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# 【1】 Introduction

## 1-1 Precautions Before Use

### Storage

Store unused FibColl® that has been opened once in a sealable container, avoiding high temperature and humidity. \*1

\* 1: Keep in a dry state, as re-drying after humidification changes the fibrous structure of atelocollagen and reduces permeability.

### Compatible 24-Well Plates

FibColl® is optimized for use with the 24-well plate included with the product (IWAKI, Product No.: 3820024), but it can also be used with 24-well plates commercially available from various manufacturers.\*2

\* 2: Some overseas-manufactured 24-well plates have fogging across the wells, which may cause FibColl® to tilt when set, or the plate lid may not close properly.

Products verified by our company are as follows

| Manufacturer   | Catalog Number |
|----------------|----------------|
| Corning        | 353047         |
| Greiner BioOne | 662160         |
| Sarstedt       | 83.3922.300    |
| TPP            | 92024          |
| TrueLine       | TR5002         |

# 【2】 Basic Usage and Evaluation Methods

## 2-1 Cell Culture

### Cell Seeding

(1) Add 600 µL of medium to the well and 100 µL to the insert, and let stand in a CO<sub>2</sub> incubator for at least 30 minutes to equilibrate the insert.

(2) Remove the medium from the well and the insert. \*1,2

\* 1: Remove the medium in the well with an aspirator and carefully remove the medium in the insert using a micropipette to avoid tearing the membrane when removing the medium.

\* 2: There are two holes in the frame for medium change, allowing access to and removal of the medium in the well through the holes without removing FibColl®.

(3) Add 600 µL of fresh medium to the well. \*3

\* 3: For co-culture, add 600 µL of the first cell suspension to the well.

(4) Add 100 µL of cell suspension to the insert.

Recommended number of cells to seed on the membrane:  $1 \times 10^4$  cells \*4 (culture area: 0.33 cm<sup>2</sup>)

\* 4: This is a reference value when using fibroblasts; the optimal number of seeded cells should be adjusted according to the cell type and experimental purpose.

(5) Place in a CO<sub>2</sub> incubator and culture

## Medium Change

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- (1) Remove the medium from the well and the insert.
- (2) Add 100  $\mu$ L of fresh medium to the insert.
- (3) Add 600  $\mu$ L of fresh medium to the well.
- (4) Place in a CO<sub>2</sub> incubator and culture.

## 2-1 Post-Culture Evaluation

### Cell Collection

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#### Required Reagents

- ① PBS (–)
- ② Trypsin solution (0.05% trypsin/EDTA, etc.)

#### Procedure

- (1) Remove the medium from the well and the insert.
- (2) Add a buffer solution such as PBS (–) that does not contain Ca, wash, and then remove.
- (3) Add 100  $\mu$ L of trypsin solution and incubate for approximately 2 minutes. \* 1

\* 1 : Consider the enzyme solution and treatment time depending on the cells used.

- (4) Collect the cells by pipetting with buffer solution or similar.

#### Viable Cell Count Measurement

Measurement is possible using commercially available viable cell count measurement kits (such as WST–8).

### RNA Extraction

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#### Required Reagents

- ① PBS (–)
- ② Commercially available RNA extraction reagents or kits

#### Procedure

- (1) After removing the medium, add 100  $\mu$ L of PBS(–) to the insert and 600  $\mu$ L to the well, rinse, and then remove .
- (2) Repeat the procedure in (1) once more.
- (3) From this step onward, use commercially available RNA extraction reagents or kits according to the instruction manual.

## Phase Contrast Microscopy and Fluorescence Microscopy

Note 1 : Although the membrane is not transparent and therefore not as clear as in normal planar culture, it is possible to observe cells using a phase contrast microscope.

Note 2: Fluorescence observation during cell culture is difficult because the membrane causes the cells to appear blurred. Therefore, when observing fluorescence, it is recommended to observe after separating the membrane from the frame using the method described below.

## Membrane Recovery from Frame and Cell Observation

### Required Equipment and Reagents

- ① Fixative (4% paraformaldehyde, etc.)
- ② PBS
- ③ Forceps
- ④ Commercially available disposable scalpel (such as Kai Industries Disposable Scalpel No. 11)
- ⑤ Microscope slide and cover slip
- ⑥ Mounting medium

### Procedure

(1) Remove the medium, wash with PBS, fix appropriately with fixative, and wash with PBS after removing the fixative. \* 1

\* 1 : Pour PBS gently, as the cell layer may detach from the membrane.

(2) Remove FibColl® with forceps and absorb the residual liquid on the back of the membrane with sterilized cotton or cotton swabs.

(3) Hold FibColl® upside down and use a tool such as a commercially available disposable scalpel to cut out the peripheral edge of the membrane along the frame by moving the scalpel up and down in small increments like a can opener (Figure 1). \*2, 3, and 4



Figure 1. Cutting out the membrane



Figure 2. Scalpel blade shape suitable for cutting

\* 2 : A disposable scalpel with a fine, pointed tip makes it easier to cut out the membrane (Figure 2). If necessary, FibColl® may be placed on the plate lid or similar surface during the procedure.

\* 3 : Operate carefully, as this may cause the cell layer to detach from the membrane. At this time, be careful not to let the cell layer dry out.

\* 4 : Forceps for handling the membrane should preferably be without hooks or grooves to avoid damaging the cell layer.

## Instructions for Use of FibColl® High Permeability Atelocollagen Insert

(4) Collect the membrane with forceps (Figure 3). For histological evaluation using immunostaining or similar methods, proceed to the next section "Frozen Section Preparation" or "Immunofluorescence Staining."

(5) When observing cells directly without processing such as immunostaining, place the membrane on a slide glass, place it on the slide glass, add PBS or mounting medium as needed, cover with a cover glass, and then observe (Figure 4). \* 5

\* 5 : Mount with the cell seeding surface facing the lens side.



Figure 3. Cut-out membrane



Figure 4. Membrane covered with cover glass

## Frozen Block Preparation

(1) Prepare frozen blocks using cryoembedding compound with standard methods. \*1, 2

\* 1 : Paraffin embedding may be selected depending on the purpose.

\* 2 : To prevent bending, tilting, or ice crystal damage to the membrane, it is recommended to first acclimate it in a small amount of cryoembedding compound before preparing the frozen block.

The following excerpts introduce only the key steps in frozen block preparation.

(1) Pour a small amount of cryoembedding compound into a flat container such as a 24-well plate lid.

(2) Place the collected membrane on the cryoembedding compound from (1) with the cell surface facing up, add cryoembedding compound to sufficiently cover the entire surface, and let stand for approximately 10 minutes to acclimate.

(3) Transfer the membrane to an embedding dish, cover with fresh cryoembedding compound, and freeze. \* 3

\* 3 : Use forceps or similar to sink the membrane to the bottom; keeping the membrane horizontal makes it easier to prepare clean sections

(1) Fill a deeper container with cryoembedding compound, place the membrane frozen in (3) perpendicular to the bottom of the container, and freeze again.

## Frozen Section Preparation

(1) Let the frozen block stand in the cryostat chamber for approximately 30 minutes, then trim and section using standard methods. \* 1

\* 1 : At our company, we set the cryostat chamber to 20° C and the specimen stage to 25° C, and prepare 7 μm thick sections using the adhesive film method (Kawamoto method). we prepare. \* 2

\* 2 : The Kawamoto method allows sections to be supported by adhesive film, enabling clean frozen sections to be prepared with short training.

## Immunofluorescence Staining

### Required Equipment and Reagents

- ① Fixative (4% paraformaldehyde, etc.)
- ② TBS-T (Tris Buffered Saline + 0.05% Tween20)

## Instructions for Use of FibColl® High Permeability Atelocollagen Insert

- ③ Commercially available blocking agents, etc.
- ④ PBS
- ⑤ Various primary antibodies and secondary antibodies
- ⑥ Nuclear staining solution \* 1

\* 1 : Since collagen itself has blue autofluorescence, using nuclear staining solutions such as DRAQ5 instead of DAPI makes observation easier.

## Procedure

- ① Following the membrane recovery from frame on the previous page, fix the cells with fixative and then cut out the membrane.
- ② Shaping the cut-out membrane as needed makes it easier to distinguish front from back (Figure 5).

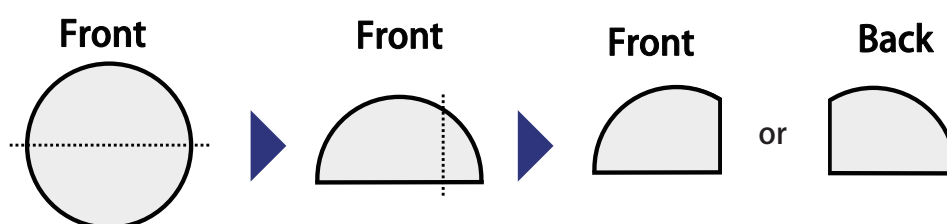


Figure 5. Example of shaping the cut-out membrane

- ③ Add TBS-T to the shaped membrane and let stand for 15 minutes.
- ④ Remove TBS-T, add blocking agent, and let stand at room temperature for 1 hour.
- ⑤ Add primary antibody solution diluted with TBS-T containing blocking agent and incubate overnight at 4° C.
- ⑥ Remove the primary antibody solution and wash 3 times with TBS-T.
- ⑦ Add secondary antibody solution diluted with TBS-T containing blocking agent and incubate at room temperature for 3 hours protected from light.
- ⑧ Remove the secondary antibody solution, wash 3 times with TBS-T, and then replace with PBS.
- ⑨ Add nuclear staining solution diluted with PBS and incubate at room temperature for 15 minutes protected from light.
- ⑩ Remove the nuclear staining solution and wash once with PBS.
- ⑪ Mount on a slide glass using mounting medium, then observe with the cell seeding surface facing the lens side.

## 【3】 Preparation and Evaluation Methods for Epidermal Models

### 3-1 Preparation of Epidermal Models

#### Precautions for Normal Human Epidermal Keratinocytes

- (1) Normal human epidermal keratinocytes (hereinafter referred to as epidermal keratinocytes) derived from neonates are recommended. \* 1, 2

\* 1 : The passage number of epidermal keratinocytes is recommended to be within 3 passages.

\* 2 : If lot-to-lot variation of cells is a concern, consider comparing lots or using cells derived from multiple donors.

- (2) For normal differentiation induction, epidermal keratinocytes need to maintain an undifferentiated state; therefore, perform operations involving stimulation, such as pipetting, as gently as possible. Additionally, to achieve a uniform epidermal layer, immediately after cell seeding, handle with particular care.

## Instructions for Use of FibColl® High Permeability Atelocollagen Insert

(3) To maintain good differentiation capacity of epidermal keratinocytes, be careful not to exceed 80% confluency during the cell proliferation stage. \*3

\* 3 : If cells become too dense, contraction may occur, which can interfere with subsequent epidermal model preparation.

### Preparation of differentiation induction medium (example: when preparing 10 mL)

#### Required Reagents

|   | Reagent name                                                            | Volume      | Final concentration |
|---|-------------------------------------------------------------------------|-------------|---------------------|
| ① | Epidermal keratinocyte growth medium<br>(hereinafter, growth medium) *1 | 9.85mL      | —                   |
| ② | 0.1 M calcium chloride dihydrate *2                                     | 150 $\mu$ L | 1.5mM               |

Add ② to ① to prepare the differentiation induction medium.

\* 1 : Use the medium recommended by the cell supplier or manufacturer.

\* 2 : Some media already contain this, so in such cases, adjust the volume to be added to achieve the final concentration.

### Cell seeding onto FibColl® and differentiation induction

(1) Add 600  $\mu$ L of differentiation induction medium to the well and 100  $\mu$ L to the insert, and allow to stand in a CO<sub>2</sub> incubator for approximately 30 minutes to equilibrate the FibColl® membrane. \*1

\* 1 : Aligning the orientation of FibColl® also aligns the two medium exchange ports on the frame, improving workability during subsequent medium changes.

(2) Wash the pre-cultured epidermal keratinocytes with 30 mM HEPES Buffer.

(3) Add 2 mL of cell dissociation enzyme TrypLE reagent, distribute it over all cells, and then allow to stand for 10 minutes in a clean bench (at room temperature). \*2

\*2 : Recommendation: Use TrypLE reagent which cause less damage to cells than Trypsin.

(4) Suspend (3) in 18 mL of growth medium and transfer to a 50 mL centrifuge tube.

(5) Count the number of cells using a hemocytometer or similar device.

(6) Centrifuge at 1,000 rpm for 3 minutes at room temperature.

(7) After removing the supernatant, suspend in differentiation induction medium and adjust to a cell concentration of  $1.0 \times 10^6$  cells/mL.

(8) Remove all medium from the well and insert added in (1) using an aspirator. \*3,4

\* 3 : If membrane damage is a concern, carefully remove it while using a micropipette.

\* 4 : The frame has medium exchange ports, allowing the medium in the well to be accessed without removing FibColl® from the plate.

(9) After seeding 180  $\mu$ L of the cell suspension prepared in (7) into the insert, and add 900  $\mu$ L of differentiation induction medium to the well.

(10) Check for air bubbles under the FibColl® membrane, and if present, remove them by tilting the plate or using similar methods. \*5

\* 5 : Ensure that the suspension does not leak from the insert when tilting the plate, and also be careful of air bubbles during subsequent medium operations.

(11) Cover the 24-well plate with a lid and place in a CO<sub>2</sub> incubator for 3 days of submerged culture. \*6,7

\* 6 : Be careful as the plate lid may not close properly if the insert is not properly fitted in the well.

\* 7 : It is recommended to change the medium in the well on day 2 of culture.

## Instructions for Use of FibColl® High Permeability Atelocollagen Insert

**Preparation of epidermal maturation medium (For preparation of 10 mL)****Required Reagents**

| Reagent                                                           | Volume     | Final concentration |
|-------------------------------------------------------------------|------------|---------------------|
| ① Differentiation induction medium                                | 9.979 mL   | —                   |
| ② 25 mg/mL ascorbic acid 2-phosphate sesquimagnesium salt hydrate | 20 $\mu$ L | 50 $\mu$ g/mL       |
| ③ 100 $\mu$ g/mL Keratinocyte Growth Factor (KGF)                 | 1 $\mu$ L  | 10ng/mL             |

Add in the order of ①, ②, ③ to prepare the epidermal maturation medium.

**Promotion of Epidermal Differentiation by Air-Liquid Interface Culture**

- (1) Tilt the plate at an angle and remove most of the medium with a micropipette.
- (2) Be careful not to damage the membrane, and completely remove the medium from the insert using an aspirator. \* 1, 2

\* 1 : If membrane damage is a concern, use a micropipette carefully and thoroughly remove the medium.

\* 2 : Since drying of the epidermal surface is important for normal epidermal differentiation, visually confirm carefully that no residual liquid remains after medium removal.

- (3) Confirm that the FibColl® membrane is completely covered with rough tissue and that there are no holes in the tissue or membrane.

- (4) Remove all medium from the well using an aspirator or similar device. \* 3

\* 3 : After removing the medium from the insert, the FibColl® may be transferred to a new well containing 500  $\mu$ L of epidermal maturation medium.

- (5) Add 500  $\mu$ L of epidermal maturation medium to the well and culture in a CO<sub>2</sub> incubator. \* 4

\* 4 : Formation of the stratum corneum can be confirmed approximately 7 days after air-liquid interface culture. Adjust the duration of air-liquid interface culture as appropriate according to the purpose.

- (6) Change the epidermal maturation medium in the well once every 2 days.

**3-2 Evaluation of Epidermal Models****Recovery of epidermal model and membrane**

- (1) Remove the medium from the well using an aspirator or similar device, and wash the top of the epidermal model and the back of the membrane with buffer solution. \* 1, 2

\* 1 : Select an appropriate buffer solution according to the subsequent experimental system.

\* 2 : Pour the buffer solution gently as the epidermal model may peel off from the membrane.

- (2) Remove the FibColl® with tweezers and absorb the residual liquid on the back of the membrane with sterilized cotton or cotton swabs.

- (3) Hold the FibColl® upside down and, using a commercially available disposable scalpel or similar tool, cut along the frame to remove the peripheral edge of the membrane by moving the scalpel up and down in small increments like a can opener (Figure 1). \* 3, 4, 5



Figure 1. Cutting out the membrane

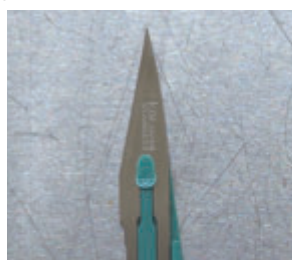


Figure 2. Scalpel blade tip shape suitable for cutting

## Instructions for Use of FibColl® High Permeability Atelocollagen Insert

- \* 3: A disposable scalpel or similar tool with a thin, pointed tip makes it easier to cut out the membrane (Figure 2). If necessary, FibColl® may be placed on the plate lid or similar surface during the procedure.
- \* 4: Operate carefully as this may cause the epidermal model to peel off from the membrane. At this time, be careful not to let the epidermal model dry out.
- \* 5: Tweezers used to handle the epidermal model and membrane should preferably be without hooks or grooves to avoid damaging the epidermal model. Also, when grasping with tweezers, be careful not to grasp only the epidermal model, but to grasp both the epidermal model and membrane together.

## RNA Extraction

Follow the extraction protocol for tissues using commercially available RNA extraction reagents or kits.\*<sup>1</sup>

\* 1 : The recovered epidermal model and membrane are stabilized by immersing into a RNA stabilization reagent. The sample is homogenized and total RNA is extracted using the Acid Guanidinium-Phenol-Chloroform (APGC) method and purified using a spin column.

## Reverse transcription-quantitative PCR (RT-qPCR)

Perform RT-qPCR using standard methods to confirm the expression of epidermal differentiation markers and other markers.

## Protein extraction

Extract proteins using a tissue lysis buffer appropriate for the purpose.\*<sup>1, 2</sup>

\* 1 : In our laboratory, we pick up only the epidermal model from the recovered epidermal model and membrane with tweezers, remove the membrane, and then immerse it in 150  $\mu$ L of tissue lysis buffer, homogenize it with a homogenizer, and then destroy nucleic acids with a sonicator.

\* 2 : When recovering the epidermal model from the membrane with tweezers, be careful as some portions may remain on the membrane.

## Frozen Block Preparation

Prepare frozen blocks using cryoembedding compound with standard methods.\*<sup>1, 2</sup>

\* 1 : Paraffin embedding may be selected depending on the purpose.

\* 2 : To prevent bending, tilting, or damage from ice crystals to the epidermal model and membrane, it is recommended to first acclimate them in a small amount of cryoembedding medium before preparing the frozen block.

The following excerpts introduce only the key steps in frozen block preparation.

- (1) Pour a small amount of cryoembedding compound into a flat container such as a 24-well plate lid.
- (2) Place the recovered epidermal model and membrane on the cryoembedding medium in (1) with the epidermal model side facing up, add cryoembedding medium to sufficiently cover the entire sample, and allow to stand for approximately 10 minutes to acclimate.
- (3) Transfer the epidermal model and membrane to an embedding dish, cover with fresh cryoembedding medium, and freeze.\*<sup>3</sup>

\* 3 : Using tweezers or similar tools, submerge the skin model and membrane to the bottom. Keeping them horizontal makes it easier to prepare clean sections.

- (4) Fill a deeper container with cryoembedding medium, insert the epidermal model and membrane frozen in (3) perpendicular to the bottom of the container, and freeze again.

## Frozen Section Preparation

Let the frozen block stand in the cryostat chamber for approximately 30 minutes, then trim and section using standard methods. \* 1

\* 1 : In our laboratory, frozen sections with a thickness of 7  $\mu\text{m}$  are prepared using the adhesive film method (Kawamoto's method), with the cryostat chamber temperature set to  $-20^{\circ}\text{C}$  and the specimen holder set to  $-25^{\circ}\text{C}$ .

\* 2 : The Kawamoto method supports sections with adhesive film, allowing clean frozen sections to be prepared with short training.

## HE staining

Perform HE staining using standard methods and confirm whether a 4-layer structure as shown below has been formed (Figure 1). \* 1

\* 1 : Individual images obtained from multiple experiments are shown, and the color tone does not change gradually. Each image is only an example, and the observed images will vary depending on the cell lot used and operational techniques.

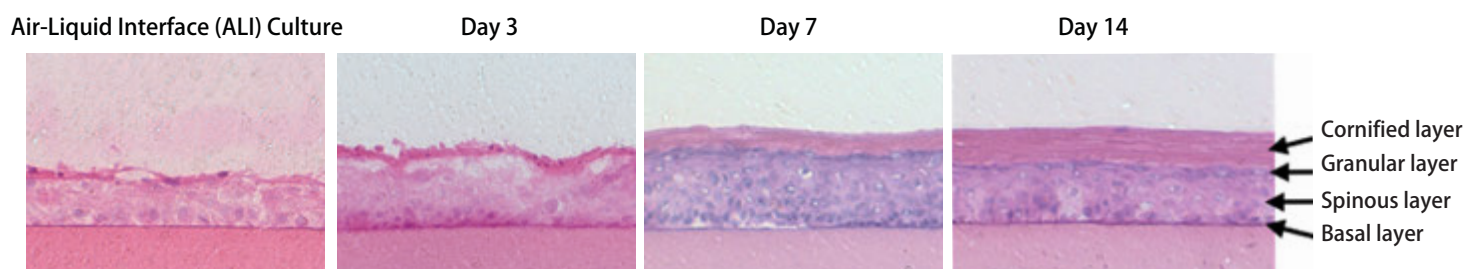


Figure 1. Examples of HE staining images of epidermal models (cross-section) at days 0, 3, 7, and 14 of air-liquid interface culture using FibColl®

## Immunofluorescence Staining

Perform immunofluorescence staining using standard methods and confirm the expression of epidermal differentiation markers and other markers as shown below (Figure 1). \* 1

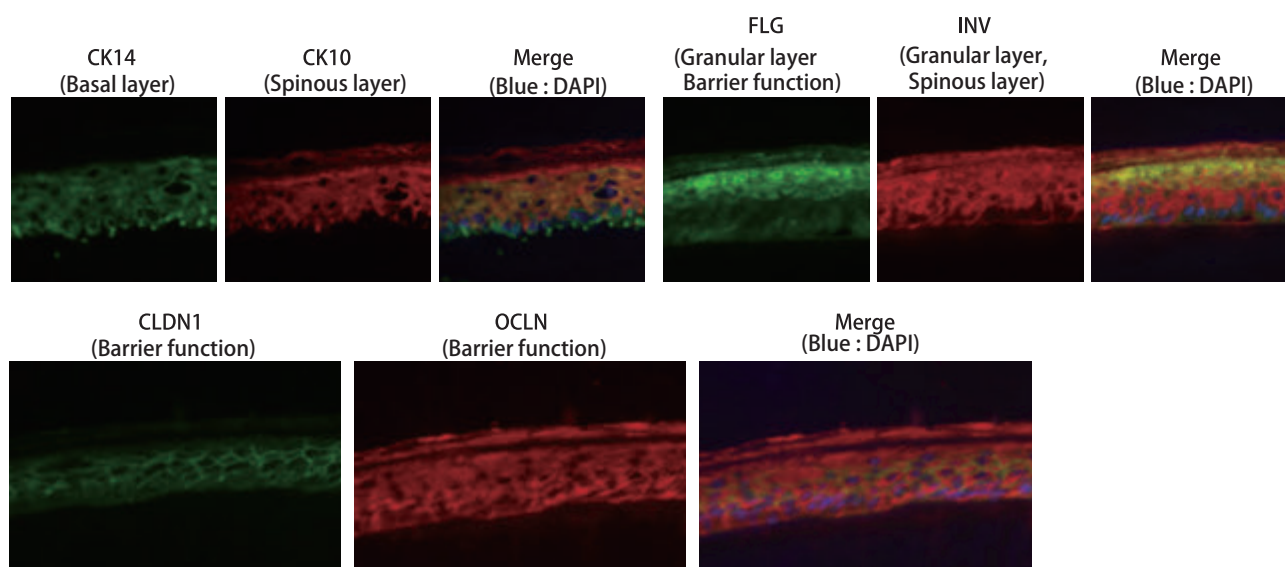


Figure 1. Example of immunofluorescence staining image of epidermal model (cross-section) at day 7 of air-liquid interface culture using FibColl®

### 【3】 Product Information

| Catalog number | Product Name                      | Dimensions of components                                                                                              | Size  |
|----------------|-----------------------------------|-----------------------------------------------------------------------------------------------------------------------|-------|
| KOU-FAI-24     | FibColl® Atelocollagen Inserts 24 | Product : $\varnothing$ 19mm $\times$ 16mm<br>Membrane : $\varnothing$ 6.4mm<br>Membrane thickness : about 35 $\mu$ m | 24 PC |

#### Related Products

| Catalog Number | Product Name                                                                 | Dimensions of components                                                                                      | Size    |
|----------------|------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------|---------|
| KOU-CM-6       | Atelocollagen Permeable Membrane (Well Format)<br>for 6-well Culture Plates  | Product : $\varnothing$ 31mm $\times$ 8mm<br>Membrane : $\varnothing$ 26mm<br>Membrane thickness : 25 $\mu$ m | 12 PC   |
| KOU-CM-24      | Atelocollagen Permeable Membrane (Well Format)<br>for 24-well Culture Plates | Product : $\varnothing$ 14mm $\times$ 8mm<br>Membrane : $\varnothing$ 9mm<br>Membrane thickness : 25 $\mu$ m  | 24 PC   |
| KOU-CLF-01     | Atelocollagen Permeable Membrane Sheet                                       | Product : 100mm $\times$ 90mm<br>Membrane thickness : 35 $\mu$ m                                              | 1 Sheet |

#### Type of Collagen

Bovine skin-derived atelocollagen

Click here for frequently asked Q&A

<https://atelocollagen.com/en/atelocell/faq/13>

Distributed worldwide by Cosmo Bio USA



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