# Type II Collagen Detection Kit

Catalog # 6018

*For Research Use Only - Not Human or Therapeutic Use*

## INTRODUCTION

Type II collagen is unique among the collagen family, and its tissue distribution is limited to avascular tissues such as cartilage and the vitreous body of the eye. Since type II collagen can induce arthritis in experimental animals, autoimmunity to type II collagen is suspected in the pathogenesis of certain autoimmune diseases in humans such as rheumatoid arthritis, eye diseases associated with rheumatoid arthritis, and relapsing polychondritis, which affects specific tissues containing type II collagen.

The Type II Collagen Detection Kit (Catalog # 6018) is designed to quantify the amount of solubilized native type II collagen from various species (such as human, monkey, porcine, bovine, rat, mouse, rabbit, equine, dog, and chick) in cell and/or tissue culture or from tissue specimens by ELISA.

*To save your time, Chondrex, Inc. recommends the 1-step assay protocol (see Part II-A, page 2). However, this kit does allow the use of a 2-step assay protocol (see Part II-B, page 5) as well. Please use the appropriate protocol for your studies. Both protocols yield comparable standard curves and identical sample results.*

## KIT COMPONENTS

| Item | Quantity | Amount | Storage |
|---|---|---|---|
| Type II Collagen Standard (9034) | 1 vial | 100 µl, 100 µg/ml | -20°C |
| Capture Antibody (60182) | 1 vial | 100 µl, 5 mg/ml | -20°C |
| Detection Antibody (60183) | 1 vial | Lyophilized | -20°C |
| Solution A - Capture Antibody Dilution Buffer (9052) | 1 bottle | 10 ml | -20°C |
| Solution B - Sample/Standard Dilution Buffer (9053) | 1 bottle | 50 ml | -20°C |
| Solution C - Detection Antibody Dilution Buffer (9054) | 1 bottle | 10 ml | -20°C |
| Solution D - Streptavidin Peroxidase Dilution Buffer (9055) | 1 bottle | 20 ml | -20°C |
| Streptavidin Peroxidase (9029) | 2 vials | 50 µl | -20°C |
| OPD (90021) | 2 vials | Lyophilized | -20°C |
| Chromogen Dilution Buffer (90022) | 1 bottle | 20 ml | -20°C |
| Stop Solution - 2N Sulfuric Acid (9016) | 1 bottle | 10 ml | -20°C |
| Wash Buffer, 20X (9005) | 1 bottle | 50 ml | -20°C |
| ELISA Plate | 1 each | 96-well (8-well strips x 12) | -20°C |



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## NOTES ON PROCESSING SAMPLES

### Part I: SOLUBILIZATION OF COLLAGEN

This ELISA kit requires that collagen from cultured cell layers and tissues be solubilized before proceeding with the assay. Pepsin digestion is recommended for solubilizing collagen from sample specimens. However, the collagen solubilization protocol should be optimized depending on the type of tissue and the level of intra- and inter-molecular cross-linkages in your sample.

In general, cartilage from younger sources can be solubilized with pepsin within 24 - 48 hours, whereas cartilage from adult or older sources require at least 7-9 days of pepsin digestion. This is because collagen from younger sources have lower levels of intra- and inter-molecular cross-linkages, whereas collagen from older sources contain more intra- and inter-molecular cross-linkages. Additionally, the more cross-linked the collagen is, the lower the yield will be, since pepsin can only digest the telopeptides located on both the N- and C-terminals of the collagen molecule, but is not capable of digesting the helical conformation region of the collagen molecule and intra- and inter-molecular cross-linkages.

In some cases, pepsin-resistant collagen (insoluble) might be solubilized with alkaline treatment. Suspend insoluble collagen in cold 0.1N NaOH solution containing 10% $Na_2SO_4$ and 0.1M amine such as Tris, and incubate at 4°C for 1-2 weeks. After the alkaline treatment, raise the pH to 5.0 with HCl, and then dilute it with 0.05M acetic acid or a neutral buffer such as 0.1M Tris-0.3M NaCl, pH 7.5.

Chondrex, Inc. recommends optimizing a solubilization protocol for each unique tissue sample before processing a large batch. Moreover, the level of collagen solubilization can be evaluated via 6% SDS-gel under non-reducing conditions (Chondrex, Inc.'s type II collagen may be used as a standard). If samples contain bands larger than the γ-chain (MW = 300 kDa), the samples must be further digested by elastase which converts polymeric collagen into monomeric collagen. On the other hand, if smaller bands or smear bands are observed beneath the α-chain (MW = 100 kDa), the samples might be over-digested. Once the collagen is solubilized, it is ready for ELISA.

**Tips for collagen solubilization can be obtained from Chondrex, Inc. customer service (support@chondrex.com).**

## NOTES BEFORE USING ASSAY

Note 1: It is recommended that the standard and samples be run in duplicate.

Note 2: Partially used reagents may be kept at –20°C.

Note 3: Crystals may form in the 20X wash buffer when stored at cold temperatures. If crystals have formed, it is necessary to warm the wash buffer by placing the bottle in warm water until crystals have dissolved completely.

Note 4: Measure exact volume of buffers using a serological pipette prior to diluting, as extra buffer is provided.

### Part II-A: 1-STEP ASSAY PROTOCOL

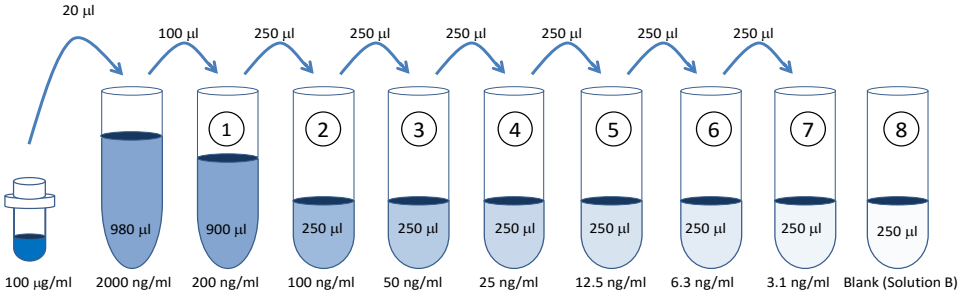
*To save your time, Chondrex, Inc. recommends the 1-step assay protocol (see Part II-A, page 2). However, this kit does allow the use of a 2-step assay protocol (see Part II-B, page 5) as well. Please use the appropriate protocol for your studies. Both protocols yield comparable standard curves and identical sample results.*

**All reagents must be at room temperature before use.**

1. **Add Capture Antibody**: Dilute one vial of Capture Antibody with 10 ml of Capture Antibody Dilution Buffer (Solution A). Add 100 µl of capture antibody solution to each well and incubate at 4°C overnight.

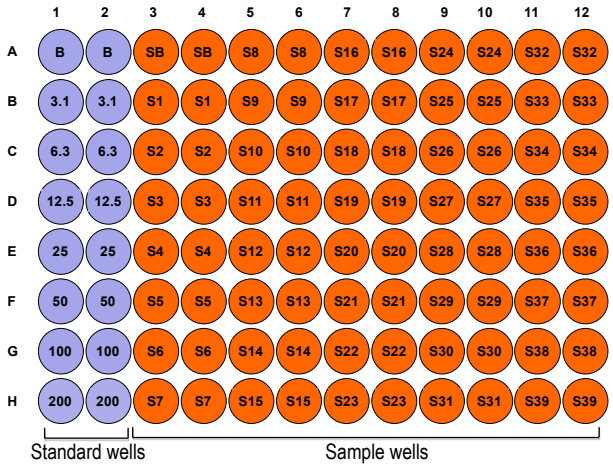
2. **Dilute Wash Buffer**: Dilute 50 ml of 20X wash buffer in 950 ml of distilled water (1X wash buffer). Wash the plate with 1X wash buffer at least 3 times using a wash bottle with manifold or an automated plate washer. Empty the plate by inverting it and blot on a paper towel to remove excess liquid. *Do not allow the plate to dry out.*

3. **Prepare Standard Dilutions**: The standard range is 3.1 - 200 ng/ml. Prepare serial dilutions of the standard by mixing 20 µl of 100 µg/ml standard with 980 µl of Sample/Standard Dilution Buffer (Solution B) - 2,000 ng/ml.Then mix 100 µl of the 2,000 ng/ml standard with 900 µl of Solution B to make 200 ng/ml. Next, mix 250 µl of the 200 ng/ml standard with 250 µl of Solution B to make 100 ng/ml. Then repeat this procedure to make five more serial dilutions of standard - 50, 25, 12.5, 6.3, and 3.1 ng/ml solutions. The 100 µg/ml standard can be stored at -20°C for use in a second assay. Fresh serial dilutions should be prepared for each assay.




4. **Prepare Sample Dilutions**: Dilute solubilized samples 1:1-1:1000 with Solution B depending on the estimated collagen content in the samples. Sample solutions (buffers used in the collagen solubilization process which contain pepsin, and other reagents) may need to be assayed depending on the final sample dilution.

5. **Add Standards and Samples**: Mix samples and standard tubes well. Add 50 µl of Solution B (blank), standards and samples to appropriate wells.




B: Blank, SB: Sample Blank, S: Sample

6. **Add Detection Antibody**: Reconstitute one vial of Detection Antibody with 50 µl of distilled water to make a Detection Antibody Stock Solution. Dilute 50 µl of the detection antibody stock solution in 5 ml of Detection Antibody Dilution Buffer (Solution C). Add 50 µl of detection antibody solution to all wells. Mix detection antibody solution and sample/standard solution wells by tapping or using a plate shaker. Then, cover the plate with a plate sealer and incubate at room temperature for 2 hours. If you plan to use less, the leftover detection antibody stock solution can be stored in its original vial at -20°C for use in a second assay.

7. **Wash**: Wash the plate with 1X wash buffer at least 3 times using a wash bottle with manifold or an automated plate washer. Empty the plate by inverting it and blot on a paper towel to remove excess liquid. *Do not allow the plate to dry out.*

8. **Add Streptavidin Peroxidase**: Dilute one vial of Streptavidin Peroxidase in 10 ml of Streptavidin Peroxidase Dilution Buffer (Solution D). Add 100 µl of streptavidin peroxidase solution to each well, cover the plate with a plate sealer, and incubate at room temperature for 1 hour.

9. **Wash**: Wash the plate with 1X wash buffer at least 3 times using a wash bottle with manifold or an automated plate washer. Empty the plate by inverting it and blot on a paper towel to remove excess liquid. *Do not allow the plate to dry out.*



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10. **OPD**: Dissolve one vial of OPD in 10 ml of Chromogen Dilution Buffer just prior to use. Add 100 µl of OPD solution to each well immediately after washing the plate. Incubate for 30 minutes at room temperature.

11. **Stop**: Add 50 µl of 2N sulfuric acid (Stop Solution) to each well.

12. **Read Plate**: Read the OD values at 490 nm. If the OD values of samples are greater than the OD values of the highest standard, re-assay the samples at a higher dilution. A 630 nm filter can be used as a reference.

## CALCULATION OF RESULTS

1. Average the duplicate OD values for the blank, standards, and test samples.

2. Subtract the "blank" (B) values from the averaged OD values in step 1.

3. Plot the OD values of standards against the concentration of collagen (ng/ml). Using a log/log plot will linearize the data. Figure 1 and Table 1 show representative experiments where the standard range is from 3.1-200 ng/ml.

4. The ng/ml of type II collagen in test samples can be calculated using regression analysis.

Figure 1 - A typical standard curve

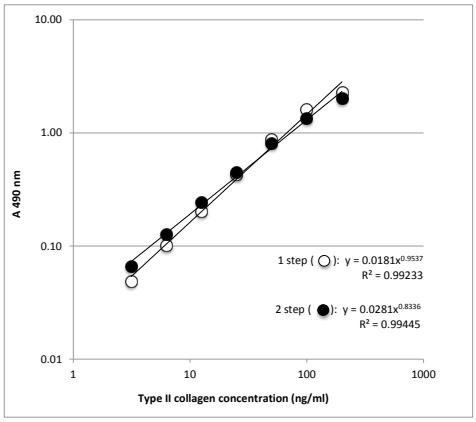


Table 1 - OD values of standards

| (ng/ml) | 1 step | 2 step |
|---|---|---|
| 200 | 2.278 | 2.007 |
| 100 | 1.597 | 1.353 |
| 50 | 0.876 | 0.809 |
| 25 | 0.427 | 0.447 |
| 12.5 | 0.203 | 0.243 |
| 6.25 | 0.101 | 0.127 |
| 3.13 | 0.049 | 0.066 |

## PRECISION

**Reproducibility of data assayed by type II collagen detection kit**

| Test At | 100 ng/ml | 25 ng/ml | 5 ng/ml |
|---|---|---|---|
| Inter-Assay CV (%) | 6.3 | 6.8 | 9.3 |
| Intra-Assay CV (%) | 2.1 | 2.2 | 4.4 |
| Spiking Test (%)* | 97 | 97 | 86 |

*Standard was mixed with known amounts of type II collagen solution.

**Reactivity of type II collagen from various species assayed by type II collagen detection kit**

| Species | Chick | Human | Mouse | Rat | Bovine | Porcine |
|---|---|---|---|---|---|---|
| Reactivity | 100% | 91% | 98% | 103% | 247% | 121% |



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## Part II-B: 2-STEP ASSAY PROTOCOL

*To save your time, Chondrex, Inc. recommends the 1-step assay protocol (see Part II-A, page 2). However, this kit does allow the use of a 2-step assay protocol (see Part II-B, page 5) as well. Please use the appropriate protocol for your studies. Both protocols yield comparable standard curves and identical sample results.*

**All reagents must be at room temperature before use.**

1. **Add Capture Antibody**: Dilute one vial of Capture Antibody with 10 ml of Capture Antibody Dilution Buffer (Solution A). Add 100 µl of capture antibody solution to each well and incubate at 4°C overnight.

2. **Dilute Wash Buffer**: Dilute 50 ml of 20X wash buffer in 950 ml of distilled water (1X wash buffer). Wash the plate with 1X wash buffer at least 3 times using a wash bottle with manifold or an automated plate washer. Empty the plate by inverting it and blot on a paper towel to remove excess liquid. *Do not allow the plate to dry out.*

3. **Prepare Standard Dilutions**: The recommended standard range is 3.1 - 200 ng/ml. Prepare serial dilutions of the standard by mixing 20 µl of 100 µg/ml standard with 980 µl of Sample/Standard Dilution Buffer (Solution B) - 2000 ng/ml . Then mix 100 µl of the 2000 ng/ml standard with 900 µl of Solution B - 200 ng/ml. Then mix 250 µl of the 200 ng/ml standard with 250 µl of Solution B - 100 ng/ml. Then repeat this procedure to make five more serial dilutions of standard - 50, 25, 12.5, 6.3, and 3.1 ng/ml solutions. The 100 µg/ml standard stock may be stored at -20°C for use in a second assay. Fresh serial dilutions should be prepared for each assay.

4. **Prepare Sample Dilutions**: Dilute solubilized samples 1:1-1:1000 with Solution B depending on the estimated collagen content in the samples. Sample solutions (buffers used in the collagen solubilization process which contain pepsin, and other reagents) may need to be assayed depending on the final sample dilution.

5. **Add Standards and Samples**: Mix samples and standard tubes well. Add 100 µl of Solution B (blank), standards and samples to appropriate wells. Then, cover the plate with a plate sealer and incubate at room temperature for 2 hours.

6. **Wash**: Wash the plate with 1X wash buffer at least 3 times using a wash bottle with manifold or an automated plate washer. Empty the plate by inverting it and blot on a paper towel to remove excess liquid. *Do not allow the plate to dry out.*

7. **Add Detection Antibody**: Reconstitute one vial of Detection Antibody with 50 µl of distilled water to make a Detection Antibody Stock Solution. Dilute 50 µl of the detection antibody stock solution in 10 ml of Detection Antibody Dilution Buffer (Solution C). Add 100 µl of detection antibody solution to all wells, cover the plate with a plate sealer, and incubate at room temperature for 2 hours. If you plan to use less, the leftover detection antibody stock solution can be stored in its original vial at -20°C for use in a second assay.

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9. **Add Streptavidin Peroxidase**: Dilute one vial of Streptavidin Peroxidase in 10 ml of Streptavidin Peroxidase Dilution Buffer (Solution D). Add 100 µl of streptavidin peroxidase solution to each well, cover the plate with a plate sealer, and incubate at room temperature for 1 hour.

10. **Wash**: Wash the plate with 1X wash buffer at least 3 times using a wash bottle with manifold or an automated plate washer. Empty the plate by inverting it and blot on a paper towel to remove excess liquid. *Do not allow the plate to dry out.*

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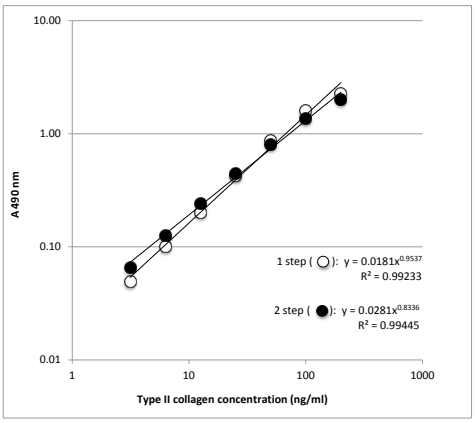


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