFC corresponds to the metabolism of one mole of Aminopeptidase N Substrate. **(b–c)** Kinetics of APN Substrate metabolism in rat kidney lysate (5 µg protein), HT-1080 cell and HepG2 cell lysates (each 10 µg protein), as well as pooled normal human serum and serum from breast cancer patients (each 10 µl undiluted serum) in the presence and absence of the included selective APN inhibitor. **(d–e)** Quantification of APN activity in tissue/cell lysates and human serum samples (mean ± SEM at least 3 replicates). Assays were performed according to the kit protocol.

VIII. RELATED PRODUCTS:

Leucine Aminopeptidase (LAP) Activity Assay Kit (K534)
Dipetidyl Peptidase-4 (DPP4) Activity Assay Kit (K779)
Dipetidyl Peptidase-4 (DPP4) Inhibitor Screening Kit (K780)
Bestatin HCl (9630)

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