Collagenase Assay Kit

Catalog # 3001 and 3002

For Research Use Only - Not Human or Therapeutic Use

INTRODUCTION

Collagenases are members of the matrix metalloproteinase (MMP) family and degrade collagen types I, II, and III. At least three distinct forms of collagenase (MMP-1, MMP-8, and MMP-13) have been identified. Collagenases are produced by many types of cells such as myeloid and fibrosarcoma cells. Increased collagenase levels have been found in both physiological conditions, such as post-partum uterine tissue or tadpole metamorphosis, and pathological conditions, such as inflammation and tumor metastasis.

These collagenases have almost identical substrate specificities. However, individual collagenases may have unique enzyme-substrate affinities, resulting in different physiological and pathological roles in the turnover of collagen depending on the tissues and cell types. For example, MMP-13 digests type II collagen ten times faster than type I collagen.

This kit is designed to assay mammalian collagenase activities in two hours using FITC-labeled telopeptide-free soluble bovine type I collagen (Catalog # 3001) or bovine type II collagen (Catalog # 3002) as a substrate (1). This kit only requires 1/10 of the assay time compared to conventional assay methods that use a collagen gel substrate and also has higher sensitivity than assays using radioisotope-labeled collagen (2).

PRINCIPLE OF RAPID ASSAY METHOD FOR MAMMALIAN COLLAGENASE

Mammalian collagenases cleave the alpha chain triple helixes of collagen, yielding 3/4 and 1/4 collagen fragments, TC^A and TC^B fragments (Figure 1). The denaturation temperature of these fragments is 34-35°C and 28-29°C respectively, whereas the denaturation temperature of intact collagen is 40°C. Therefore, these cleaved fragments selectively denature into single random coils at 35°C which can be extracted with Extraction Buffer. To shorten the denaturation process, which normally takes 60 minutes (2), Enhancer is used to further digest collagenase-degraded products into small peptides as shown in Figure 1.

Note: When using FITC-labeled type II collagen as a substrate, the denaturation temperature of intact type II collagen and its TC^A fragment is 41°C and 38°C respectively, higher than those of type I collagen. Therefore, when type II collagen is used as a substrate, increase the denaturation temperature of collagenase-degradation products from 35°C to 38°C. To shorten the denaturation process using collagen as a substrate, add a proteinase such as an Enhancer (elastase) and incubate at 38°C for 20 minutes to digest TC^A and TC^B fragments into smaller fragments (See Collagenase Activity Assay, step 4, page 4).

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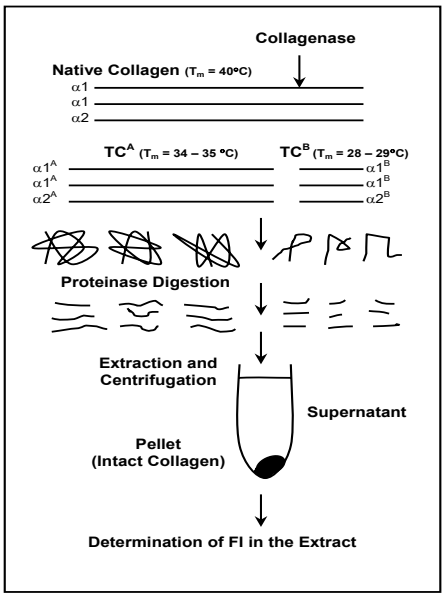
Figure 1 - Principle of the Rapid Collagenase Assay Kit (Type I Collagen)



This assay consists of four steps:

1. Activate latent collagenase in sample specimens with an Activator.
2. React the activated samples with FITC-labeled soluble collagen for 10-120 minutes.
3. Denature and further digest the cleaved collagen fragments into small peptide fragments by Enhancer.
4. Extract the fragments with Extraction Buffer and determine the fluorescent intensity (FI) of the extract.

KIT COMPONENTS

Item	Quantity	Amount	Storage
Reference - Recombinant Human MMP-8 (30043)	1 vial	25 µl, 100 units/ml	-80°C
Solution A - FITC-Collagen Dilution Buffer (30041)	1 bottle	10 ml	4°C
Solution B - Sample Dilution and Reaction Buffer (30042)	1 bottle	50 ml	4°C
FITC-Collagen - 2X FITC-Labeled Bovine Type I or Type II Collagen (4001 or 4002)	1 bottle	10 ml, 1 mg/ml	-20°C
Activator 1 - 20X APMA (Toxic - Handle With Care) (30049)	1 vial	1 ml	RT
Activator 2 - Trypsin (30045)	1 vial	1 mg lyophilized	-20°C
Proteinase Inhibitor (30046)	1 vial	3 mg lyophilized	-20°C
Enhancer - Elastase (30047)	1 vial	1 mg lyophilized	-20°C
Stop Solution - o-Phenanthroline (300410)	1 vial	1 ml, 10 mM	RT
Extraction Buffer (30048)	2 bottles	25 ml	RT
ELISA Plate (Black)	2 each	96-well	RT

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ACTIVATION OF INACTIVE COLLAGENASE

In general, collagenases are secreted as latent proenzymes and require proteolytic conversion for activation. However, collagenase activity is strictly regulated by tissue inhibitors of metalloproteinase (TIMP) and by α2 macroglobulins (α2M) in serum. Thus, different conditions are required to activate collagenase depending on the experimental purpose, source of enzyme, and levels of proteinase inhibitors in individual specimens.

The following is a brief description of activation methods for collagenase and is intended to be used as a guide. See "Tips for Assaying Collagenase Activity" for more detailed information.

1. APMA (4-aminophenylmercuric acetate) is widely used to activate latent pro-collagenase (3).

2. Trypsin activates both latent pro-collagenases and collagenases bound by inhibitors such as α2M and low molecular weight collagenase inhibitors (4, 5). However, soybean trypsin inhibitor (SBTI) must be added to neutralize the added trypsin before assaying collagenase activity.

3. Potassium thiocyanate (KSCN) or potassium iodide (KI) reactivate collagenases bound by inhibitors such as α2M (6). These reagents may be useful for denaturing collagenase inhibitors in sample specimens prior to activating procollagenase by APMA (7).

4. Both dithiothreitol (DTT) and iodoacetamide have been reported to reactivate collagenases bound by TIMPs (8). However, these reagents also may inactivate collagenase by reducing the disulfide bonds and alkylating glutamic acid at the active site. Therefore, the limitations of this method must be taken into consideration.

SAFETY PRECAUTIONS

Activator 1 (APMA) contains mercury which is very toxic if inhaled, ingested, or makes contact with skin. Neurological hazard target organs include the kidneys and nerves. Wear suitable protective gloves, clothing, and eyewear.

ASSAY PROCEDURE

ACTIVATE COLLAGENASE

A collagenase activation method should be chosen depending on the experimental purpose, source of collagenase, and enzyme type. Pro-collagenases, such as recombinant human neutrophil collagenase, can be activated by either APMA (Activator 1) or trypsin (Activator 2). We recommend selecting one suitable method and optimizing the activation conditions.

1. Prepare 1.5 ml microcentrifuge tubes for (1) Buffer, (2) 100% Control, (3) Blank, (4) Reference MMP-8, and (5) Test Samples as shown on the collagenase assay sheet, page 6.

   Note: Proteins in sample specimens may cause quenching, and consequently, fluorescent intensity (FI) determined in sample tubes might be underestimated. For example, if the collagenase activity is very low in a sample solution which contains a certain amount of contaminant proteins, the FI in the samples will be lower than the Blank value. In order to correct these underestimated results, the identical sample mixed with Stop Solution should be added to the Blank tubes and 100% Control tubes. This quenching is mainly caused by turbidity formed by the proteins in the Extraction Buffer. Similarly, colors or dyes in cell culture media also causes quenching. In this case, add the same culture medium to Blank and 100% Control tubes.

2. Add the proper amounts of Solution B, reference MMP-8, and test samples to adjust the final volume to 180 µl as shown on the assay sheet. The buffer tube should only have 380 µl of Solution B.

   Note: The sample volume may be 5 -180 µl. However, the final volume should be adjusted to 180 µl with Solution B.

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3. Activate collagenase with Activator 1 or Activator 2.

   To activate collagenase, choose one of the methods described in step 3a OR 3b. In general, it is not necessary to activate collagenase using both Activator 1 and Activator 2 as the collagenases activated by APMA will be digested and inactivated by trypsin.

   APMA-activated collagenase may be immediately inhibited by proteinase inhibitors coexsiting in samples, such as α2M. In these cases, trypsin activation may be more effective than APMA. The trypsin concentration will need to be optimized. 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 5mM $CaCl_2$.

   a) Add 10 µl of Activator 1 to the test tubes and incubate for 60 minutes at 35°C. **Do not add Activator 1 to undiluted samples, because it is a strong alkaline solution.**

   OR

   b) Dissolve one vial of trypsin (Activator 2) in 1 ml of Solution B. Add 10 µl of Activator 2 to the test tubes and incubate for 60 minutes at 35°C.

      Note: Trypsin concentration should be optimized for individual samples.

4. Dissolve one vial of proteinase inhibitor in 1 ml of Solution B. Add 10 µl of proteinase inhibitor into all test tubes to neutralize non-collagenolytic proteinases in sample solutions.

COLLAGENASE ACTIVITY ASSAY

1. Prepare a 1X FITC-collagen solution by mixing an equal volume of the 2X FITC-collagen and Solution A at 4°C (200 µl of the mixture is required for each sample to be tested) in a container protected from light, such as an amber colored tube or bottle (FITC is light sensitive).

2. Add 200 µl of the 1X FITC-collagen solution into the activated collagenase samples (200 µl). Mix well and incubate at 35°C for 10-120 minutes. Separately incubate the 100% control tube in boiling water for 5 minutes to denature the FITC-collagen.

   Note: Incubate reference MMP-8 for 60 minutes at 35°C. However, the incubation time for samples will vary depending on the collagenase activity in sample specimens. Do not incubate samples longer than 120 minutes to avoid high background levels.

3. Stop the collagenase reaction by adding 10 µl of Stop Solution to each tube and mix well.

4. Dissolve one vial of Enhancer in 1 ml of Solution B. Add 10 µl of Enhancer to each tube and incubate at 35°C for 10 - 20 minutes. This will further digest the collagenase-degradation products into smaller fragments).

   Note: If type II collagen is used as a substrate (catalog # 3002), increase the denaturation temperature from 35°C to 38°C. Add 10 µl of Enhancer to each tube and incubate at 38°C for 20 minutes.

5. Cool samples to room temperature. Add 400 µl of Extraction Buffer to each tube. Do not use cold buffer. Mix vigorously and centrifuge at 10,000 rpm for 10 minutes.

6. Carefully transfer 200 µl of each supernatant into the black 96-well plates provided in the kit and determine the fluorescence intensity (FI) at λem = 520 nm and λex = 490 nm.

   Note 1: Colored samples such as cell culture media may reduce FI approximately 5-10% by quenching, thus the same culture media must be added to the 100% control tubes for accurate results.

   Note 2: Supernatants contaminated with pellets in the 96-well plate will lead to high FI values, resulting in overestimated assay results

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CALCULATION OF COLLAGENASE ACTIVITY

One unit of collagenolytic activity is defined as the cleavage of 1 μg of collagen per minute (1 units = 1 μg/minutes). Because this kit uses 100 μg of collagen as a substrate per an assay, collagenolytic activity is calculated by the following equation:

$$\text{Collagenase Activity (units/ml)} = \frac{(FI_{sample} - FI_{blank}) \times 100\ \mu g}{(FI_{control} - FI_{blank}) \times \text{Reaction Time (minute)} \times \text{Sample Volume (ml)}}$$

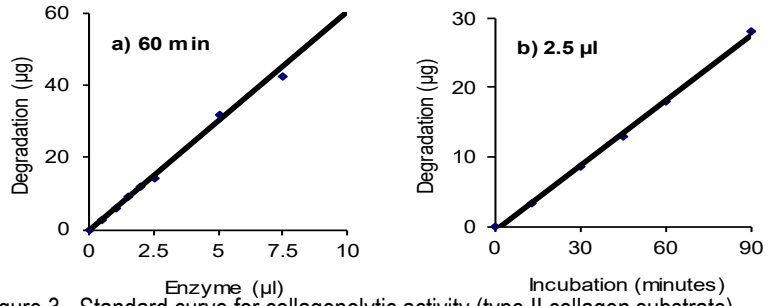
FI_{blank} = FI in blank

$FI_{control}$ = FI in 100% control

FI_{sample} = FI in test samples

Note: Reference MMP-8 works to check assay acuracy. Collagenase activities in individual samples must be calculated using the equation above.

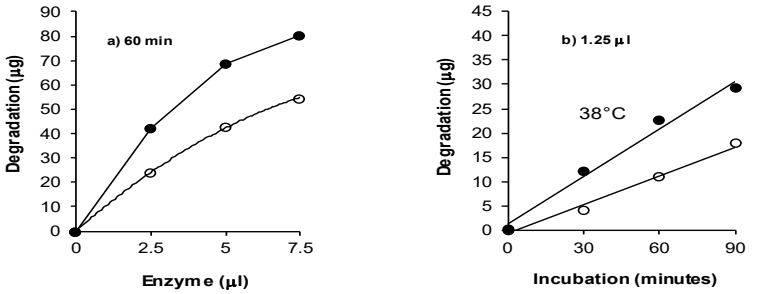
Figure 2 - Standard curve for collagenolytic activity (type I collagen substrate)



a) MMP-8 activity dose response using FITC-type I collagen as a substrate - Various amounts (2.5 – 10 μl) of reference MMP-8 (100 units/ml) were reacted with 100 μg of FITC-type I collagen at 35°C for 60 minutes. The mixtures were further incubated at 35°C for 10 minutes after adding 10 μl of Enhancer.

b) MMP-8 activity time couse using FITC-type I collagen as a substrate - 2.5 μl of MMP-8 (100 units/ml) was reacted with 100 μg of FITC-type I collagen at 35°C for 90 minutes.

Figure 3 - Standard curve for collagenolytic activity (type II collagen substrate)



a) MMP-8 activity dose response using type II collagen as a substrate - Various amounts (2.5-7.5 μl) of reference MMP-8 (100 units/ml) were reacted with 100 μg of FITC-labeled type II collagen for 60 minutes at 35°C and 38°C. The mixtures were further incubated at 38°C for 20 minutes after adding 10 μl of Enhancer.

b) MMP-8 activity time course using FITC-type II collagen as a substrate - 1.25 μl of MMP-8 (100 units/ml) was reacted with 100 μg of FITC-type II collagen at 35°C and 38°C for 90 minutes.

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Collagenase Assay Sheet

This assay sheet is provided as a guideline. Researchers will need to optimize the assay for their individual needs.

A) Activation

	Buffer	Control (100%)	Blank	Ref 1	Ref 2	Ref 3	Ref 4	Test 1	Test 2
Step 1 Add Reference rhMMP-8 or Test Sample (µl)	0	0	0	2.5	5	7.5	10	100	100
Step 2 Solution B (µl)	380	180	180	177.5	175	172.5	170	80	80
Step 3a Add Activator 1 - APMA (µl)	10	10	10	10	10	10	10	10	10
OR									
Step 3b Add Activator 2 - Trypsin (µl)	10	10	10	10	10	10	10	10	10
Incubate at 35°C for 60 minutes									
Step 4 Add Proteinase Inhibitor (µl)	10	10	10	10	10	10	10	10	10
Total Volume (µl)	400	200	200	200	200	200	200	200	200

B) Enzyme Assay - Prepare the 1X FITC-collagen solution. Then, add 200 µl of the 1X FITC-collagen solution (100 µg of collagen) to the activated enzyme solution.

	Buffer	Control (100%)	Blank	Ref 1	Ref 2	Ref 3	Ref 4	Test 1	Test 2
Activated Enzyme from Step 4 (µl)	400	200	200	200	200	200	200	200	200
Step 5 Add 1X FITC-Collagen (µl)	0	200*	200	200	200	200	200	200	200
React at 35°C (type I substrate) or 38°C (type II substrate) for 0-120 minutes									
Step 6 Add Stop Solution (µl)	10	10	10	10	10	10	10	10	10
Step 7 Add Enhancer Solution (µl)	10	10	10	10	10	10	10	10	10
Incubate at 35°C (type I substrate) or 38°C (type II substrate) for 10-20 minutes									
Step 8 Add Extraction Buffer (µl)	400	400	400	400	400	400	400	400	400
Mix well and centrifuge at 10,000 rpm for 5 minutes.									
Transfer 200 µl of supernatant into a 96-well flat bottom black plate									
Step 9 Determine FI at Em 520/Ex 490	FI_{blank}	$FI_{control}$ = 100 µg collagen	$FI_{(0)}$	$FI_{(2.5)}$	$FI_{(5)}$	$FI_{(7.5)}$	$FI_{(10)}$	$FI_{(test\ 1)}$	$FI_{(test\ 2)}$
Calculate collagenase activity by comparing FI_{test} and $FI_{control}$.									

*Heat-denatured 1X FITC Collagen

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