MMP-13 Inhibitor Assay Kit

Catalog # 3003

For Research Use Only - Not Human or Therapeutic Use

INTRODUCTION

MMP-13 (collagenase 3) is a newly discovered matrix metalloproteinase found in various tissues such as malignant tumors, osteoarthritic cartilage, rheumatoid synovium and wounds. MMP-13 production in chondroctyes and synoviocytes is upregulated by stimulation with inflammatory mediators such as IL-1, TNF and retinoic acid (reference 1). MMP-13 has been shown to degrade type I and II collagen and the degradation of type II collagen occurs approximately ten times faster than that of type I collagen (reference 2). The typical 3/4 and 1/4 fragements of collagen are produced as with MMP-1 (collagenase 1). However, MMP-13 also generates a second cleavage of type II collagen that removes three amino acids from the amino terminus of the 1/4 fragement. In addition, MMP-13 degrades aggrecan, the major proteoglycan of cartilage (reference 3).

Inhibitors of MMPs may be useful therapeutics to prevent metastasis of certain cancer cells and tissue damage in inflammatory diseases. This MMP-13 inhibitor assay kit is ideal for assessing inhibitors of MMP-13 and related enzymes. MMP-13 inhibitors can be easily screened and evaluated by simply adding potential inhibitory compounds to the activated recombinant human MMP-13 (rhMMP-13), see MMP-13 inhibitor assay sheet, page 5. This kit contains a truncated, rhMMP-13, which cleaves a fluorogenic peptide substrate included in this kit, but is not capable of digesting native collagen molecules. This kit contains all necessary reagents to perform 100 reactions.

Furthermore, this kit is useful for assaying total collagenase-like proteinase activity in tissue fluids or extracts and cell culture supernatants. Since the fluorogenic peptide substrate used in this kit does not hold a triple helical structure, this substrate is not specific to collagenase and might be susceptible to non-collagenolytic proteinases. However, it is important to know the total activity of collagenase-like proteinases in specimens before proceeding with further experiments.

KIT COMPONENTS

Item	Quantity	Amount	Storage
Recombinant Human MMP-13 (Truncated)	1 vial	100 µL, 100 µg/mL	-80°C
100X Substrate - Fluorogenic Peptide	1 vial	100 µL, 2 mM in DMSO	-20°C
Solution A - Substrate Dilution Buffer	1 bottle	10 mL	-20°C
Solution B - Sample Dilution and Reaction Buffer	1 bottle	50 mL	-20°C
Proteinase Inhibitor	1 vial	3 mg lyophilized	-20°C
Stop Solution - o-Phenanthroline	1 vial	1 mL, 10 mM in Ethanol	RT
Activator 1 - 20X APMA (Toxic - Handle With Care)	1 vial	1 mL	RT
ELISA Plate (Black)	2 each	96-well	RT

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SAFETY PRECAUTIONS

Activator 1 (APMA) contains mercury and is very toxic by inhalation, contact with skin or if swallowed. This compound may be irritating to the eyes, respiratory system and skin. Neurological hazard target organs include the kidneys and nerves. Wear suitable protective gloves, clothing, and eyewear.

ASSAY PROCEDURE

Activation of Recombinant Human MMP-13 (rhMMP-13)

1. Designate reactions to be performed in the 96-well microtiter plate for (1) blank, (2) standards and (3) inhibitor samples. An example is shown on the MMP-13 Inhibitor Assay Sheet, page 5.

2. Dilute the reference rhMMP-13 (100 μg/mL) with Sample Dilution and Reaction Buffer (Solution B) 1:10. Add the proper amounts of diluted rhMMP-13 (2-8 μL : 20-80 ng/well) to the wells. Add Solution B to adjust the final volume to 95 μL.

3. Add 5 μL of Activator 1 (APMA) to each well and incubate for 60 minutes at 35°C. **Do not add Activator 1 to undiluted reference rhMMP-13 solution, since it is a strong alkaline solution.**

4. Dissolve 1 vial of Proteinase Inhibitor in 1 mL of Solution B. Add 10 μL of proteinase inhibitor solution into all wells to neutralize non-collagenolytic proteinases in sample solutions.

Activation of Tissue Collagenase (MMP-1, MMP-8 and MMP-13)

To activate latent collagenases in tissue fluids or extracts and cell culture supernatant, choose one of these activation methods: APMA, trypsin or 3M KSCN. In general, it may not be necessary to activate enzymes using a combination of two activators, APMA and trypsin, since the collagenase activated by APMA will be digested and inactivated by trypsin.

APMA activated-collagenases may be inhibited by excess amounts of proteinase inhibitors, such as alpha 2-macroglobulin (α2M) contained in the sample specimens. In these cases, trypsin activation may be more effective than APMA. For example, add 5 μL of trypsin solution (1 mg/mL in neutral buffer) to 95 μL of sample solution. Incubate for 10 minutes at 35°C. The trypsin concentration needs to be optimized for individual samples. Another activation method to consider is to dialyze samples against 3M KSCN dissolved in 0.05M Tris-HCl buffer, pH 7.5, at 4°C overnight. Then remove the KSCN by dialyzing against 0.05M Tris-HCl buffer, pH 7.8, containing 0.2M NaCl and 5 mM $CaCl_2$. Add 10 μL of proteinase inhibitor such as soybean trypsin inhibitor (3 mg/mL) to neutralize non-collagenolytic proteinases in sample solutions. Proceed with the assay by adding the substrate solution. Dilute the samples as necessary with Sample Dilution and Reaction Buffer (Solution B).

Preparation of Inhibitor Solution

Most synthetic, organic compounds are water insoluble. Dissolving test compounds in water is required for screening and testing biological and pharmacological activities. Solvents such as alcohol, DMSO, PEG and PPG may be used to dissolve water insoluble compounds. These test samples must then be diluted with the Sample Dilution and Reaction Buffer (Solution B) prior to incubation. The pH of the sample solution should be 7.5 - 8.0.

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Collagenase Activity Assay

1. Activate the rhMMP-13 described on page 2 (shown on the MMP-13 Inhibitor Assay Sheet, page 5).

2. Add 1-50 μL of inhibitor test samples to the appropriate wells.

3. Add appropriate amounts of Sample Dilution and Reaction Buffer (Solution B) to the wells to adjust the final volume to 160 μL.

4. Prepare substrate solution (20 μM) by diluting the 100X Substrate 1:100 with Substrate Dilution Buffer (Solution A). Add 100 μL (2 nmole) of diluted substrate solution to each well. Incubate at room temperature (25°C) for approximately 30 minutes. Incubation time depends on the amount of rhMMP-13 used.

5. Stop the collagenase reaction by adding 10 μL of Stop Solution to each well.

6. Determine the fluorescence intensity (FI) at λem = 450 nm and λex = 365 nm or at λem = 440 nm and λex = 340 nm.

CALCULATION OF COLLAGENASE ACTIVITY

1. Subtract the blank value from the fluorescence intensity (FI) values of standards and test samples to obtain corrected FI values of standards ($FI_{Standard}$) and test samples ($FI_{Test\ Sample}$).

   $FI_{Standard}$ = FI (Standard) - FI (Blank)

   $FI_{Test\ Sample}$ = FI (Test Sample) - FI (Blank)

2. Plot the $FI_{Standard}$ values of standards against the enzyme dose on a cross-sectional graph paper or Excel worksheet (see Figure 1).

3. Calculate the inhibition % of test samples. Inhibition % of test samples is simply calculated by comparing $FI_{Test\ Sample}$ to $FI_{Standard}$ as follows:

   Inhibition % = $(1 - FI_{Standard} / FI_{Test\ Sample}) \times 100$

   Note: For the calculation of inhibitor activity, use an appropriate amount of rhMMP-13, such as 40 ng/tube, that is within the linear range of the dose response curve shown in Figure 1.

Figure 1 - Dose response curve of rhMMP-13. rhMMP-13 (0, 20, 40 and 60 ng) was reacted with the fluorogenic peptide substrate (2 nmole) at 25°C for 30 minutes.

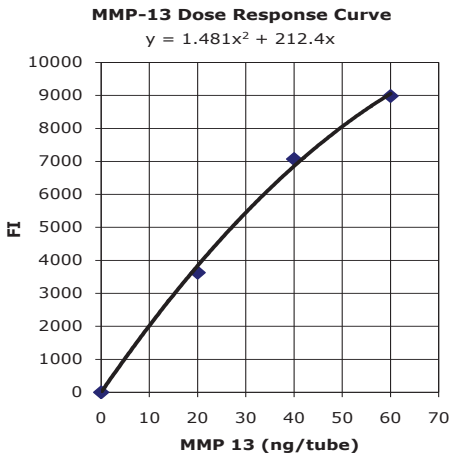

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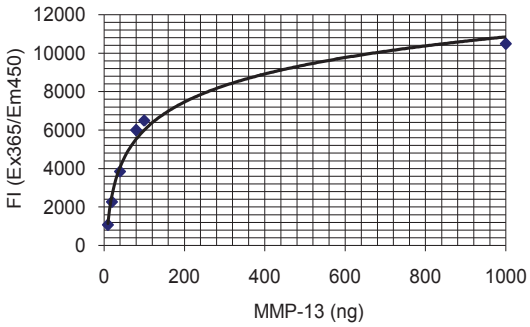
Figure 2 - Maximum digestion of fluorogenic peptide substrate by rhMMP-13. Fluorogenic peptide substrate (2 nmole) was completely digested by the reaction with 1000 ng of rhMMP-13 at 25°C for 30 minutes. This FI value will reflect 2 nmole of substrate and can be used for calculation of actual amounts of substrate digested by the enzyme.



CALCULATION OF SPECIFIC ACTIVITY OF rhMMP-13

React 1 μg of rhMMP-13 (10 μL of undiluted rhMMP-13, 100 μg/mL) with 100 μL of substrate solution (20 μM), which contains 2 nmole of substrate at 25°C for 30 minutes. The fluorescence intensity (FI) obtained will reflect 100% of substrate (2 nmole) digested. By comparing the FI obtained for individual test samples to FI of 2 nmole substrate (Figure 2), the amounts of substrate (nmole) digested can be simply calculated.

Example: If FI of 100% substrate digested was 10495, whereas FI observed in test sample 1 and test sample 2 were 1062 and 3845 respectively, actual amounts of substrate digested by samples are:

1062/10495 x 2 nmole = 0.202 nmole

3845/10495 x 2 nmole = 0.432 nmole

If the protein concentration in sample solution is known, divide the amount of substrate digested by the amount of protein in sample (μg or ng), so specific activity of rhMMP-13 will be obtained as nmole of substrate per μg or ng of protein.

REFERENCES

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MMP-13 Inhibitor Assay Sheet

A) Activation of rhMMP-13

	Standard Curve					Inhibitor Assay		
	Blank	Standard 1	Standard 2	Standard 3	Standard 4	Inhibitor 1	Inhibitor 2	Inhibitor 3
Step 1 Reference rhMMP-13 1:10 Diluted rhMMP-13 10 μg/mL (μL)	0	2	4	6	8	4	4	4
Step 2 Solution B (μL)	95	93	91	89	87	91	91	91
Step 3 Activator 1 - APMA (μL)	5	5	5	5	5	5	5	5
Incubate at 35°C for 60 minutes								
Step 4 Proteinase Inhibitor (μL)	10	10	10	10	10	10	10	10
Total Volume (μL)	110	110	110	110	110	110	110	110

B) Collagenase Activity Assay

	Standard Curve					Inhibitor Assay		
	Blank	Standard 1	Standard 2	Standard 3	Standard 4	Inhibitor 1	Inhibitor 2	Inhibitor 3
Step 1 Activated rhMMP-13 from Part A (μL)	110	110	110	110	110	110	110	110
Step 2 Inhibitor Test Sample (μL)	0	0	0	0	0	12.5	25	50
Step 3 Solution B (μL)	50	50	50	50	50	37.5	25	0
Step 4 Substrate Solution (μL)	100	100	100	100	100	100	100	100
Incubate at 25°C (room temperature) for 30 minutes. Note: Incubation time depends on the amount of rhMMP-13 used.								
Step 5 Stop Solution (μL)	10	10	10	10	10	10	10	10
Total Volume (μL)	270	270	270	270	270	270	270	270
Read fluorescence intensity (FI) at λem = 450 nm and λex = 365 nm or at λem = 440 nm and λex = 340 nm.								

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