

Status: Currently Official on 14-Feb-2025
 Official Date: Official as of 01-Aug-2020
 Document Type: USP Monographs
 DocId: GUID-8C8B8ECA-A1C3-41D5-8DB1-6092D892B90C_4_en-US
 DOI: https://doi.org/10.31003/USPNF_M481_04_01
 DOI Ref: bg1pb

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Construct Human Fibroblasts in Bilayer Synthetic Scaffold

DEFINITION

Construct Human Fibroblasts in Bilayer Synthetic Scaffold is a nonliving monolayer skin substitute derived from neonatal foreskins. It is composed of fibroblasts, an extracellular matrix, and a nylon mesh bonded to a transparent, semi-permeable silicone membrane. Human fibroblasts are seeded onto the nylon mesh. The fibroblasts proliferate within the nylon mesh and secrete human matrix proteins. Following freezing, no cellular metabolic activity remains.

The fibroblast-cell banks, from which Construct Human Fibroblasts in Bilayer Synthetic Scaffold is derived, test negative for human and animal viruses and retroviruses, and are also tested for normal cell morphology, human karyology, and isoenzymes. Maternal blood sera are tested for evidence of infection with human immunodeficiency virus types 1 and 2, hepatitis B and C viruses, syphilis, and human T-lymphotropic virus type 1, and are found negative for the purpose of donor selection.

Construct Human Fibroblasts in Bilayer Synthetic Scaffold is manufactured with sterile components under aseptic conditions within the final package. All materials derived from bovine sources originate from countries free of bovine spongiform encephalopathy. During subsequent screening of the fibroblast cell strain at various stages in the manufacturing process, testing for these same viruses, as well as Epstein-Barr virus and human T-lymphotropic virus type 2, is carried out and found to be negative. The final product is inspected and tested to ensure that the product meets specifications.

SPECIFIC TESTS

• HISTOLOGICAL CHARACTERIZATION

Buffered formalin: Prepare a solution containing 10% (w/v) formaldehyde solution and 1.0%–1.5% methanol in a suitable buffer, adjusted to a pH of 6.8–7.2.¹

Preparation of tissue for staining: Cut Construct Human Fibroblasts in Bilayer Synthetic Scaffold into 3-mm × 3-mm sections. Place three sections into suitable histological tissue cassettes,² and insert the cassettes into suitable histological cassette basket(s).³

Embedding and sectioning: At a temperature of 40°, sequentially immerse the histological cassette basket(s) in separate solutions of *Buffered formalin* (2 h), two changes of 80% alcohol (30 min/step), alcohol (30 min), three changes of dehydrated alcohol (30 min/step), suitable histological xylene substitute (30 min),⁴ and two changes of suitable xylene substitute (30 min/step). Immerse the histological cassette basket(s) into molten paraffin⁵ that is at a temperature of 60° for 30 min. Remove the cassette basket(s), and immerse in a fresh container of molten paraffin at 60° for 60 min. Remove the histological tissue cassette from the container and basket, and disassemble. Fill preheated embedding molds with molten paraffin heated to 56°–60°, and place on top of a preheated warming platform that is designed for histology work. Transfer Construct Human Fibroblasts in Bilayer Synthetic Scaffold specimens from the cassettes using forceps, and place specimens into individual molds. Orient the specimens in molds so as to cut cross-sections. Cool the paraffin by sliding the mold down the platform to its cool side until the paraffin has solidified. Maintain specimen orientation with forceps during cooling, removing the forceps when the paraffin becomes translucent. Slide the paraffin block onto a histological cold plate to rapidly cool the block. Trim the paraffin block with a new single-edged razor blade to form a rectangle or slight trapezoid to within 5 mm of the tissue mass, if necessary. Cool the block at 4° for 15–30 min. Clamp the tissue block into the block holder of the microtome. Fill a histological tissue flotation water bath with fresh water, add an appropriate amount of a suitable histological adhesive,⁶ and heat to 5° less than the melting point of the paraffin. Properly mount and adjust the tissue and paraffin block into a microtome. Set the microtome to make cuts 5 µm thick with a blade angle of 5 ± 2°. Insert a sharp stainless steel microtome knife that has been properly honed, or a new disposable microtome knife, into the knife holder. Cut a ribbon that contains 6–10 sections of Construct Human Fibroblasts in Bilayer Synthetic Scaffold. Pick up the ribbon with forceps, and stretch it across the tissue flotation water bath. Separate 2–3 adjacent sections from the ribbon on the water bath. The selected sections should not be compressed, wrinkled, or scratched. Pick up the selected sections by dipping a microscope slide into the water bath under the floating sections, and gently lift the slide out of the water. For each staining procedure, prepare three slides from each of the three starting Construct Human Fibroblasts in Bilayer Synthetic Scaffold 3-mm × 3-mm sections. Allow the mounted sections to air-dry completely, or dry the slide in a 60° oven for 1 h.

Hematoxylin–eosin staining

Hematoxylin–alcohol solution: Dissolve 2.5 g of hematoxylin in 25.0 mL of dehydrated alcohol with heating.

Potassium alum solution: Dissolve 50.0 g of potassium alum in 500 mL of water with heating.

Hematoxylin staining solution: Mix *Hematoxylin–alcohol solution* and *Potassium alum solution*. Bring to a boil as rapidly as possible with constant stirring. Do not heat for more than 1 min. Slowly add 0.185 g of sodium iodate. Reheat to a simmer until the solution becomes a deep purple. Remove from heat, and quickly cool. Filter daily before use.

10% acid alcohol: Add 5.0 mL of hydrochloric acid to 495 mL of 70% alcohol.

Eosin solution: Dissolve 1 g of eosin Y in 100 mL of alcohol. Filter daily before use.

Analysis: Sequentially immerse the microscope slide with affixed tissue, as prepared in *Preparation of tissue for staining*, in three changes of a suitable histological, aliphatic xylene substitute (2 min/step), three changes of dehydrated alcohol (1 min/step), alcohol (20 s), running tap water rinse (1 min), *Hematoxylin staining solution* (4–5 min), running tap water rinse (1 min), *10% acid alcohol* (15 s), running tap water rinse (1 min), a suitable bluing reagent⁷ (20–30 s), running tap water rinse (1 min), alcohol (20 s), *Eosin solution* (10–20 s, until a reddish-brown color is obtained in the tissue), three changes of dehydrated alcohol (10 s/step), and three changes of a suitable histological xylene substitute (10 s/step). Adjust the above immersion times as needed to suitably stain the tissue. Remove the slide from the last histological xylene substitute wash, and blot dry the back of the slide. Do not allow the tissue to dry. Affix a coverslip over the tissue using a coverslip mountant.

Acceptance criteria: Using USP Construct Human Fibroblasts in Bilayer Synthetic Scaffold Reference Photomicrograph 1 (hematoxylin–eosin stained)⁸ for comparison, the nylon-scaffold mesh, silicone membrane, and secreted collagen-based matrix are present, and the tissue contains normal human fibroblast distributed throughout the secreted matrix, and resembles normal human papillary dermis. The fibroblasts appear elongated and spindle shaped. The tissue contains about 10^6 cells/cm² and about 500 cells/mm along the section.

Collagen staining

Bouins' solution: Mix 75.0 mL of 1.22% picric acid solution, 25.0 L of dimethoxymethane, and 5.0 L of acetic acid.

Weigert's iron hematoxylin solution A: Dissolve 1.0 g of hematoxylin in 100 mL of alcohol.

Weigert's iron hematoxylin solution B: Mix 4.0 mL of 29% ferric chloride, 95.0 mL of water, and 1.0 mL of hydrochloric acid.

Weigert's iron hematoxylin working solution: Combine *Weigert's iron hematoxylin solution A* and *Weigert's iron hematoxylin solution B* (1:1). Pass the solution through a suitable filter of 0.45-μm pore size. Prepare fresh as needed.

Gomori's trichrome solution: Mix 1.0 mL of acetic acid and 100 mL of water. Dissolve 0.6 g of chromotrope 2R, 0.3 g of Fast Green FCF, and 0.6 g of phosphotungstic acid.

1% acetic acid: Dilute 1 mL of glacial acetic acid with water to make 100 mL of solution.

Analysis: Sequentially immerse the microscope slide with affixed tissue, as prepared in *Preparation of tissue for staining*, in three changes of a suitable histological, aliphatic xylene substitute (2 min/step), three changes of dehydrated alcohol (1 min/step), alcohol (20 s), and running tap water rinse (1 min). Immerse the slide in *Bouins' solution*, and place in a 42° water bath for 1 h. Rinse the slide in water for 10 s. Sequentially immerse the slide in *Weigert's iron hematoxylin working solution* (10 min) and running tap water rinse (10 min). Rinse the slide in water for 10 s, and immerse in *Gomori's trichrome solution* (3–5 min). Rinse the slide in *1% acetic acid* for 20 s. Sequentially immerse the slide in three changes of alcohol (10 s/step) and three changes of a suitable histological, aliphatic xylene substitute (10 s/step). Affix a coverslip over the tissue using a suitable coverslip mountant. Nuclei will stain black; cytoplasm, keratin, and muscle fibers will stain red; and collagen and mucin will stain blue.

Acceptance criteria: Using USP Construct Human Fibroblasts in Bilayer Synthetic Scaffold Reference Photomicrograph 2 for comparison, collagen is found throughout the extracellular matrix. The tissue contains normal human fibroblast distributed throughout the secreted matrix and resembles normal human papillary; dermis, muscle fibers, and keratin are absent.

Distribution of fibronectin

Tris-saline buffer: Combine 0.1 M tris-(hydroxymethyl)aminomethane hydrochloride and 0.15 M sodium chloride, and adjust to a pH of 7.8.

3% Hydrogen peroxide: Dilute 30 mL of hydrogen peroxide with water or methanol.

Diaminobenzidine solution: Use a suitable solution.⁹

Hematoxylin staining solution: Prepare as directed for *Hematoxylin–eosin staining*.

Analysis: The microscope slide with affixed tissue as prepared in *Preparation of tissue for staining* is dried either overnight at 37° or for 1 h at 60°. The microscope slide with affixed tissue as prepared in *Preparation of tissue for staining* is sequentially immersed in three changes of a suitable histological, aliphatic xylene substitute (2 min/step), three changes of dehydrated alcohol (1 min/step), alcohol (20 s), and running tap water rinse (1 min). Sequentially immerse the slide in *Tris-saline buffer* (10 min), *3% hydrogen peroxide* (30 min), three changes of *Tris-saline buffer* (1 min/step), a suitable normal rabbit serum¹⁰ (30 min), water (5 min), and three changes of *Tris-saline buffer* (1 min/step). Incubate the slide with a suitable solution of rabbit anti-human fibronectin antibody,¹¹ diluted using a suitable antibody diluent¹² to an antibody concentration of 21.0 ± 2.1 mg/L for 1 h. Sequentially immerse the slide in water (5 min) and three changes of *Tris-saline buffer* (1 min/step). Place enough drops of a biotinylated goat anti-rabbit antibody solution¹³ to cover the tissue section, and incubate for 30 min. Sequentially immerse the slide in water (5 min) and three changes of *Tris-saline buffer* (1 min/step). Place enough

drops of a streptavidin conjugated horseradish peroxidase solution¹⁴ to cover the tissue section, and incubate for 30 min. Sequentially immerse the slide in water (5 min) and three changes of *Tris-saline buffer* (1 min/step). Incubate the slide with *Diaminobenzidine solution* for 1–5 min, until a suitable difference in staining is seen by comparison with a control in which the fibronectin (primary) antibody is omitted. Sequentially immerse the slide in water (1 min), *Hematoxylin staining solution* (4–5 min), and water (1 min). Do not allow the tissue to dry. Affix a coverslip over the tissue using a low-viscosity, aqueous, synthetic-resin coverslip mountant.

Acceptance criteria: Using USP Construct Human Fibroblasts in Bilayer Synthetic Scaffold Reference Photomicrograph 3 (diaminobenzidine–hematoxylin stained) for comparison, fibronectin binds to collagen and is found throughout the extracellular matrix. Fibronectin is found colocalizing with the collagen fibers. The intensity of staining may vary from region to region of the slide.

• **METABOLIC ACTIVITY ASSESSMENT**

DPBS solution A: Dissolve 1.32 g of calcium chloride and 1.21 g of magnesium sulfate heptahydrate in 2 L of water.

DPBS solution B: Dissolve 80.0 g of sodium chloride, 2.0 g of potassium chloride, 11.5 g of dibasic sodium phosphate, 2.0 g of monobasic potassium phosphate, 10.0 g of glucose, 0.36 g of sodium phosphate, 0.5 g of streptomycin sulfate, and 1,000,000 USP Units of penicillin G sodium in 8 L of water.

DPBS working solution: Mix *DPBS solution B* and *DPBS solution A* (8:2). Pass the solution through a filter of 0.22-µm pore size.

Dulbecco's modified Eagle's tissue culture medium: Prepare a solution that contains the components listed in [Table 1](#).

Table 1

Component	mg/L
Calcium chloride	264.9
Ferric nitrate nonahydrate	0.10
Potassium chloride	400.0
Magnesium sulfate heptahydrate	200.0
Sodium chloride	6,400.0
Sodium bicarbonate	3,700.0
Sodium phosphate, monobasic (monohydrate)	125.0
Dextrose	4,500.0
Phenol red	15.0
Sodium pyruvate	110.0
L-Arginine hydrochloride	84.0
L-Cystine	48.0
Aminoacetic acid	30.0
L-Histidine hydrochloride monohydrate	42.0
L-Isoleucine	104.8
L-Leucine	104.8
L-Lysine hydrochloride	146.2
L-Methionine	30.0
L-Phenylalanine	66.0