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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 paraffin[5] 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 reagent[7] (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/cm^2 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 serum[10] (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 diluent[12] 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 solution[13] 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 solution[14] 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

Component	mg/L
L-Serine	42.0
L-Threonine	95.2
L-Tryptophan	16.0
L-Tyrosine	72.0
L-Valine	93.6
D-Calcium pantothenate	4.0
Choline chloride	4.0
Folic acid	4.0
Inositol	7.0
Nicotinamide	4.0
Pyridoxine hydrochloride	4.0
Riboflavin	0.40
Thiamine hydrochloride	4.0

L-Glutamine solution: Prepare 100 mL of a solution containing 2.92 g of L-glutamine.

Sodium pyruvate solution: Prepare 100 mL of a solution containing 1.10 g of sodium pyruvate.

Antibiotic-antimycotic solution: Prepare 100 mL of a solution containing 0.85 g of sodium chloride, 10,000 USP Units of penicillin G sodium, 10,000 µg of streptomycin (base), and 25 µg of amphotericin B in water.

Assay stock medium: Mix 1000 mL of *Dulbecco's modified Eagle's tissue culture medium,* 10 mL of *L-Glutamine solution*, 10 mL of *Sodium pyruvate solution*, 10 mL of *Antibiotic-antimycotic solution*, and 20 mL of fetal bovine serum.[15]

MTT–assay solution: Dissolve 0.50 g of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide in 1 L of *Assay stock medium*, using constant stirring.

Sterilize the solution by passing it through a filter of 0.2-µm pore size.

MTT formazan stock solution: 100 µg/mL of 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan in isopropyl alcohol

MTT formazan calibration solutions: 15, 30, 45, 60, and 75 µg/mL of MTT formazan, using *MTT formazan stock solution* and diluting with isopropyl alcohol

Analysis: Thaw Construct Human Fibroblasts in Bilayer Synthetic Scaffold by placing the tissue, still in its ethyl vinyl acetate bag, in a water bath heated to between 34° and 37° for 2–3 min. The minimum amount of water in the water bath is 2 L/Construct Human Fibroblasts in Bilayer Synthetic Scaffold unit. Cut three 11-mm × 11-mm sections of Construct Human Fibroblasts in Bilayer Synthetic Scaffold, and immerse the sections into separate, 3.0-mL portions of *MTT–assay solution*. Incubate for 2 h at 37 ± 2° in a 3%–7% CO_2–air environment with shaking on an orbital shaker at 150–200 rpm. After incubation, remove from the 37°, 3%–7% CO_2–air environment. Remove the *MTT–assay solution*, and rinse twice with *DPBS working solution*. Immerse the Construct Human Fibroblasts in Bilayer Synthetic Scaffold in 2 mL of isopropyl alcohol, and incubate at ambient temperature for 1 h with shaking on an orbital shaker at approximately 125 rpm. Transfer 200-µL aliquots of the five *MTT formazan calibration solutions*, in triplicate, and 200-µL aliquots of the three isopropyl alcohol extracts of Construct Human Fibroblasts in Bilayer Synthetic Scaffold to a suitable 96-well, flat-bottom plate. Read the absorbance of each aliquot at 540 nm, using 200 µL of isopropyl alcohol as the blank.

Plot the responses of the *MTT formazan calibration solutions* versus concentration, in µg of MTT formazan/mL, and calculate the regression line using the least-squares method.

System suitability requirements: The test is considered valid if the regression line has a square of the correlation coefficient NLT 0.95.

Acceptance criteria: The absorbance value of individual Construct Human Fibroblasts in Bilayer Synthetic Scaffold sections at 540 nm is less than 0.1.

- **DNA Content**

Cell culture water: Sterile water containing NMT 0.005 USP Endotoxin Unit/mL

DNA extraction buffer: Transfer 850 mL of *Cell culture water* to a sterile, 1-L graduated container. Dissolve 12.110 g of 2-amino-2-hydroxymethyl-1,3-propanediol, 3.802 g of edetate disodium, 23.380 g of sodium chloride, and 0.080 g of sodium dodecyl sulfate, with stirring. Adjust with 1 N hydrochloric acid or 1 N sodium hydroxide to a pH of 7.0. Dilute with *Cell culture water* to 1 L.

Proteinase K solution: Prepare a solution of Tritirachium album proteinase K in 10 mM of 2-amino-2-hydroxymethyl-1,3-propanediol, adjusted to a pH of 7.5, having an activity of 600 units/mL.[16]

Working DNA extraction buffer: Add 1.22 mL of *Proteinase K solution* to 38.78 mL of *DNA extraction buffer*, and mix.

Dilution buffer: Transfer 850 mL of *Cell culture water* to a sterile, 1-L graduated container. Add 1.211 g of 2-amino-2-hydroxymethyl-1,3-propanediol, 3.802 g of edetate disodium, and 5.844 g of sodium chloride, with stirring. Adjust with 1 N hydrochloric acid or 1 N sodium hydroxide to a pH of 7.0. Dilute with *Cell culture water* to 1 L.

DPBS without Ca^{++}, Mg^{++} solution: Prepare a solution containing 8.00 g/L of sodium chloride, 1.15 g/L of dibasic sodium phosphate (anhydrous), 0.20 g/L of potassium chloride, and 0.20 g/L of monobasic potassium phosphate in water.

Calf thymus DNA solution: Prepare a solution containing 1 mg/L of calf thymus DNA in *DPBS without Ca^{++}, Mg^{++} solution*, mixing thoroughly for 12–24 h at ambient temperature. Dilute the resulting solution with *DPBS without Ca^{++}, Mg^{++}solution* to prepare a solution containing 50 µg/mL of calf thymus DNA, mixing thoroughly on a vortex mixer for 10 min.

Calf thymus DNA calibration solutions: Prepare four calibration solutions containing 5, 10, 15, and 20 µg/mL of calf thymus DNA, using *Calf thymus DNA solution*, and diluting with *DPBS without Ca^{++}, Mg^{++} solution*.

DNA staining solution: Prepare a solution containing 0.5 µg of 2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi(1H-benzimidazole) trihydrochloride pentahydrate/mL of *Dilution buffer*. Store in low-actinic glassware.

Analysis: Thaw Construct Human Fibroblasts in Bilayer Synthetic Scaffold by placing the tissue, still in its ethyl vinyl acetate bag, in a water bath heated to between 34° and 37° for 2–3 min. The minimum amount of water in the water bath is 2 L/Construct Human Fibroblasts in Bilayer Synthetic Scaffold unit. Cut three 11-mm × 11-mm sections of Construct Human Fibroblasts in Bilayer Synthetic Scaffold. To each of three microcentrifuge tubes add 1 mL of *DPBS without Ca^{++}, Mg^{++} solution*. Immerse a single Construct Human Fibroblasts in Bilayer Synthetic Scaffold 11-mm × 11-mm section into each microcentrifuge tube to remove the cryopreservative. Aspirate the *DPBS without Ca^{++}, Mg^{++} solution* from each tube, and replace with 1 mL of *Working DNA extraction buffer*, making sure that each Construct Human Fibroblasts in Bilayer Synthetic Scaffold is completely submerged in the extraction buffer. Incubate the samples in a 56°–60° water bath for 4–18 h. Sonicate for 10–15 s using an ultrasonic cell disrupter to achieve complete cellular disruption of the tissue and to mix the contents of the tube. Centrifuge the microcentrifuge tubes at 12,000–15,000 × g to pellet non-DNA material. Transfer three 50-µL aliquots of each sample supernatant to individual wells of a 96-well black plate suitable for performing fluorescent analysis. Transfer triplicate 50-µL aliquots of each of the *Calf thymus DNA calibration solutions* to the 96-well plate, as well as a 50-µL aliquot of *DPBS working solution* for the blank. Add 150 µL of *DNA staining solution* to all wells containing the tissue samples, *Calf thymus DNA calibration solutions*, and the blank. Cover with aluminum foil, and place in a dark cabinet for 30–45 min at 15°–30°. Read the fluorescence of each well, using an excitation wavelength of 355 nm and an emission wavelength of 460 nm, blanking against the *DPBS without Ca^{++}, Mg^{++} solution* well. Plot the responses of the *Calf thymus DNA calibration solutions* versus concentration, in µg/mL of calf thymus DNA, and calculate the regression line using the least-squares method. The test is considered valid if the %CV of the replicate values is less than 15%, the slope is 4.48–6.27, the *y*-intercept is between −2.04 and 3.65, and the square of the correlation coefficient is NLT 0.990. From the regression line so obtained, determine the amount of DNA, in µg/11-mm × 11-mm sample.

Acceptance criteria: The amount of DNA of an individual Construct Human Fibroblasts in Bilayer Synthetic Scaffold 11-mm × 11-mm section is between 6 and 14 µg.

- **Total Collagen Content**

DPBS without Ca^{++}, Mg^{++} solution: Proceed as directed for *DNA Content*.

DPBS with Ca^{++}, Mg^{++} solution: 8.00 mg/mL of sodium chloride, 1.15 mg/mL of dibasic sodium phosphate (anhydrous), 0.20 mg/mL of potassium chloride, 0.20 mg/mL of monobasic potassium phosphate, 0.10 mg/mL of magnesium chloride hexahydrate, and 0.10 mg/mL of calcium chloride (anhydrous) in water

Collagenase extraction solution: At least 250 Units/mL of *Clostridium histolyticum* collagenase, type 2, in *DPBS with Ca^{++}, Mg^{++} solution*

2% Acetic acid solution: Mix 10 mL of acetic acid with 490 mL of water.

Collagen standard stock solution: 2 mg/mL of collagen, type I, in *2% Acetic acid solution*

Collagen calibration standards: Cut polyglactin mesh[17] into seventeen 11-mm × 11-mm squares, and place one square into 17 individual wells of a 24-well plate. Each well of the 24-well plate has a surface area of 220 mm^2 and a volume of 3.5 mL. In quadruplicate, prepare wells containing 0.050, 0.100, 0.200, and 0.400 mg of collagen by adding 25, 50, 100, and 200 µL, respectively, of the *Collagen standard stock solution*. The remaining well to which no *Collagen standard stock solution* has been added is used as the blank. Allow the wells to air dry.

Sirius red solution: 1 mg/mL of Direct Red 80 in saturated picric acid

1% (*p*-*tert*-Octylphenoxy) polyethoxyethanol solution: Mix 10 mL of (*p*-*tert*-Octylphenoxy) polyethoxyethanol in 990 mL of *DPBS with Ca^{++}, Mg^{++} solution*.

Analysis: Thaw Construct Human Fibroblasts in Bilayer Synthetic Scaffold by placing the tissue, still in its ethyl vinyl acetate bag, in a water bath heated to between 34° and 37° for 2–3 min. The minimum amount of water in the water bath is 2 L/Construct Human Fibroblasts in Bilayer Synthetic Scaffold unit. Cut three 11-mm × 11-mm sections of Construct Human Fibroblasts in Bilayer Synthetic Scaffold. Place each test section into separate wells of a 24-well plate. Add 200 µL of *1% (p-tert-Octylphenoxy) polyethoxyethanol solution* to each sample. Shake on a rotating platform shaker at 100–150 rpm for 60–70 min at room temperature. Aspirate off the *1% (p-tert-Octylphenoxy)polyethoxyethanol solution*, and rinse three times with 2 mL of *DPBS without Ca^{++}, Mg^{++} solution*. Transfer each of the collagen standards to individual wells of the 24-well plate. Add 0.5 mL of *Sirius red solution* to each test sample and collagen standards. Shake on a rotating platform shaker at 100–150 rpm for 60 min at room temperature. Aspirate off the *Sirius red solution* from each well. Rinse each well twice with 2 mL of *DPBS without Ca^{++}, Mg^{++} solution*. Add an additional 2 mL of *DPBS without Ca^{++}, Mg^{++} solution* to each well, and allow to stand for 2 min. Aspirate off the *DPBS without Ca^{++}, Mg^{++} solution*, and rinse twice more with 2 mL of *DPBS without Ca^{++}, Mg^{++} solution*. Aspirate off all traces of *DPBS without Ca^{++}, Mg^{++} solution*. Add 0.5 mL of *Collagenase extraction solution* to each well containing the *Collagen calibration standards*. Add 2.0 mL of *Collagenase extraction solution* to each well containing test samples. Rotate the plate on an orbital rotator at 150 rpm for 90 min at 37°. Transfer 200 µL from each well, to a suitable 96-well, flat-bottom plate. Read the absorbance of each aliquot at 540 nm. Dilute the Construct Human Fibroblasts in Bilayer Synthetic Scaffold sample preparation further with *DPBS without Ca^{++}, Mg^{++} solution* if the absorbance is greater than the absorbance of the highest of the *Collagen calibration standards*.

Plot the responses of the *Collagen calibration standards* versus the amount, in mg of collagen, and calculate the regression line using the least-squares method.

System suitability requirements: The test is considered valid if the slope is 3.00–5.00 and the square of the correlation coefficient is ≥ 0.950.

Determine the collagen content, in mg, of an 11- × 11-mm section of Construct Human Fibroblasts in Bilayer Synthetic Scaffold from the regression line, and by using the following equation:

$$\text{Result} = D \times A \times SC_{SR}$$

D = dilution factor (normally 4, unless the sample had to be further diluted)

A = absorbance at 540 nm

SC_{SR} = slope of the regression line of the standards calculated above

Acceptance criteria: The amount of collagen in individual Construct Human Fibroblasts in Bilayer Synthetic Scaffold 11- × 11-mm samples is 0.50–4.0 mg.

• **Bacterial Endotoxins Test ⟨85⟩**

Sample solution: Thaw Construct Human Fibroblasts in Bilayer Synthetic Scaffold by placing the tissue, still in its polycarbonate cassette contained in a plastic covering bag, in a water bath heated to a maximum of 37° for 15–20 min until no visible ice remains in the cassette. The minimum amount of water in the water bath is 2 L/Construct Human Fibroblasts in Bilayer Synthetic Scaffold unit.

Analysis: Remove the unit from the polycarbonate cassette, and immerse in 25 mL of LAL Reagent Water. Extract for 60 min at 37° with shaking on an orbital shaker set at 125 revolutions/min. Remove a 4-mL aliquot of the extract for testing.

Acceptance criteria: NMT 0.5 USP Endotoxin Unit/mL

• **Sterility Tests ⟨71⟩**

Sample solution: Thaw Construct Human Fibroblasts in Bilayer Synthetic Scaffold by placing the tissue, still in its polycarbonate cassette contained in a plastic covering bag, in a water bath heated to a maximum of 37° for 15–20 min until no visible ice remains in the cassette. The minimum amount of water in the water bath is 2 L/Construct Human Fibroblasts in Bilayer Synthetic Scaffold unit.

Analysis: Perform the test on 20 mL of the cryopreservative.

Acceptance criteria: Meets the requirements

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Construct Human Fibroblasts in Bilayer Synthetic Scaffold is aseptically packaged and supplied frozen in a clear plastic cassette containing two, approximately 12.5- × 19-cm units. The solution within the cassette is a phosphate-buffered cryoprotectant solution used to facilitate long-term storage. A clear plastic bag surrounds the cassette for its protection. Construct Human Fibroblasts in Bilayer Synthetic Scaffold should be stored at a temperature of −70° to −20° for no longer than 18 months.

• **Labeling:** The label indicates the dimensions of the Construct Human Fibroblasts in Bilayer Synthetic Scaffold material enclosed. It contains the expiry date, required storage conditions, and the lot number. The label cautions that Construct Human Fibroblasts in Bilayer Synthetic

Scaffold is not to be used if the package shows signs of damage. Additional labeling requirements include instructions on the proper thawing and handling of Construct Human Fibroblasts in Bilayer Synthetic Scaffold and the time frame for use after package opening.

Change to read:

- **▲USP Reference Standards ⟨11⟩**

USP Authentic Visual References RS▲ (CN 1-Aug-2020)

USP Construct Human Fibroblasts in Bilayer Synthetic Scaffold Reference Photomicrographs. These three photomicrographs represent examples of passing units, prepared as directed in *Hematoxylin–eosin staining*, *Collagen staining*, and *Distribution of fibronectin*. They are specified to assist in ascertaining histological quality. The fibroblasts are embedded in an extracellular matrix that they have secreted (USP Construct Human Fibroblasts in Bilayer Synthetic Scaffold Reference Photomicrograph 1). The collagen (USP Construct Human Fibroblasts in Bilayer Synthetic Scaffold Reference Photomicrograph 2) and fibronectin (USP Construct Human Fibroblasts in Bilayer Synthetic Scaffold Reference Photomicrograph 3) are to be found throughout the extracellular matrix. The nylon fibers (yellow in USP Construct Human Fibroblasts in Bilayer Synthetic Scaffold Reference Photomicrograph 1) and the silicone backing (gray in USP Construct Human Fibroblasts in Bilayer Synthetic Scaffold Reference Photomicrograph 1) are frequently visible, although easily lost during processing.

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[1] A suitable *Buffered formalin* can be obtained from VWR International, 1310 Goshen Pkwy., West Chester, PA 19380.

[2] A suitable histological tissue cassette can be obtained from Sakura Finetek U.S.A., Inc., 1750 West 214th St., Torrance, CA 90501.

[3] A suitable histological tissue cassette basket can be obtained from Sakura Finetek U.S.A., Inc., 1750 West 214th St., Torrance, CA 90501.

[4] A suitable histological xylene substitute is Citrosolve® Clearing Agent, available from Fisher Scientific, 200 Park Ln., Pittsburgh, PA 15275.

[5] A suitable paraffin for use is Tissue Prep* 2 Embedding Media, available from Fisher Scientific, 200 Park Ln., Pittsburgh, PA 15275.

[6] A suitable histological adhesive for use is Histoslide® Adhesive, which can be obtained from Poly Scientific Research Corp., 70 Cleveland Ave., Bay Shore, NY 11706-1282.

[7] A suitable bluing reagent can be obtained from Sigma-Aldrich Corp., P.O. Box 14508, St. Louis, MO 63178.

[8] These photomicrographs are available as a CD from the USP Reference Standards collection, available to the user through USP Customer Services. To order these and other Reference Standards, call 1-800-227-8772 (U.S. and Canada), +1-301-881-0666 or 00-800-4875-5555 (select Europe); or go online to www.usp.org. Order item number 1535857.

[9] A suitable Diaminobenzidine solution can be obtained from Sigma-Aldrich Corp., P.O. Box 14508, St. Louis, MO 63178; catalog number D-6815.

[10] A suitable normal rabbit serum can be obtained from Dako Corp., 6392 Via Real, Carpinteria, CA 93013.

[11] Suitable rabbit anti-human fibronectin antibodies can be obtained from Dako Corp., 6392 Via Real, Carpinteria, CA 93013.

[12] Suitable antibody diluent can be obtained from Dako Corp., 6392 Via Real, Carpinteria, CA 93013.

[13] Suitable biotinylated goat anti-rabbit antibody solution can be obtained from BioGenex, 4600 Norris Canyon Rd., San Ramon, CA 94583.

[14] A suitable streptavidin conjugated horseradish peroxidase solution can be obtained from BioGenex, 4600 Norris Canyon Rd., San Ramon, CA 94583.

[15] A suitable fetal bovine serum can be obtained from HyClone, 925 West 1800 South, Logan, UT 84321; catalog number SH30070.03.

[16] A suitable Proteinase K solution can be obtained from Roche Diagnostics Corp., Roche Applied Sciences, P.O. Box 50414, 9115 Hague Rd., Indianapolis, IN 46250-0414.

[17] A suitable polyglactin mesh can be obtained from Ethicon Co., Johnson & Johnson Corp., 425 Hoes Ln., P.O. Box 6800, Piscataway, NJ 08855.

Topic/Question	Contact	Expert Committee
CONSTRUCT HUMAN FIBROBLASTS IN BILAYER SYNTHETIC SCAFFOLD	Rebecca C. Potts Associate Scientific Liaison	BIO52020 Biologics Monographs 5 - Advanced Therapies

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