Chymotrypsin Activity Assay Kit (Fluorometric)

(Catalog #NBP2-59744 Store at -20°C)

I. Introduction:

Chymotrypsin (EC 3.4.21.1) is a key serine protease involved in dietary protein digestion in mammals. It is primarily produced by the pancreas, but may be expressed in other tissues, including spleen and liver. In the pancreas, chymotrypsin is initially expressed as the inactive proenzyme chymotrypsinogen, which is cleaved by other proteases to chymotrypsin, its active form. Chymotrypsin specifically cleaves peptide bonds at the C-terminal end of bulky hydrophobic or aromatic amino acids (such as tyrosine, tryptophan or phenylalanine). Novus' Chymotrypsin Activity Assay kit uses a synthetic fluorogenic substrate, enabling kinetic measurement of chymotrypsin activity in cell and tissue lysates. A chymotrypsin activator cleaves chymotrypsinogen to form active chymotrypsin, which then hydrolyzes the non-fluorescent substrate to release a stable fluorophore (Ex/Em = 380/460 nm). The kit includes a selective chymotrypsin inhibitor that can be used to measure specific chymotrypsin activity in samples containing non-specific proteases and endopeptidases that may also metabolize the substrate. The assay can detect as low as 0.01 mU of Chymotrypsin.

Chymotrypsinogen —(*Chymotrypsin Activator*)→ Chymotrypsin

Chymotrypsin Substrate —(*Chymotrypsin*)→ **Fluorescent Product (λ_{em} = 460 nm)**

II. Applications:

- Measurement of chymotrypsin activity in tissues / cultured cells.

III. Sample Type:

- Cultured cell lysates (*e.g.* HeLa, MCF-7 cells)
- Tissue lysate (*e.g.* liver, pancreas, spleen)

IV. Kit Contents:

Components	NBP2-59744	Cap Code
Chymotrypsin Assay Buffer	25 ml	WM
Chymotrypsin Substrate	200 µl	Red
Chymotrypsin Activator	1 vial	Green
Chymotrypsin Inhibitor	80 µl	Purple
Coumarin Standard (1 mM)	100 µl	Yellow
Chymotrypsin Positive Control	1 vial	Blue

V. User Supplied Reagents and Equipment:

- 96-well clear or white plate with flat bottom
- Multi-well spectrophotometer
- Anhydrous DMSO

VI. Storage Conditions and Reagent Preparation:

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

- **Chymotrypsin Assay Buffer:** Store at -20°C. Warm to room temperature before use.
- **Chymotrypsin Activator:** Reconstitute with 220 µl Chymotrypsin Assay Buffer immediately before use. Aliquot remainder and store at -80ºC. Once reconstituted, use within 2 months.
- **Chymotrypsin Substrate, Chymotrypsin Inhibitor** and **Coumarin Standard:** Store at -20ºC. Thaw completely before use. Mix well.
- **Chymotrypsin Positive Control**: Reconstitute with 22 µl Chymotrypsin Assay Buffer immediately before use. Remaining positive control should be aliquoted and stored at -80°C. Once reconstituted, use within 2 months.

VII. Chymotrypsin Activity Assay Protocol:

1. **Sample Preparation:** Homogenize cells (1 x 10^6) or tissue (20 mg) with 100 µl ice-cold Chymotrypsin Assay Buffer, and keep on ice for 10 min. Centrifuge at 10,000 x *g* for 10 minutes at 4°C and transfer the supernatant to a fresh tube. Determine protein concentration. Protein concentration should range between 5-20 mg/ml. Concentrated samples may be diluted with Chymotrypsin Assay Buffer. Aliquot and store lysates at -80°C unless being used immediately. Use 5-20 µl sample per well using a clear 96-well plate. Prepare two identical wells for each sample labelled “Sample Background Control” (SBC), and “Sample” (S). An additional well called “Sample + Inhibitor” (SI) may be prepared for samples in which nonspecific chymotrypsin-like protease activity is likely to be present. For SI, add 2 µl of chymotrypsin inhibitor in addition to sample. Adjust volume in each well to 50 µl with Chymotrypsin Assay Buffer. For positive control (PC), add 1-4 µl of Chymotrypsin Positive Control into desired well(s) and adjust the final volume to 50 µl with Chymotrypsin Assay Buffer. For reagent background control (BC), add 50 µl of Chymotrypsin Assay Buffer to a well. *For unknown samples, we suggest testing several concentrations to ensure the readings are within the Standard Curve range.*
2. **Coumarin Standard Curve Generation:** Add 0, 2, 4, 6, 8 and 10 µl from the provided 1 mM Coumarin Standard stock solution into a series of wells in a clear 96-well plate. Bring the total volume up to 100 µl per well with Chymotryspin Assay Buffer to generate 0, 2, 4, 6, 8 and 10 nmol/well of Coumarin Standard. Mix by pipetting, making sure that no bubbles are introduced in the wells. If sample activity is low (outside standard curve RFU values), another standard curve ranging from 0.1 to 1 nmol/well may be generated. For this, dilute the provided Coumarin Standard 1:10 in DMSO to obtain a 100 µM Coumarin Standard solution. Add 0, 2, 4, 6, 8 and 10 µl of the 100

µM solution into a series of wells in a 96 well plate and bring the total volume up to 100 µl with Chymotrypsin Assay Buffer to generate 0, 0.2, 0.4, 0.6, 0.8 and 1 nmol/well of Coumarin Standard

3. **Reaction Mix**: Prepare reaction mixes for test sample and corresponding sample background control wells according to the table below. Mix enough reagents for the number of assay to be performed (50 µl/well). Add 50 µl of the SBC Mix to each of the "Sample Background Control" wells and 50 µl of the Reaction Mix to wells containing samples (S), sample + inhibitor (SI), positive control (PC) and reagent background control (BC) for a final volume of 100 µl per well. *Turbidity upon addition of Chymotrypsin Substrate to Chymotrypsin Assay buffer is normal and will disappear following vortexing.*

	SBC Mix	Reaction Mix
Chymotrypsin Assay Buffer	48 µl	46 µl
Chymotrypsin Substrate	—	2 µl
Chymotrypsin Activator	2 µl	2 µl

4. **Measurement:** Immediately start recording the fluorescence (Ex/Em = 380/460 nm) in kinetic mode (*i.e.* at 30 second intervals) for 30-60 min at 25°C. Ideal measurement time depends on the chymotrypsin activity in samples. We recommend running the assay in kinetic mode to ensure that the linear reaction phase is recorded. *The Coumarin Standard curve can be read in endpoint mode.*

5. **Calculation:** Subtract the 0 pmol/well reading from all other Coumarin Standard readings and plot the standard curve. 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 curve, obtain the corresponding fluorescence values at those points (RFU_1 and RFU_2) and determine the change in fluorescence over the time interval: $\Delta F = RFU_2 - RFU_1$. Subtract the reagent background control (BC) ΔF value from the respective sample (S) ΔF values. *If sample background control (SBC) ΔF values are higher than BC, subtract the SBC from the corresponding sample (rather than subtracting the reagent BC)*. Chymotrypsin specific activity is obtained by applying the background-corrected ΔF values to the Coumarin Standard curve to get B pmol of substrate metabolized during the reaction time.

Chymotrypsin Specific Activity = ΔB / (Δt x p) = pmol/min/mg ≡ µU/mg

Where: **ΔB =** change in coumarin concentration during reaction (in pmol)
Δt = $t_2 - t_1$ (in min)
p = sample protein content added to well (in mg)

If chymotrypsin inhibitor is being used, calculate chymotrypsin activity as follows:
Chymotrypsin activity = Total activity in sample – Activity in presence of Chymotrypsin Inhibitor

Unit Definition: One unit of Chymotrypsin is the amount of enzyme that generates 1.0 µmol of coumarin per minute at pH 8 at 25°C.

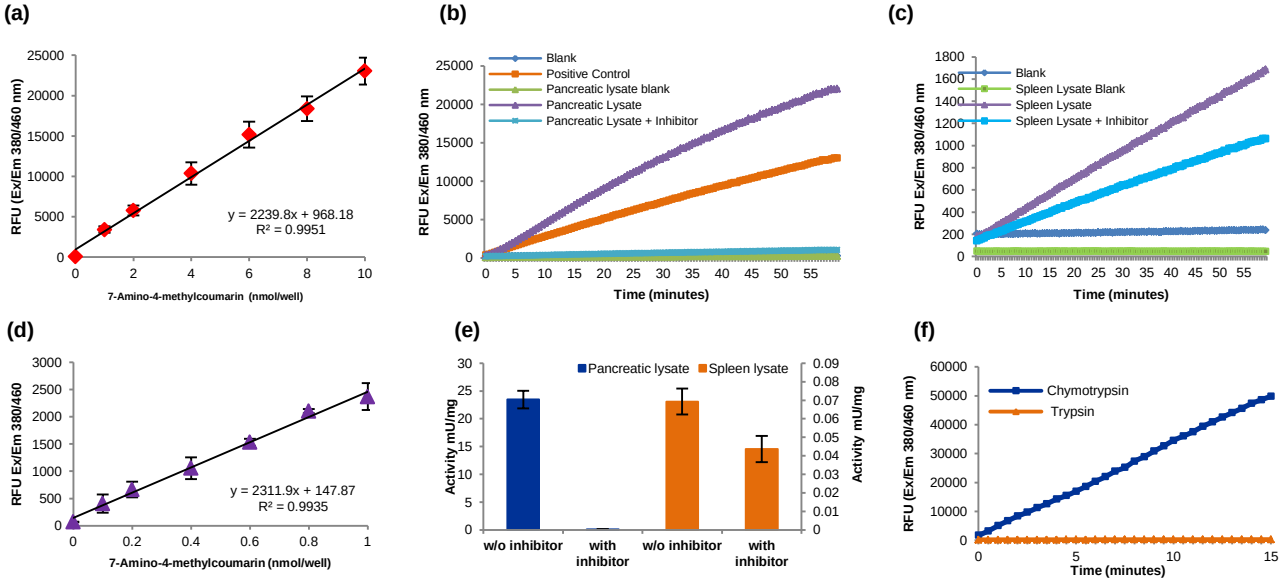

Figure: (a) Coumarin standard curves showing a range of 0-10 nmol/well. **(b)** Reaction kinetics for positive control and rat pancreatic lysate (9 µg). **(c)** Reaction kinetics for rat spleen lysate (160 µg). **(d)** Coumarin standard curves showing a range of 0-1 nmol/well. **(e)** Chymotrypsin activity with and without inhibitor in rat pancreas and rat spleen. The presence of non-specific chymotrypsin-like proteases in spleen leads to some activity in presence of the selective chymotrypsin inhibitor. **(f)** Reaction kinetics using substrate in the presence of Chymotrypsin or Trypsin. The substrate is cleaved by chymotrypsin but not trypsin, making the assay free from trypsin interference.