Cat. # MK310

For Research Use

TaKaRa

Cartilage Staining Kit
(Chondrocyte and Cartilage Tissue Staining Kit)

Product Manual

v201009

Table of Contents

I. Description

The main constituents of articular cartilage include aggrecan and type II collagen, both of which are proteolyzed when cartilage degenerates. Aggrecan, the major proteoglycan in cartilage, is a macromolecule consisting of an approximately 210-kDa core protein bound to a large number of sulfated chondroitin-side chains and multiple sulfated keratin-side chains. The core protein also has a domain capable of binding to hyaluronate produced by synoviocytes.

Type II collagen is a fibrillary element responsible for maintaining the special mesh structure in healthy cartilage and forms the structure that takes into its pores water-retentive proteoglycan. Type II collagen, unlike types I and III, exhibits a foreign-body antigenicity. Anti-type II collagen antibody, once generated in the body, induces rheumatism or arthritis.

This kit can be used to easily detect aggrecan and type II collagen in cartilage. It includes three stains: Toluidine blue, Safranin-O and Fast Green.

Toluidine blue and Safranin-O are cationic stains (basic dyes) that stain acidic proteoglycan present in cartilage tissues. Toluidine blue, also called metachromasia dye, shows subtle color changes depending on the tertiary structure of the sample. Cytoplasm stains light blue, nuclear region dark blue, and mast cell purple.

Safranin-O, which binds to glucosaminoglucan and shows an orange color, is often used to stain articular cartilage. Fast green, the contrast stain of Safranin-O, is a sulfate-group containing acidic substrate, which binds strongly to the amino group on protein and thereby strongly stains the non-collagen sites.

This kit also contains an Anti-Rat type II Collagen monoclonal Antibody to detect type II collagen of rat chondrocyte and cartilage tissue by immunostaining.

Note : Safranin-O, Fast green and Toluidine blue may be used to stain samples from any animal species for detection. The Anti-Rat collagen Type II monoclonal antibody may only be used for the detection of rat samples.

II. Kit Components

(1) Fixation solution Citrate buffer (pH5.4) containing 45% Acetone and 10% methanol	30 ml
(2) 1% Acetic acid	10 ml
(3) 0.1% Safranin O	10 ml
(4) 0.1% Fast green	10 ml
(5) 0.1% Toluidine blue (pH 4.1)	10 ml
(6) Anti-Rat Collagen type II monoclonal Antibody 0.25 mg/ml	100 µl
(7) Diluent for Antibody	10 ml

Note : Please check the attached Material Safety Data Sheet (MSDS)

III. Materials Required but not Provided

1. Reagents for colorimetric method
   - TBS : TBS (Tris-Buffered Saline) powder (Cat. #T903)
   - PBS : PBS (Phosphate Buffered Salts)Tablets (Cat. #T900)
   - Blocking reagent : Blocker™ BSA in TBS (10 ×) (Thermo Fisher Cat. #37520) or Blocker™ BSA in PBS (10 ×) (Thermo Fisher Cat. #37525), etc.
   - Secondary antibody : ImmunoPure® Goat Anti-Mouse IgG (H+L), (min x BvHnHs Sr Prot), Peroxidase Conjugated (Thermo Fisher Cat. #31432), etc.
   - DAB substrate : DAB Substrate Kit (Thermo Fisher Cat. #34002), etc.
   - Methyl green : Methyl Green (Dako Cat. #S1962), etc.
   - Proteinase K : Proteinase K (Dako Cat. #S3020), etc.
   - 0.3% hydrogen peroxide-containing methanol
   - Absolute ethanol

   Note : if using fluorescent antibody technique, then fluorescent–labeled secondary antibody (e.g., Alexa488 anti-mouse IgG by Invitrogen) will be required

2. Reagents for Immunofluorescence
   - PBS: PBS (Phosphate Buffered Salts)Tablets (Cat. #T900)
   - Blocking reagent: PBS containing 25% BlockAce (DS Pharma Biomedical) and 0.1% Proclin 300 (Sigma-Aldrich).
   - Fluorescent-labeled secondary antibody: Alexa 488 anti-mouse IgG (Invitrogen), etc.

IV. Storage — 20℃

V. Preparation of Solutions

- Fixation solution is supplied ready-to-use as is.
- 1% Acetic acid and Stain solutions (3), (4) and (5) are supplied ready-to-use after thawing at room temperature.
- Stain solutions (3), (4) and (5) and Antibody (6) are stable for up to 1 month at 4°C. For long-term storage, dispense them into the required aliquots and store at − 20°C.

VI. Protocol

1. Staining by Safranin-O, Fast green and Toluidine blue

<Cell staining>

A. Cells cultured in 24-well plates

1. Culture cells in 24-well plates.
2. Gently remove the culture medium.
3. Add 250 µl of (1) Fixation solution to each well and let stand at room temperature for 10 minutes to fix the cells.

4. Remove the Fixation solution in each well after diluting it with 2 ml of sterile PBS. Wash each well twice with 2 ml of sterile PBS again and remove the final wash.
5. Add 250 µl of (2) 1% Acetic acid to each well, allow to react for 10 - 15 seconds and then remove the 1% Acetic acid.
6. Add a 250-µl aliquot of Stain solution (3), (4) or (5) to separate wells and let stand at room temperature for 5 minutes to stain the cells.
7. Wash 3 times with sterile PBS.
   - Adjust the color level by altering the number of washes.
8. Microscopic observation may be done in the presence of PBS.

Note : Because the stain is water soluble, the glycerol solution used to prevent desiccation of stained samples in storage may cause discoloration.

B. Cells cultured in 96-well plates

The procedure is the same as that for 24-well plates except use 50 µl of (1) Fixation solution per well and 250 µl of sterile PBS for washing.

<Tissue section staining>

1. Prepare frozen sections or paraffin sections. Paraffin sections require deparaffinization.
   - Deparaffinization procedure

Paraffin sections

Xylene I	5 min	Deparaffinization
Xylene II	5 min	
Xylene III	5 min	
100% Ethanol	5 min	Hydration
100% Ethanol	5 min	
90% Ethanol	5 min	
80% Ethanol	5 min	
Wash under running water	2 min	Rinse

After last wash, soak in distilled water.

2. Add 100 - 200 µl of (1) Fixation solution to each tissue section and let stand at room temperature for 10 minutes. Remove the Fixation solution.
3. Add 200 µl of (2) 1% Acetic acid to each tissue section, allow to react for 10 - 15 seconds and then remove acetic acid.
4. Add a 200-µl aliquot of Stain solution (3), (4) or (5) to each tissue section and let stand at room temperature for 5 minutes to stain the tissue. Remove stain solution.
5. Add absolute ethanol for destaining.
   - Destain until the color density reaches an appropriate level.
6. Perform dehydration, clearing and mounting.

Note : Adjust the volume of the Fixation solution and Stain solution depending on the size of the tissue section.

<Double staining of paraffin section>

(double staining with 0.1% Fast green and 0.1% Safranin-O)

1. Prepare frozen sections or paraffin sections. (Paraffin sections require deparaffinization.)
2. Add 100 - 200 µl of (1) Fixation solution to each tissue section and let stand at room temperature for 10 minutes. Remove the Fixation solution.
3. Add 200 µl of (4) 0.1% Fast green to each tissue section and let stand at room temperature for 5 minutes to stain the section.
4. Soak in water for 10 minutes to destain.
5. Add 200 µl of (2) 1% Acetic acid to each tissue section and allow to react for 10 - 15 seconds. Remove 1% Acetic acid solution.
6. Add 200 µl of (3) 0.1% Safranin-O to each tissue section and let stand at room temperature for 5 minutes to stain the section.
7. Add absolute ethanol to destain.
   - Destain until the color density reaches an appropriate level.
8. Perform dehydration, clearing and mounting.

   Note : Adjust the volume of the Fixation solution and stain depending on the size of the tissue section.

2. Tissue Staining Using Anti-Type II Collagen Antibody

<Tissue section staining (colorimetric method)>

1. Prepare frozen sections or paraffin sections.
   (For paraffin sections, carry out deparaffinization followed by a distilled-water wash, a 5-minute treatment at room temperature with 0.4 mg/ml proteinase K, and a PBS wash.)
2. Perform endogenous peroxidase blocking (0.3% hydrogen peroxide-containing methanol) for 30 minutes.
3. After the reaction, wash with TBS or PBS.
4. Perform non-specific block using the blocking reagent (Blocker™ BSA in TBS or Blocker™ BSA in PBS) for 30 minutes and then discard the reagent.
5. Add the primary antibody onto the tissue section and allow the antibody reaction to take place at room temperature for 30 - 60 minutes.

   Note : Prepare 10-fold dilution (25 µg/ml) of (6) Anti-Rat Collagen type II monoclonal Antibody using (7) Diluent.
6. Rinse with TBS or PBS and wash 3 times (5 minutes each) with TBS or PBS.
7. Add the secondary antibody (peroxidase-labeled anti-mouse IgG antibody) onto the tissue section and allow the reaction to take place. Use dilutions prepared following the antibody's protocol.
8. Following the reaction, rinse with TBS or PBS and wash for 5 minutes 3 times with TBS or PBS.
9. Place the DAB substrate solution on the tissue section and allow color to develop (5 - 15 minutes).
10. Wash under running distilled water and allow distilled water to penetrate.

11. Perform counter staining (methyl green) for 1 to 5 minutes.
12. Destain with absolute ethanol until reaching an appropriate color.
13. Perform dehydration, penetrating and mounting.

Note : Using a cover slip over the blocking solution and antibody solution during blocking and antibody reaction will allow even staining.

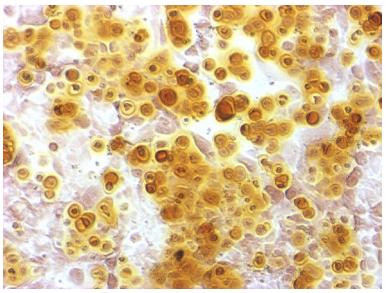
<Cell immunostaining on culture plates (immunofluorescence technique)>

1. Culture cartilage cells in 24-well plates.
2. Gently remove the culture medium.
3. Add 250 µl of (1) Fixation solution to each well and let stand at room temperature for 10 minutes to fix cells. Remove the fixation solution.
4. Wash once with PBS.
5. Add 500 µl of Blocking agent (25% BlockAce/0.1% Proclin 300 in PBS), incubate at 37°C for 1 hour, and then discard the blocking reagent.
6. Add 250 µl of the primary antibody to each well and incubate at 37°C for 1 hour.
   Note : Prepare 25- to 50-fold dilution (5 - 10 µl/ml) of (6) Anti-Rat Collagen type II monoclonal Antibody using (7) Diluent.
7. Remove the reaction mixture and wash once with PBS.
8. Add 250 µl of the secondary antibody to each well and incubate at 37°C for 1 hour.
   Note : Use fluorescent-labeled secondary antibody (e.g., Alexa488 anti-mouse IgG). Use dilutions prepared following the antibody's protocol.
9. Remove the reaction mixture and wash once with PBS.
10. Add 500 µl of PBS before observation.

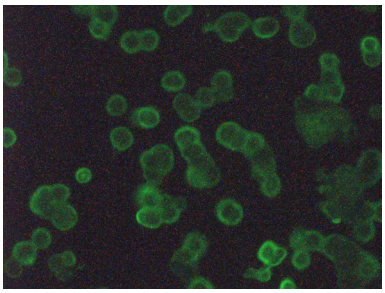
VII. Experimental Examples

1. Safranin-O stain and fluorescent stain (using Anti-collagen antibody) of primary cultured cartilage cells

Cartilage cells prepared from the costal epiphyseal plate of a 4-week old rat were cultured on a 24-well plate and stained by Safranin-O stain or type-II collagen antibody.



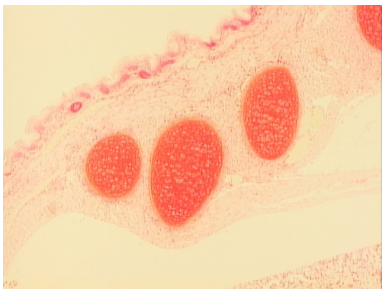
Safranin-O



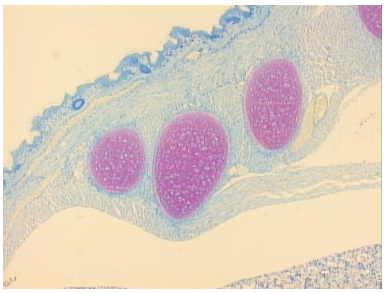
Type II collagen antibody

2. Mouse paraffin tissue slide staining

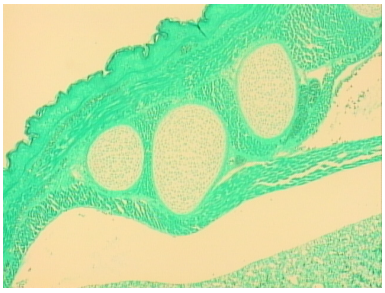
(C57BL6 day1, including bone tissue, partial lateral)



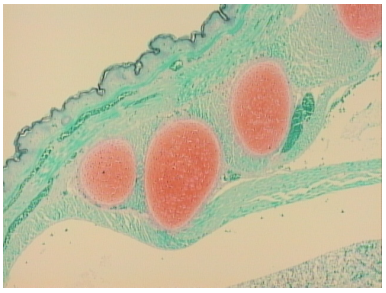
0.1% Safranin-O



0.1% Toluidine blue (pH 4.1)



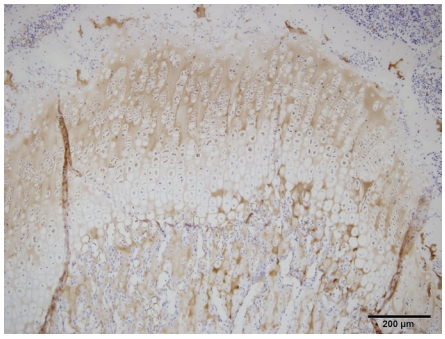
0.1%Fast green



Double staining with 0.1% Fast green + 0.1% Safranin-O

3. Type II collagen antibody staining using rat paraffin tissue slide

A formaldehyde-fixed, paraffin-embedded rat tibia section was stained with Anti-Rat type II Collagen Antibody. The growth plate cartilage showed excellent stain.



Detection: Histofine Mouse Stain Kit (Nichirei)

VIII. Related Products

TRACP & ALP double-stain Kit (Cat. #MK300)

TRACP & ALP Assay Kit (Cat. #MK301)

IX. References

Davit Tran, *et al*. (2000) Hematoxylin and Safranin O staining of frozen sections. *Dermatol Surg* (2000) **26**: 3

K.L. Camplejohn and S.A.Allard (1988) Limitation of safranin O staining in proteoglycan-depleted cartilage demonstrated with monoclonal antibodies. *Histochemistry* (1988) **89**: 185-188

Moskowitz R.W. and Goldberg VM. (1987) Studies of osteophyte pathogenesis in experimentally induced osteoarthritis. *J Rheumatol*. **14**: 311-20

Simon WH., *et al*. (1976) Long-term effects of chondorocyte death on rabbit articular cartilage *in vivo*. *J Bone Joint Surg Am*. **58**: 517

Rosenberg L (1971) Chemical basis for the histological use of safranin O in the study Articular cartilage. *J Bone Joint Surg Am*. **53**: 69-82

Alfert, M. and Geschwind, I. (1951) A selective staining method for the basic proteins of cell nuclei. *Proc. Natl. Acad. Sci. USA*. **39**: 991