Collagenase
Activity Assay Kit

Cat. No. ECM487

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

USA & Canada Phone: +1(800) 437-7500 Fax: 1-800-437-7502
www.millipore.com

Introduction

The Collagenases or Matrix Metalloproteinases are collagen decomposing enzymes which are involved in normal and pathophysiological processes such as inflammation, metastasis and tumor growth. Collagenases are produced by many myeloid and non-myeloid cell types. A clear understanding of the cellular stimuli and control mechanisms of these enzymes may provide a key to the cause and treatment of matrix metalloproteinase-associated pathologies.

Application

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 chondrocytes and synoviocytes is uprefulated by stimulation with inflammatory mediators, such as IL-1, TNF and retinoic acid.(1)

MMP-13 has been shown to degrade types I and II, although the degradation of type II collagen occurs approximately 10 times faster than that of type I collagen (2). Although typical ¾ and ¼ collagen fragments are produced as with MMP-1 (collagenase 1), MMP-13 generates a second cleavage of type II collagen that removes three amino acids from the amino terminus of the ¼ fragment. MMP-13 also degrades aggrecan; the major proteoglycan of cartilage (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 (rh-MMP-13), see MMP-13 inhibitor assay sheet, page 7. This kit contains a truncated, rh-MMP-13, which cleaves a fluorogenic peptide substrate included in this kit, but is not capable of digesting native collagen molecules.

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.

This kit contains all necessary reagents to perform 100 reactions.
Note that the substrate peptide (Dnp-Pro-beta-cyclohexyl-Ala-Gly-Cys(Me)-His-Ala-Lys(N-Me-Abz)-NH2 is also cleaved by other MMPs, including MMP-1 and MMP-9. (See reference 4). MMP-13 enzyme is provided as a positive control for enzyme cleavage, and additional experimental controls should be incorporated to ensure integrity of experimental results.

The Millipore® Collagenase Activity Assay Kit is intended for research use only; not for diagnostic or therapeutic applications.

Kit Components

1. Substrate Stock Solution (100X Fluorogenic Peptide, 2mM in DMSO): 100 µL. Store at -20°C.
2. Buffer Solution A (Dilution buffer for Substrate): 10 mL. Dilute 10 µL of Substrate Stock Solution into 1 mL Buffer A. Store at -20°C.
3. Buffer Solution B (Sample dilution and reaction buffer): 50 mL. Store at -20°C.
4. Stop Solution (10 mM o-phenanthroline in EtOH): 1 mL. Store at RT.
5. Recombinant Human MMP-13 (truncated form): 1 vial; 10 µg, {100µg/ml, 100 µL}. Dilute 1:10 with Buffer B before use. Store at –70°C.
6. Activator-1 (20X APMA Solution of a mercury compound): 1 mL. Store at room temperature. **Safety Precaution -** 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**.
7. Inhibitor (Proteinase inhibitor): Reconstitute with 1 mL of Buffer B: 1 vial, 3 mg; Store at –20°C.
8. Plastic 96-well Black microtiter plates: 2 each. Store at room temperature.

Materials Not Supplied

1. 30-50°C water bath or incubator
2. Fluorometric plate reader

Collagenase Activation

Activation of Recombinant Human MMP-13 (rh-MMP-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 7.
2. Dilute the reference rh-MMP-13 (100 µg/ml) with Sample Dilution and Reaction Buffer (Solution B) 1:10. Add the proper amounts of diluted rh-MMP-13 (2.5 - 10 µl: 25 - 100 ng) 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 rh-MMP-13 solution, since it is a strong alkaline solution.**
4. Add 10 µl of proteinase inhibitor 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 APMA will be digested and inactivated by trypsin. APMA activated collagenases may be inhibited by excess amounts of proteinase inhibitors, such as 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 CaCl2. 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.

Collagenase Activity Assay:

1. Activate the recombinant human MMP-13 described on page 3 (shown on the MMP-13 inhibitor assay sheet, page 6).
2. Add 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 by diluting the 100X substrate 1:100 with Substrate Dilution Buffer (Solution A). Add 100 µl of diluted substrate solution to each well. Incubate at room temperature (25°C) for approximately 30 minutes. Incubation time depends on the amount of MMP-13 used. Note serum samples and other samples with low levels of activity can be incubated for longer periods of time (15-120 minutes).
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 = 340nm.

Storage

IMPORTANT NOTE: The items in this kit require a variety of different storage conditions. Improper storage of these items may cause damage to components.

Activator-1 and Stop Solution should be stored at **room temperature**. Long term storage at 4°C may cause precipitation of Activator-1.

Recombinant Human MMP-13 should be stored at **–70°C**.

If necessary, all kit components kit may be stored at **–20°C** for short term periods (less than 2 weeks).

Calculation of Collagenase Activity:

1. Correct fluorescence intensity (FI) values:

   Corrected FI (standard) = FI (standard) - FI (blank)
   Corrected FI (inhibition) = FI (inhibition) - FI (blank)

2. Plot the corrected FI values against the enzyme dose or the incubation time using a log/log plot.

   Figure 1 shows a dose response curve where MMP-13 (10 - 1000 ng) was activated with Activator 1 and then reacted with the substrate for 30 minutes at room temperature.

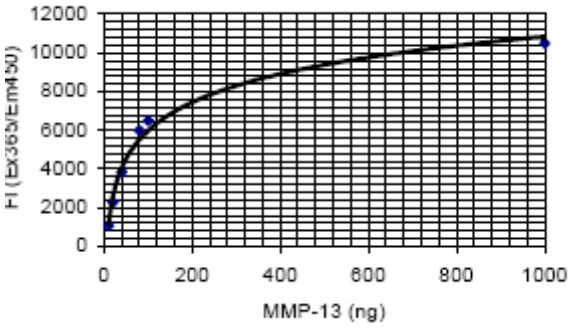

Figure 1 - A typical standard curve for MMP-13

Calculation of Specific Activity of MMP-13:

React 1 µg of MMP-13 (10µg of undiluted MMP-13, 100 µg/ml) with 100 µl of substrate solution (2 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 samples to FI of 2 nmole substrate, the amounts of substrate (nmole) digested can be simply calculated.

Example: If FI of 100% substrate digested was 10495 whereas FI observed in sample 1 and 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 MMP-13 will be obtained as nmole of substrate per µg or ng of protein.

MMP-13 Inhibitor Assay Sheet

A) Activation of MMP-13

	Standard Curve					Inhibitor Assay		
	Blank	Standard 1	Standard 2	Standard 3	Standard 4	Inhibitor 1	Inhibitor 2	Inhibitor 3
Step 1 Reference rh-MMP-13 1:10 Diluted rh-MMP-13 10 ug/ml (ul)	0	2.5	5	7.5	10	5	5	5
Step 2 Solution B (ul)	95	92.5	90	87.5	85	90	90	90
Step 3 Activator 1 (ul)	5	5	5	5	5	5	5	5
Incubate at 35°C for 60 minutes.								
Step 4 Proteinase Inhibitor (ul)	10	10	10	10	10	10	10	10
Total Volume (ul)	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 MMP-13 from Part A (ul)	110	110	110	110	110	110	110	110
Step 2 Inhibitor Test Sample (ul)	0	0	0	0	0	12.5	25	50
Step 3 Solution B (ul)	50	50	50	50	50	37.5	25	0
Step 4 Substrate Solution (ul)	100	100	100	100	100	100	100	100
Incubate at room temperature (25°C) for approximately 30 minutes. (Note: Incubation time depends on the amount of MMP-13 used.)								
Step 5 Stop Solution (ul)	10	10	10	10	10	10	10	10
Total Volume (ul)	270	270	270	270	270	270	270	270
Read Fluorescence Intensity at Ex365/Em450 or Ex340/Em440.								

References

1. Vincenti, M.P. (1988) Biochem. J. **331**: 341-346.
2. Mitchell, P.G. (1996) J.Clin. Invest. **97**: 761-768.
3. Fosang, A.S. (1996) FEBS Lett. **380**: 17-20.
4. Bickett, D.M. et al (1993) Anal. Biochem. **212 (1)**: 58-64.

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Cat. No. ECM487

July 2009
Revision F: 41078