Collagenase Assay Kits

Catalog # 3001 & 3002

For Research Use Only - Not Human or Therapeutic Use

PRODUCT SPECIFICATIONS

DESCRIPTION:	Assay kit to assess collagenase activity
	3001: Rapid Collagenase Assay Kit with Type I Collagen Substrate
	3002: Rapid Collagenase Assay Kit with Type II Collagen Substrate
FORMAT:	96-well ELISA Plate with non-removeable strips
ASSAY TYPE:	Enzyme Assay/Fluorescence-based Assay
ASSAY TIME:	2.5 hours
STANDARD RANGE:	Depends on incubation time
NUMBER OF SAMPLES:	Up to 44 (duplicate) samples/plate
SAMPLE TYPES:	Culture Media and Tissue Homogenate
RECOMMENDED SAMPLE DILUTIONS:	Depends on enzyme activity in samples
CHROMOGEN:	N/A (Read at Emission 520 nm/Excitation 490 nm)
STORAGE:	-20°C for 12 months (Reference Standard is stored at -80°C)
VALIDATION DATA:	N/A
NOTES:	Uses FITC

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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 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 approximately 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 of conventional assay methods that use a collagen gel substrate and it also has higher assay sensitivity than assays using radioisotope-labeled collagen (2).

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	-20°C/*
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	-20°C/*
Extraction Buffer (30048)	2 bottles	25 ml	-20°C/*
Black ELISA Plate	2 each	96-well	-20°C/*

*These reagents can also be stored at room temperature

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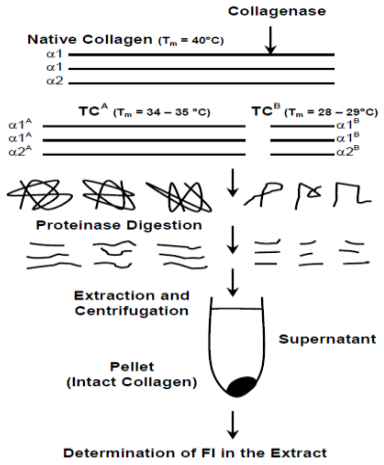
PRINCIPLE OF THE RAPID COLLAGENASE ASSAY KIT (TYPE I COLLAGEN)

Mammalian collagenases cleave the alpha chain triple helixes of collagen, yielding 3/4 and 1/4 collagen fragments, TC^A and TC^B fragments. 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, an enhancer is used to further digest collagenase-degraded products into small peptides (2).



This assay consists of four steps:

1. Activate latent collagenase in samples 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.

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 when using collagen as a substrate, add a proteinase such as elastase and incubate at 38°C for 20 minutes to digest TC^A and TC^B fragments into smaller fragments.

NOTES BEFORE USING ASSAY

NOTE 1: 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.

NOTE 2: APMA (4-aminophenylmercuric acetate) is widely used to activate latent pro-collagenase (3). 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.

NOTE 3: 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.

NOTE 4: Potassium thiocyanate (KSCN) or potassium iodide (KI) reactivates collagenases bound by inhibitors such as α2M (6). 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 $CaCl_2$. These reagents may be useful for denaturing collagenase inhibitors in sample specimens prior to activating pro-collagenase by APMA (7).

NOTE 5: 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.

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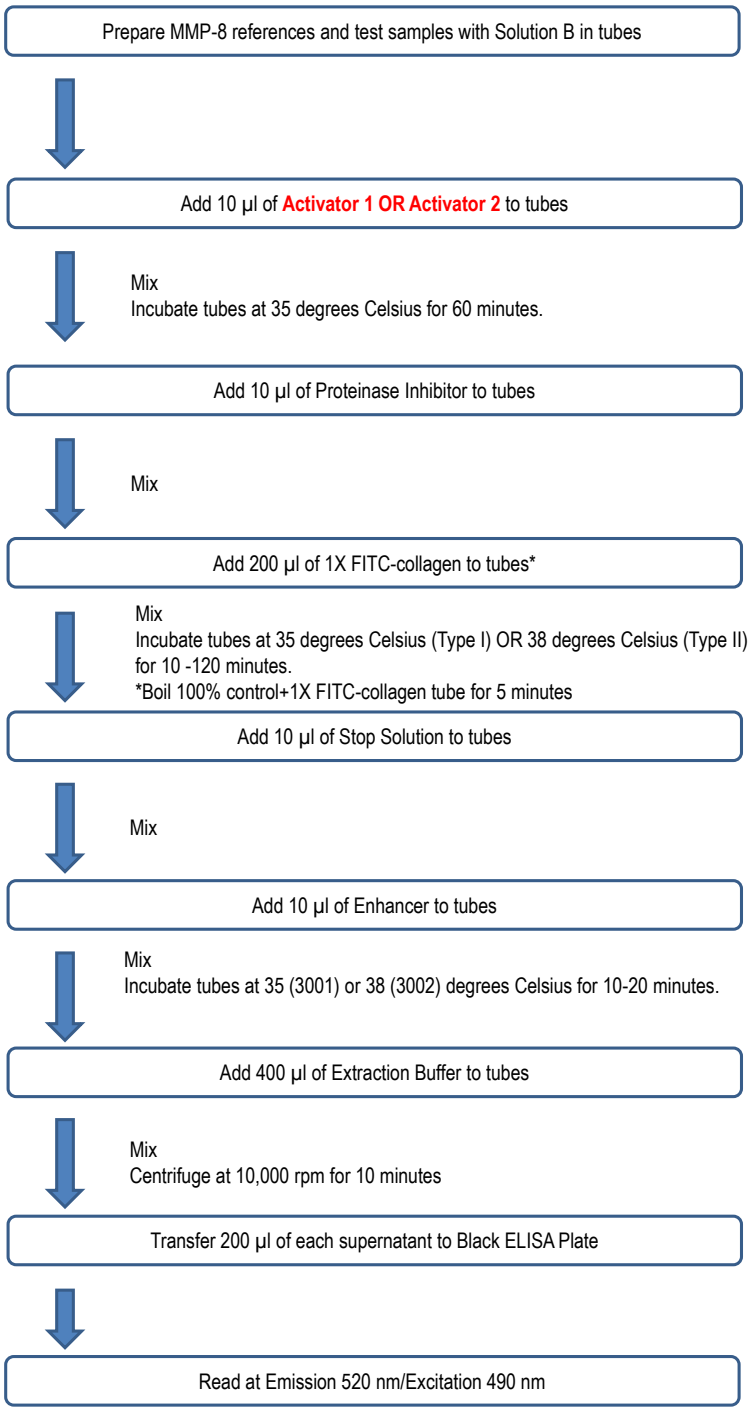
ASSAY OUTLINE



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ASSAY PROCEDURE

1. **Prepare Microcentrifuge Tubes**: Prepare 1.5 ml microcentrifuge tubes for Buffer, 100% Control, Blank, Reference MMP-8, and Test Samples as shown on the Collagenase assay sheet at the end of this protocol.

   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 under-estimated 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 References and Samples**: Add the proper amounts of Solution B, reference MMP-8, and test samples to tubes and 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.

3. **Activate collagenase with Activator 1 or Activator 2**: To activate collagenase, choose one of the methods described in step 3a OR 3b depending on the experimental purpose, source of collagenase, and enzyme type. 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.

   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

   Dissolve one vial of 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 1: APMA (Activator 1) is recommended as a general activator for latent collagenase activation. However, APMA-activated collagenase may be immediately inhibited by proteinase inhibitors coexisting in samples, such as α2M. In these cases, trypsin activation may be more effective than APMA.

   NOTE 2: Trypsin concentration should be optimized for individual samples.

   NOTE 3: Collagenase activity is strictly regulated by tissue inhibitors of metalloproteinase (TIMP) and by α2 macroglobulins (α 2M). For re-activation of inactivated collagenase, Tips for Assaying Collagenase Activity can be obtained from Chondrex, Inc. customer service (support@chondrex.com)

4. **Add Proteinase Inhibitor**: 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 solution.

5. **Prepare 1X FITC**: Prepare a 1X FITC-collagen solution by mixing an equal volume of the 2X FITC-collagen and cold Solution A (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).

6. **Add 1X FITC**: Add 200 µl of the 1X FITC-collagen solution into the activated tubes (200 µl). Mix well and incubate at 35°C for Type I Collagen (Cat # 4001) OR 38°C for Type II Collagen (Cat # 4002) 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 test samples longer than 120 minutes otherwise they may yield high background levels. Background refers to the degradation of collagen due to extended exposure to high temperatures.

7. **Add Stop Solution**: Stop the collagenase reaction by adding 10 µl of Stop Solution to each tube and mix well.

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8. **Add Enhancer**: Dissolve one vial of Enhancer in 1 ml of Solution B. Add 10 µl of Enhancer to each tube and incubate at 35°C (Cat # 3001 Type I collagen) or 38°C (Cat # 3002 Type II collagen) for 10 - 20 minutes. This will further digest the collagenase-degradation products into smaller fragments.

9. **Add Extraction Buffer**: 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.

10. **Transfer**: Carefully transfer 200 µl of each supernatant (in duplicate) 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

CALCULATING RESULTS

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

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

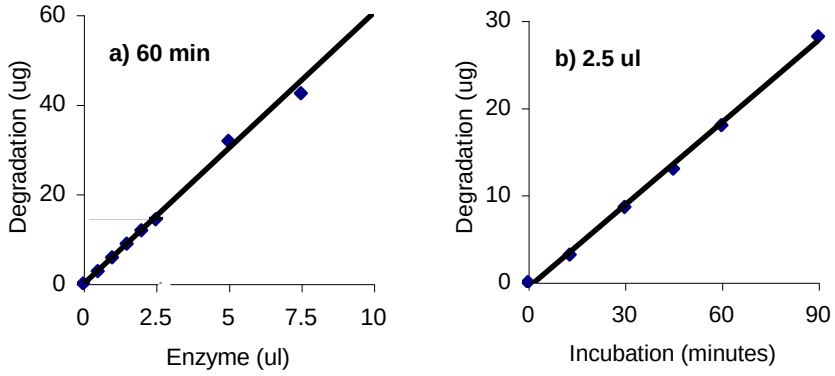
FI_{blank}= FI in Blank

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

FI_{sample}=FI in Test Samples

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

Figure 1a - Standard Curves 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 course using FITC-Type I collagen as a substrate - 2.5 µl of MMP-8 (100 units/ml) was reacted with 100 µg FITC-Type I collagen at 35°C for 90 minutes.

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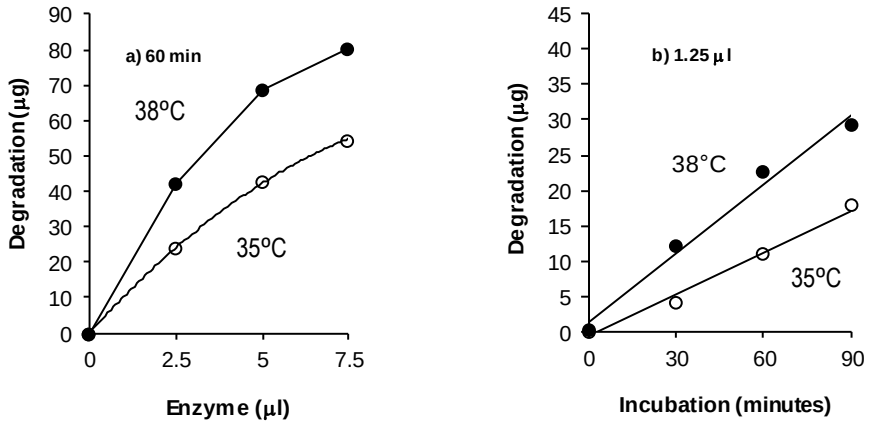
Figure 1b - Standard Curves for Collagenolytic Activity (Type II Collagen Substrate)



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.

TROUBLESHOOTING

For frequently asked questions about assays and ELISAs, please see Chondrex, Inc.'s Assay FAQ for more information.

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COLLAGENASE ASSAY SHEET

This assay sheet is provided as a guide. 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 and Test Samples (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

	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 10 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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