Bacterial Collagenase Assay Kit

Catalog # 3014

For Research Use Only - Not Human or Therapeutic Use

PRODUCT SPECIFICATIONS

DESCRIPTION:	Assay kit to assess collagenase activity and inhibitory activity to collagenase
FORMAT:	96-well ELISA plate with non-removeable strips
ASSAY TYPE:	Enzyme Assay/Fluorescence-based Assay
ASSAY TIME:	Approximately 2 hours
STANDARD RANGE:	Depends on incubation time
NUMBER OF SAMPLES:	Activity Assay: Up to 41 (duplicate) samples/plate
	Inhibitor Assay: Up to 45 (duplicate) samples/plate
SAMPLE TYPES:	Culture Media and Tissue Homogenate
RECOMMENDED SAMPLE DILUTIONS:	Depends on enzyme activity in samples
CHROMOGEN:	N/A (Read Fluorescence Intensity at Emission 520 nm/Excitation 490 nm)
STORAGE:	-20°C for 12 months
VALIDATION DATA:	N/A
NOTES:	Uses FITC

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16928 Woodinville-Redmond Rd NE Suite B-101
Woodinville, WA 98072
Phone: 425.702.6365 or 888.246.6373
Fax: 425.882.3094
www.chondrex.com
support@chondrex.com

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INTRODUCTION

Collagenase produced by *Clostridium histolyticum*, isolated by Mandl *et al*. in 1953 (1), has been widely used in research and clinical fields for isolating cells and digesting connective tissues (2). Importantly, bacterial collagenases differ from mammalian collagenases in their substrate specificity, as bacterial collagenases cleave the native collagen molecule into multiple fragments, whereas mammalian collagenases cleave the collagen molecule at a single site into two fragments. *Clostridium* species infection causes severe tissue necrosis leading to gas gangrene (3). At the infection site, collagenases from *Clostridium* species facilitate extensive unregulated destruction of the extracellular matrix because of the absence of bacterial collagenase inhibitors in animal and human sera, whereas mammalian collagenase activity is strictly regulated by mammalian serum inhibitors (4-6).

Chondrex, Inc. provides a rapid bacterial collagenase assay kit using soluble FITC-labeled type I collagen as a substrate instead of radio-labeled collagen (7). This kit can be used not only for assaying collagenase activity, but also for inhibitor assay and includes protocols for both.

Note: The reference collagenase provided in this kit is a positive control, not a standard. Collagenase activity in samples should be determined based on the amounts of collagen substrate digested.

KIT COMPONENTS

Item	Quantity	Amount	Storage
Reference *Clostridium histolyticum* Collagenase (30141)	2 vials	100 units lyophilized	-20°C
Solution A - Substrate Dilution Buffer (30041)	1 bottle	10 ml	-20°C
Solution B - Sample Dilution and Reaction Buffer (30042)	1 bottle	50 ml	-20°C
2X FITC-Labeled Bovine Type I Collagen (4001)	1 bottle	10 ml, 1 mg/ml in 0.01M acetic acid	-20°C
Proteinase Inhibitor (30046)	1 vial	3 mg lyophilized	-20°C
Stop Solution - o-Phenanthroline (300410)	1 vial	1 ml, 10 mM in ethanol	-20°C/*
Extraction Buffer (30048)	2 bottles	25 ml	-20°C/*
Black ELISA Plate	1 plate	96-well	-20°C/*

*These reagents can also be stored at room temperature

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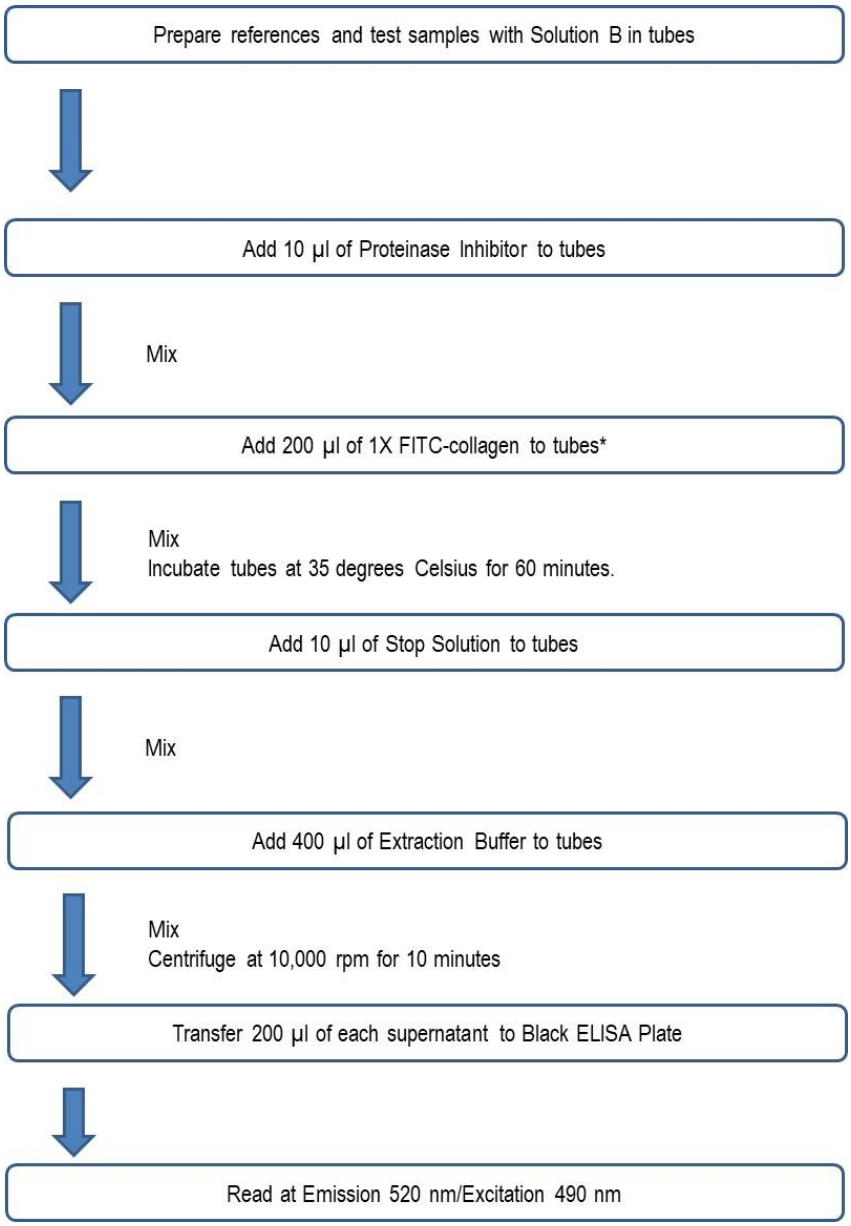
ASSAY OUTLINE (FOR BOTH COLLAGENASE ACTIVITY AND INHIBITOR ASSAY)



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ASSAY PROCEDURE: COLLAGENASE ACTIVITY ASSAY

1. **Prepare Microcentrifuge Tubes and Reference**: Prepare amber 1.5 ml microcentrifuge tubes for Buffer, 100% Control, Blank, Reference Collagenases, and Test Samples as shown on the Collagenase Activity assay sheet. Dissolve one vial of Reference collagenase in 1 ml of Solution B. Partially used Reference collagenase stock solution can be kept at - 20°C for future assays.

   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 bacteria culture media also causes quenching. In this case, add the same culture media to Blank and 100% Control tubes.

2. **Add References and Samples**: Add the proper amounts of Solution B, Reference Collagenase, and test samples to tubes and adjust the final volume to 190 µl as shown on the assay sheet. The buffer tube should have 390 µl of Solution B.

   NOTE 1: *Because bacterial collagenase exists as an active form, collagenase activation is not necessary.*

   NOTE 2: The test sample volumes may vary from 1 -100 µl. However, the final assay sample volume should be adjusted to 190 µl with Solution B.

3. **Add Proteinase Inhibitor**: Dissolve one vial of proteinase inhibitor in 1 ml of Solution B. Add 10 µl of proteinase inhibitor into all tubes to neutralize non-collagenolytic proteinases in solution.

4. **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).

5. **Add 1X FITC**: Add 200 µl of the 1X FITC-collagen solution into all tubes (200 µl) except for the Buffer tube. Mix well and incubate at 35°C for 10-120 minutes.

   NOTE: Incubate the reference and 100% control tubes for 60 minutes at 35°C. The incubation time for test 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.

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

7. **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.

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

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CALCULATING COLLAGENASE ACTIVITY

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:

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

FI_{blank}= FI in Blank

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

FI_{sample}=FI in Test Samples

COLLAGENASE ACTIVITY ASSAY SHEET

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

	Buffer	Control (100%)	Blank	Ref 1	Ref 2	Ref 3	Ref 4	Test Sample
Step 1 Add Reference Collagenase (µl)	0	50	0	2.5	5	7.5	10	0
Step 2 Add Test Sample (µl)	0	0	0	0	0	0	0	1-100
Step 3 Add Solution B (µl)	390	140	190	187.5	185	182.5	180	189-90
Step 4 Add Proteinase Inhibitor (µl)	10	10	10	10	10	10	10	10
Total Enzyme Solution (µl)	400	200	200	200	200	200	200	200
Step 5 Add 1X FITC-Collagen (µl)	0	200	200	200	200	200	200	200
React at 35°C for 10-120 minutes.								
Step 6 Add Stop Solution (µl)	10	10	10	10	10	10	10	10
Step 7 Add Extraction Buffer (µl)	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 8 Determine FI at Em 520/Ex 490	FI_{buffer}	$FI_{control}$	FI_{blank}	$FI_{(2.5)}$	$FI_{(5)}$	$FI_{(7.5)}$	$FI_{(10)}$	$FI_{(sample)}$
Calculate collagenase activity by comparing FI.								

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ASSAY PROCEDURE: COLLAGENASE INHIBITOR ASSAY

1. **Prepare Microcentrifuge Tubes and Reference**: Prepare amber 1.5 ml microcentrifuge tubes for 100% Control, Blank, Reference Collagenase, and Test Samples as shown on the Collagenase Inhibitor assay sheet. Dissolve one vial of Reference collagenase in 1 ml of Solution B. Partially used Reference collagenase stock solution can be kept at - 20°C for future assays.

   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 bacteria culture media also causes quenching. In this case, add the same culture media to Blank and 100% Control tubes.

2. **Prepare References and Samples**: Add the proper amounts of Solution B, Reference Collagenase, and test samples and adjust the final volume to 190 µl as shown on the assay sheet.

   NOTE 1: *Because bacterial collagenase exists as an active form, collagenase activation is not necessary.*

   NOTE 2: The test sample volumes may vary from 1-100 µl. However, the final assay sample volume should be adjusted to 190 µl with Solution B.

3. **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.

4. **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).

5. **Add 1X FITC**: Add 200 µl of the 1X FITC-collagen solution to all tubes (200 µl). Mix well and incubate at 35°C for 60 minutes.

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

7. **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.

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

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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:

$$\text{Collagenase Activity (units/ml)}: \frac{(FI_{sample} - FI_{blank}) \times 100\ \mu g}{(FI_{control} - FI_{blank}) \times 60\ (\text{minutes}) \times 0.1\ (\text{ml})}$$

FI_{blank}= FI in Blank

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

FI_{sample}=FI in Test Samples

$$\text{Percent Inhibition}: \frac{\text{Collagenase Activity (sample)}}{\text{Collagenase Activity (reference)}} \times 100$$

COLLAGENASE INHIBITOR ASSAY SHEET

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

	100% Control	Blank	Reference	Test Sample
Step 1 Add Reference Collagenase (µl)	50	0	10	10
Step 2 Add Test Sample (µl)	0	0	0	1-100
Step 3 Add Solution B (µl)	140	190	180	179-80
Step 4 Add Proteinase Inhibitor (µl)	10	10	10	10
Total Enzyme Solution (µl)	200	200	200	200
Step 5 Add 1X FITC-Collagen (µl)	200	200	200	200
React at 35°C for 60 minutes.				
Step 6 Add Stop Solution (µl)	10	10	10	10
Step 7 Add Extraction Buffer (µl)	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 8 Determine FI at Em 520/Ex 490	$FI_{control}$	FI_{blank}	$FI_{reference}$	FI_{sample}
Calculate collagenase activity by comparing FI.				

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TROUBLESHOOTING

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

REFERENCES

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7. K. Terato, Y. Nagai, K. Kawanishi, S. Yamamoto, A rapid assay method of collagenase activity using 14C-labeled soluble collagen as substrate. Biochim Biophys Acta 445, 753-62 (1976).

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