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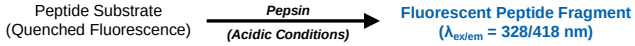
Pepsin/Pepsinogen Activity Assay Kit (Fluorometric)

6/18

(Catalog # K446-100; 100 Reactions; Store at -20°C)

I. Introduction:

Pepsin (EC 3.4.23.1) is a peptidase A1 family aspartic protease that serves as a primary digestive enzyme in the mammalian stomach. Pepsin is synthesized in the chief cells of the gastric mucosa as the inactive pro-enzyme pepsinogen, which contains a 44 amino acid autoinhibitory domain. The proenzyme is packaged into granules and stored inside the gastric mucosal cells. When food is consumed, neuronal signals traveling along the vagus nerve trigger secretion of HCl and pepsinogen into the stomach. In the highly acidic environment of the stomach, pepsinogen unfolds and undergoes autocatalytic cleavage, forming the active protease pepsin. Infection by the bacterium *H. pylori* induces hypersecretion of both stomach acid and pepsin, contributing to the formation of gastric and duodenal ulcers. In addition, excessive amounts of pepsin can play a role in the degradation of the esophageal and laryngeal tissue observed in chronic gastroesophageal reflux disease. While the majority of pepsinogen is expressed in the gastric mucosa, a small amount of the inactive enzyme is also released into the blood. Serum pepsinogen levels correlate with functional changes to gastric mucosa and are often measured for the screening of gastric cancer and chronic *H. pylori*-induced gastritis. BioVision's Pepsin Assay Kit is a homogenous assay that allows for quantification of pepsin activity in gastric tissue and various biological fluids. The assay utilizes a synthetic peptide substrate bearing both a fluorophore and a fluorescence quencher. Upon cleavage by pepsin, the fluorophore-bearing peptide fragment is unquenched to produce a bright fluorescent signal (Ex/Em = 328/418 nm). Lysosomal aspartic proteases in the peptidase A1 family (Cathepsin D and E) do not interfere with the assay. The assay is rapid, simple to perform and is vastly more sensitive than the classical hemoglobin degradation assay, with a detection limit of 500 µU pepsin activity per well.



II. Applications:

- Estimation of pepsin activity in various biological samples

III. Sample Type:

- Mammalian gastric tissues (stomach, duodenum, *etc.*)
- Human biological fluids (serum/plasma, gastric juice, vomit)

IV. Kit Contents:

Components	K446-100	Cap Code	Part Number
Pepsin Assay Buffer	25 ml	WM	K446-100-1
Pepsin Substrate	200 µl	Amber	K446-100-2
Pepsin Inhibitor (Pepstatin A)	20 µl	Blue	K446-100-3
Pepsin Positive Control	1 vial	Violet	K446-100-4
MCA Standard	25 µl	Yellow	K446-100-5

V. User Supplied Reagents and Equipment:

- Multiwell microplate spectrofluorometer
- White 96-well plates with flat bottom

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 Pepsin Assay Buffer to warm to room temperature prior to use. Read entire protocol before performing the assay procedure.

- **Pepsin Substrate:** Provided as a stock solution in DMSO. Divide into aliquots and store at -20°C, **protected from light**. Prior to use, warm solution to room temperature and vortex thoroughly.
- **Pepsin Inhibitor (Pepstatin A):** Provided as a 1 mM stock solution in DMSO. Prior to use, warm solution to room temperature and vortex thoroughly. Store at -20°C, stable for at least 3 freeze/thaw cycles.
- **Pepsin Positive Control:** Reconstitute with 110 µl ddH_2O. Divide into aliquots and store at -20°C. Avoid repeated freeze/thaw cycles.
- **MCA Standard:** Provided as a 1 mM stock solution in DMSO. Warm solution to room temperature prior to use. Store at -20°C, **protected from light**. Stable for at least 3 freeze/thaw cycles.

VII. Pepsin Activity Assay Protocol:

1. Sample Preparation:

a. For Mammalian Tissue Samples*:* Homogenize fresh or frozen tissue with Pepsin Assay Buffer (100 µl per ~10 mg of wet tissue) on ice and vortex thoroughly. Centrifuge the homogenate at 4°C for 10 min at 10,000 x *g*. Transfer the clarified supernatant to a fresh pre-chilled microfuge tube and keep on ice during use. Add 5-20 µl of clarified homogenate to desired well(s) in a white, flat-bottomed 96-well plate.

b. For Serum and Plasma Samples*:* Collect serum or plasma samples by standard methods. Samples exhibiting lipemia or excessive turbidity should be clarified by centrifugation at 10,000 x *g* for 5 min in order to separate lipid globules. Add 5-20 µl of undiluted serum/plasma sample to desired well(s).

c. For Pepsin Positive Control*:* Dilute the reconstituted Pepsin Positive Control at 1:10 ratio by mixing 10 µl of the reconstituted stock with 90 µl of Pepsin Assay Buffer. Incubate the diluted Pepsin Positive Control solution at room temperature for 5 min to allow for acid-mediated pepsin auto-activation, then add 10 µl of diluted Pepsin Positive Control solution to desired well(s).

d. Prepare assay reaction wells according to the table below. In addition to the test sample wells, prepare a background control (substrate only) well to correct for potential non-enzymatic substrate hydrolysis. For further verification of pepsin activity, you may prepare inhibitor control wells (sample + 1 µM pepstatin A). To prepare inhibitor control wells, dilute the Pepsin Inhibitor (Pepstatin A)

stock solution at 1:100 ratio in Pepsin Assay Buffer to produce a 10 µM working solution and add 10 µl of the working solution per well. Adjust the volume of all reaction wells to 80 µl per well with Pepsin Assay Buffer.

	Test Sample	+ Inhibitor	Background Control	Positive Control
Test Sample	5–20 µl	5–20 µl	—	—
Diluted Pepsin Positive Control	—	—	—	10 µl
Pepstatin A Solution (10 µM)	—	10 µl	—	—
Pepsin Assay Buffer	to 80 µl	to 80 µl	80 µl	70 µl

Note: Pepsin undergoes autolysis (self-cleavage) in solution at pH ≤ 4. To prevent pepsin autolysis, we recommend storing tissue homogenate samples at -80°C if they are to be used in future experiments.

2. **Standard Curve Preparation:** Prepare a 50 µM working solution of MCA by adding 5 µl of the 1 mM MCA Standard to 95 µl of Pepsin Assay Buffer. Add 0, 2, 4, 6, 8, and 10 µl of the 50 µM working solution into a series of wells in a white 96-well plate, generating 0, 100, 200, 300, 400 and 500 pmol of MCA/well. Adjust the volume of all standard curve wells (including the 0 pmol/well reagent blank) to 100 µl with Pepsin Assay Buffer.

3. **Reaction Preparation:**
   a. Preincubate the plate for 10 min at 37°C to allow sample temperature to equilibrate. During the preincubation, prepare Pepsin Substrate working solution by diluting the Pepsin Substrate stock solution with Pepsin Assay Buffer at a 1:10 ratio. Prepare 20 µl of substrate working solution for each reaction to be performed (for example, for 10 reaction wells, mix 20 µl of Pepsin Substrate stock with 180 µl Pepsin Assay Buffer).
   b. Start the reaction by adding 20 µl of the diluted substrate working solution to each reaction well, yielding a final volume of 100 µl per well. *Do not add Pepsin Substrate solution to the MCA Standard curve wells.*

4. **Measurement:** Measure the fluorescence (Ex/Em = 328/418 nm) of all sample wells in kinetic mode for 60 min at 37°C. We strongly recommend reading in kinetic mode in order to ensure that the measurements recorded are within the linear range of the reaction. Ideal measurement time for the linear range may vary depending upon the sample.

   Note: The MCA Standard curve wells may be read in endpoint mode (Ex/Em = 328/418 nm).

5. **Calculations:** For the MCA Standard curve, subtract the fluorescence obtained for the reagent blank (0 pmol/well standard) from all of the standard readings, plot the background-subtracted values and calculate the slope of the standard curve. For all reaction wells (including background control), 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: $\Delta F = RFU_2 - RFU_1$. If the ΔF value for the background control well is significant, it should be subtracted from each test sample to obtain the corrected fluorescence: $F_C = \Delta F_{sample} - \Delta F_{BC}$. If ΔF_{BC} is negative, background subtraction should be ignored: $F_C = \Delta F_{sample}$. Apply the F_C values to the MCA Standard curve to get B pmol of unquenched MCA-peptide in the well.

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

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

Pepsin Unit Definition: One unit of pepsin activity is the amount of enzyme that generates 1 µmole of unquenched 7-methoxycoumarin-4-acetate (MCA) per min by hydrolysis of 1 µmole peptide substrate at 37°C and pH 2.

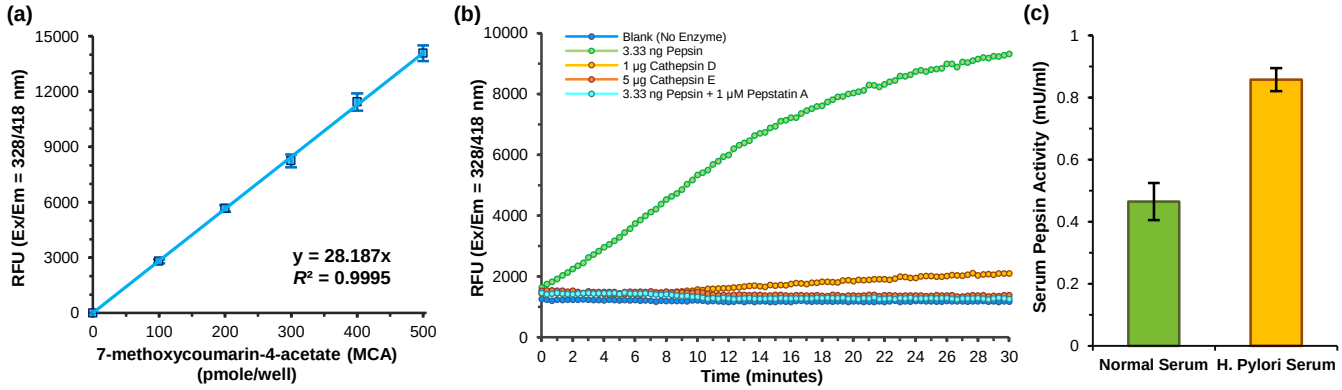


Figure: (a) 7-methoxycoumarin-4-acetate (MCA) Standard curve. One mole of MCA corresponds to the cleavage of one mole of fluorogenic Pepsin Substrate. **(b)** Kinetics of Pepsin Substrate metabolism by porcine gastric mucosal pepsin (3.33 ng purified enzyme) and specificity of substrate metabolism by pepsin versus other aspartic proteases. The acid-activated proteases Cathepsin D and E exhibit minimal assay interference, even when present at ≥300-fold excess by mass. **(c)** Estimation of pepsinogen activity in pooled normal human serum and single-donor serum from a gastric ulcer patient with confirmed *H. Pylori* infection (each 10 µl of undiluted serum). Data are mean ± SEM of 3 replicates, assayed according to the kit protocol.

VIII. RELATED PRODUCTS:

Cathepsin D Activity Assay Kit (K143)	Cathepsin D Inhibitor Screening Kit (K148)	Chymotrypsin Activity Assay Kit (K352)
Cathepsin E Activity Assay Kit (K165)	Cathepsin E Inhibitor Screening Kit (K166)	Pepstatin A (1732)

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