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Scaffold Porcine Small Intestinal Submucosa

DEFINITION

Scaffold Porcine Small Intestinal Submucosa is a collagen-based scaffold, translucent and off-white in color. It is obtained from the small intestinal submucosa layer of the domestic pig (*Sus scrofa* L.). This layer has been mechanically separated from the adjoining layers of the intestine to remove the serosal, mucosal, and muscular elements. The isolated submucosa is chemically cleaned, decellularized, freeze-dried, and terminally sterilized. Scaffold Porcine Small Intestinal Submucosa also undergoes a viral inactivation; the inactivation method is validated using parvovirus, reovirus, pseudorabies virus, and leukemia retrovirus as the test viruses. By dried weight, Scaffold Porcine Small Intestinal Submucosa consists of about 70% protein, about 20% carbohydrate, and about 7% lipid. The protein component is primarily collagen type I, with minor amounts of elastin and collagen type III, collagen type IV, and collagen type VI. In addition to these components, additional extracellular matrix components, such as glycosaminoglycans and fibroblast growth factor-2, are also retained.

SPECIFIC TESTS

- **Fibroblast Growth Factor-2 Content**

Sterile PBS solution: 8.065 g/L of sodium chloride and 0.2 g/L of potassium chloride in 0.01 M sodium phosphate buffer, pH 7.4

Sample solution: Obtain a 1-cm^2 sample of Scaffold Porcine Small Intestinal Submucosa, weigh, and submerge in 400 µL of *Sterile PBS solution*. Pulverize the tissue for 90 s using a tissue grinder, intermittently checking to be sure that the tissue remains immersed in the *Sterile PBS solution* and becomes homogenized. Centrifuge at 12,000 × *g* for 5 min at 4°. Use immediately upon preparation.

Analysis: Examine duplicate aliquots of the *Sample solution* by a suitably sensitive ELISA method.[1]

System suitability: The analysis is considered valid if the ELISA kit generates a linear standard curve with the square of the correlation coefficient (r^2) NLT 0.95, and if the duplicate aliquots of the *Sample solution* yield results that are within 20% of each other.

Acceptance criteria: The average content of fibroblast growth factor-2 is NLT 10,000 pg/g of Scaffold Porcine Small Intestinal Submucosa.

- **Glycosaminoglycan Content**

1,9-Dimethylmethylene blue solution: Mix 95 mL of 0.1 M hydrochloric acid in 500 mL of water. Add 16 mg of 1,9-dimethylmethylene blue, 3.04 g of aminoacetic acid, and 2.37 g of sodium chloride. Dilute with water to 1 L, and adjust to a pH of 3.0 with sterile solutions of either 1.0 M sodium hydroxide or 1.0 M hydrochloric acid. Store in low-actinic glassware.

Sterile PBS solution: 8.065 g/L of sodium chloride and 0.2 g/L of potassium chloride in 0.01 M sodium phosphate buffer, pH 7.4

Proteinase K solution: 600 Units/mL of *Tritirachium album* proteinase K

Stock heparin standard solution: 1 mg/mL of heparin

Heparin standard curve solutions: Use *Stock heparin standard solution* to prepare three solutions containing 20, 50, and 100 µg/mL of heparin.

Sample solution: Prepare samples in duplicate. Accurately weigh about 25 mg of Scaffold Porcine Small Intestinal Submucosa and cut into small pieces (roughly 2 mm × 2 mm). Transfer to a 1.5-mL microcentrifuge tube, and add 180 µL of *Sterile PBS solution* and 20 µL of *Proteinase K solution*. Mix, and incubate the sample at 56° for 15 min; during the incubation, mix intermittently on a vortex mixer. Cool the sample to room temperature. Dilute with water to obtain a concentration of 12.5 mg/mL of digested Scaffold Porcine Small Intestinal Submucosa.

Blank solution: Water

Collagen control solution: Accurately weigh about 25 mg of a bovine collagen, type I, that contains less than 1 µg of glycosaminoglycan/mg. Transfer to a 1.5-mL microcentrifuge tube, and add 180 µL of *Sterile PBS solution* and 20 µL of *Proteinase K solution*. Mix, and incubate the sample at 56° for 15 min; during the incubation, mix intermittently on a vortex mixer. Cool the sample to room temperature. Dilute with water to obtain a concentration of 12.5 mg/mL of digested bovine collagen.

Analysis

(See *Ultraviolet-Visible Spectroscopy ⟨857⟩*.)

Add 2.5 mL of *1,9-Dimethylmethylene blue solution* to triplicate 100-µL aliquots each of *Heparin standard curve solutions*, *Sample solution*, *Blank solution*, and *Collagen control solution*. Mix on a vortex mixer for 1 s and immediately read the absorbance at 525 nm. Generate a standard curve of absorbance versus concentration using the averages of each *Heparin standard curve solution*, correcting for the blank,

and calculate the regression line and regression coefficient. The concentration of glycosaminoglycan in the *Sample solution* and the *Collagen control solution* is determined directly from the regression line. If the absorbance of the *Sample solution* is greater than the highest *Heparin standard curve solution*, then dilute the *Sample solution* appropriately, and repeat the *Analysis* detailed in this section.

System suitability: The test is considered valid if the regression curve has a square of the correlation coefficient (r^2) NLT 0.95; the triplicate aliquots of the *Sample solution* and *Collagen control solution* yield results that are within 20% of each other, respectively; and the average glycosaminoglycan content of the *Sample solution* is statistically greater than the *Collagen control solution* using one-tailed, unequal variances, *t*-test at $\alpha = 0.05$.

Acceptance criteria: The average glycosaminoglycan content of the *Sample solution* is NLT 2 µg/mg.

- **Bioactivity**

[Note—Aseptic cell culture techniques must be used throughout the performance of this test.]

Modified RPMI-1640 culture medium: Prepare a sterile solution that contains the components included in *Table 1*.

Table 1

Component	Content (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
Glucose	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
L-Serine	42.0

Component	Content (mg/L)
L-Threonine	95.2
L-Tryptophan	16.0
L-Tyrosine	72.0
L-Valine	93.6
L-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
Sodium 1-heptanesulfonic acid	2383.0

Penicillin–streptomycin solution: 10,000 USP Penicillin Units of penicillin/mL and 10 mg of streptomycin/mL in a suitable buffer[2]

PC12 cell line culture medium: Mix 420 mL of *Modified RPMI-1640 culture medium*, 50 mL of horse serum,[3] 25 mL of fetal bovine serum,[4] and 5 mL of *Penicillin–streptomycin solution*. Sterilize by passing through a 0.22-µm filter.

Sterile PBS solution: 8.065 g/L of sodium chloride and 0.2 g/L of potassium chloride in 0.01 M sodium phosphate buffer, pH 7.4

Rat tail collagen solution: Prepare a suspension containing 0.2 mg/mL of rat tail collagen, type I, in sterile water.

Cell culture apparatus: Prepare by adding a sufficient volume of *Rat tail collagen solution* to completely cover the bottom of each well of a 12-well cell culture plate (dimension of each well is about 22–23 mm in diameter and about 17–18 mm in depth). Incubate under sterile conditions for 2 h at 37° or overnight at room temperature. Remove the *Rat tail collagen solution* by aspiration. Rinse with *Sterile PBS solution* that has been preheated to 37°.

PC12 cells: Use cultured rat pheochromocytoma cells (ATCC CRL-1721).

Cultivation of PC12 cells: Prewarm *PC12 cell line culture medium* to 37°. Add 15 mL of prewarmed *PC12 cell line culture medium* to a T-75 culture flask. Place a single vial containing the frozen *PC12 cells* in a 37° water bath with gentle agitation until they start to thaw (about 1 min). Complete the thawing procedure by slowly rotating the vial between the hands. Rinse the outside of the vial with 70% alcohol. Transfer the contents of the vial to the T-75 flask, and mix. Incubate the cells overnight at 37° in a 5% carbon dioxide atmosphere. Transfer the contents of the T-75 culture flask to a sterile centrifuge tube, centrifuge at 200 × *g* for 5 min at 37°, and discard the supernatant. Resuspend the cells in 15 mL of *PC12 cell line culture medium*, and transfer the contents back to the T-75 culture flask. Incubate the cells at 37° in a 5% carbon dioxide atmosphere for 3 days.

Cell feeding: At the end of 3 days, the cells will need to be fed for optimal growth. To feed the cells, remove a flask of cells from the incubator, tightening the cap in the process. Examine the T-75 flask under the microscope and check for microbial contamination and confluency. If there is microbial contamination, then discard the flask. If the cells appear confluent, follow the instructions below for perpetuating the PC12 cell line (see *Culture perpetuation*). Otherwise, harvest the cells from the flask by pipetting the contents of the flask across the bottom of the flask several times. Transfer the cell suspension to a sterile 50-mL centrifuge tube. Centrifuge the cells at 200 × *g* for 5 min at 37°, and discard the supernatant. Resuspend the cells in 13 mL of *PC12 cell line culture medium*, prewarmed to 37°. Transfer the cell suspension back to the T-75 flask, and mix. Loosen the cap of the flask, and return to the incubator; incubate the cells at 37° in a 5% carbon dioxide atmosphere for another 3–7 days.

Culture perpetuation: To perpetuate a line of *PC12 cells* for culture, examine under the microscope a T-75 flask containing cells and check for microbial contamination and confluency. If there is microbial contamination, discard the flask and use another. If the cells do not appear

confluent, then follow the instructions above for feeding the PC12 cell line (see *Cell feeding*), beginning with "Otherwise, harvest the cells from the flask by pipetting the contents of the flask across the bottom of the flask several times." If the cells are confluent and there is no contamination, harvest the cells from the flask by pipetting the contents of the flask across the bottom of the flask several times to loosen up the cells from their attachment to the bottom of the flask and to break up cell clusters. Check under the microscope before proceeding to ensure that most of the cells have detached from the plastic. Transfer the cell suspension to a sterile 50-mL centrifuge tube, and centrifuge the cells at 200 × *g* for 5 min at 37°. Discard the supernatant and resuspend the cells with 10 mL of *PC12 cell line culture medium*, prewarmed to 37°. Dispense an equal amount of the cell suspension into each of 3–5 T-75 flasks, each flask containing 10 mL of *PC12 cell line culture medium*, prewarmed to 37°, and mix. Return the passaged cells to the incubator, being sure to loosen the cap of the flasks. Incubate the cells at 37° in a 5% carbon dioxide atmosphere. Feed the cells after 3 days as directed above, beginning with "To feed the cells, remove a flask of cells from the incubator, tightening the cap in the process." [NOTE—Do not use cells that have undergone more than 15 passages after obtaining them from ATCC.]

Positive control solution: Prepare a solution containing about 10 ng of fibroblast growth factor-2/mL of *PC12 cell line culture medium*.

Negative control solution: Use *PC12 cell line culture medium*.

Sample solution: Immerse 70 cm^2 of Scaffold Porcine Small Intestinal Submucosa in sterile water for 5 min. Remove the Scaffold Porcine Small Intestinal Submucosa, and blot excess water using sterile gauze. Weigh the rehydrated Scaffold Porcine Small Intestinal Submucosa to the nearest 0.1 g and add *Modified RPMI-1640 culture medium* at a ratio of 7.5 mL of *Modified RPMI-1640 culture medium* for each 1.0 g of Scaffold Porcine Small Intestinal Submucosa. Incubate for 24 h at 37° with constant shaking. Remove the Scaffold Porcine Small Intestinal Submucosa, and pass the solution through a 0.22-µm filter. Add sufficient quantities of sterile horse serum and sterile fetal bovine serum to concentrations of 10% and 5%, respectively, and add a sufficient quantity of *Penicillin–streptomycin solution* such that there are 100 USP Penicillin Units and 0.1 mg of streptomycin/mL. Adjust the pH of the *Sample solution* to 7.4, using a sterile solution of either 1.0 M sodium hydroxide or 1.0 M hydrochloric acid.

Analysis: Harvest a flask of confluent *PC12 cells* by pipetting the contents of the flask across the bottom of the flask to loosen the cells, then transfer the cell suspension to a centrifuge tube and centrifuge at 200 × *g* for 5 min. Remove the supernatant by aspiration, and resuspend the pellet to obtain a concentration of about 1×10^6 cells/mL of *PC12 cell line culture medium*. Add to each of three wells of the *Cell culture apparatus* 1.0 mL of *Negative control solution*. To a second set of three wells, add to each well 1.0 mL of *Positive control solution*, and to a third set of three wells, add to each well 1.0 mL of *Sample solution*. Add to each well about 20,000 cells, mix by gentle rocking, and incubate for 48 h at 37°. For each well, count three random microscopic fields of cells using a microscope with a 10× ocular lens and a 20× objective lens. Each field should have at least 20 cells; avoid large clumps of cells where individual cell bodies cannot be ascertained. Determine the total number of cells in the field and, using USP Cultured Rat Pheochromocytoma Reference Photomicrographs of normal and differentiated rat pheochromocytoma cells for comparison, determine the total number of cells that have formed at least one neurite-like extension at least twice the diameter of a normal, undifferentiated cell body. For each experimental group, record the total number of cells counted and the total number of cells differentiated across all three wells, and calculate the total percentage of cells that have differentiated.

System suitability: For a test to be valid, the following criteria must be met: (1) none of the wells are microbially contaminated; (2) the weighted percentage of differentiated cells across the *Negative control solution* wells is NMT 5%; (3) the weighted percentage of differentiated cells across the *Positive control solution* wells is NLT 6%; and (4) the weighted percentage of differentiated cells across the *Negative control solution* wells is statistically less than the weighted percentage of differentiated cells across the *Positive control solution* wells, using a one-sided, two-sample test for proportions at $\alpha = 0.05$.

Acceptance criteria: The weighted percentage of differentiated cells incubated in the *Sample solution* wells is statistically greater than those incubated in the *Negative control solution* wells, using a one-sided, two-sample test for proportions at $\alpha = 0.05$.

- **METABOLIC ACTIVITY ASSESSMENT**

Dulbecco's modified Eagle's tissue culture medium: Prepare a solution that contains the components included in *Table 2*.

Table 2

Component	Content (mg/L)
Calcium nitrate, tetrahydrate	100.0
Ferric nitrate, nonahydrate	0.10
Potassium chloride	400.0
Magnesium sulfate, anhydrous	48.840

Component	Content (mg/L)
Sodium chloride	6,000.0
Sodium bicarbonate	1,500.0
Sodium phosphate, dibasic (anhydrous)	800.0
Glucose	4,500.0
Glutathione (reduced)	1.0
Phenol red	5.0
Sodium pyruvate	110.0
L-Arginine (free base)	200.0
L-Asparagine, monohydrate	56.620
L-Aspartic Acid	20.0
L-Cystine dihydrochloride	65.20
Aminoacetic acid	10.0
L-Histidine (free base)	15.0
Hydroxy-L-proline	20.0
L-Isoleucine	50.0
L-Leucine	50.0
L-Lysine hydrochloride	40.0
L-Methionine	15.0
L-Phenylalanine	15.0
L-Proline	20.0
L-Serine	30.0
L-Threonine	20.0
L-Tryptophan	5.0
L-Tyrosine, disodium, dihydrate	28.830
L-Valine	20.0
D-Biotin	0.20
D-Calcium pantothenate	2.50
Choline chloride	3.0

Component	Content (mg/L)
Folic acid	1.0
Inositol	35.0
Nicotinamide	1.0
p-Aminobenzoic acid	1.0
Pyridoxine hydrochloride	1.0
Riboflavin	0.20
Thiamine hydrochloride	1.0
Cyanocobalamine	0.0050

MTT reagent: A suitable solution of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide.[5]

Detergent reagent: A suitable sodium dodecyl sulfate detergent solution.[6]

Analysis: Remove three 12-mm diameter circular sections of Scaffold Porcine Small Intestinal Submucosa, using the appropriate size biopsy punch. Immerse each section into individual wells of a 12-well cell culture plate (dimension of each well is about 22–23 mm in diameter and about 17–18 mm in depth), each containing 1 mL of *Dulbecco's modified Eagle's tissue culture medium*. Prepare a positive control by harvesting a full-thickness section of porcine jejunum immediately following slaughter. Rinse the section of jejunum at 37° in isotonic sodium chloride solution for 5 min to remove intestinal debris. Using scissors, split open the section of jejunum to form a sheet. Remove three 12-mm diameter circular sections of jejunum, using the appropriate size biopsy punch. Immerse each section into individual wells of a 12-well cell culture plate, each well containing 1 mL of *Dulbecco's modified Eagle's tissue culture medium*. Treat these positive control wells in the same manner as the sample wells. Prepare a *Blank solution* using 1 mL of *Dulbecco's modified Eagle's tissue culture medium*. Allow sections to hydrate for 5 min, add 50 µL of *MTT reagent* to each of the sections and the blank, and mix. Incubate for 3 h at 37° in an atmosphere containing 5% carbon dioxide. Add 100 µL of *Detergent reagent* to each well, and mix. Leave the samples at ambient temperature in the dark for 2 h. Measure the absorbance of the resulting solution at 570 nm, adjusting for the blank.

System suitability: The average absorbance in the positive control wells is greater than 0.100.

Acceptance criteria: The average absorbance reading for the Scaffold Porcine Small Intestinal Submucosa wells is less than 0.100.

• **Sterility Tests ⟨71⟩:** Meets the requirements

• **Bacterial Endotoxins Test ⟨85⟩**

Sample: 70 cm^2 of Scaffold Porcine Small Intestinal Submucosa

Analysis: Immerse the *Sample* in 40 mL of LAL Reagent Water. Extract for 60 min at 37° with shaking. Remove a 100-µL aliquot to measure the amount of bacterial endotoxins.

Acceptance criteria: NMT 20.0 USP Endotoxin Units/70 cm^2

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Package in single-use, peel-open pouches that are gas permeable for sterilization purposes. Store under clean, dry conditions at 25°, excursions permitted between 15° and 30°.

• **Labeling:** The package is labeled to indicate the dimensions of the enclosed Scaffold Porcine Small Intestinal Submucosa, the expiry date, required storage conditions, and the lot number. The label indicates that the Scaffold Porcine Small Intestinal Submucosa is sterile if the package is intact, and that the Scaffold Porcine Small Intestinal Submucosa is designed for single patient, one-time use.

Change to read:

• ▲**USP Reference Standards ⟨11⟩**

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

USP Cultured Rat Pheochromocytoma Reference Photomicrographs

These photomicrographs represent examples of normal and differentiated rat pheochromocytoma cells and are used to assist in ascertaining bioactivity.

[1] A suitably sensitive ELISA test kit for the quantitation can be obtained from R&D Systems Inc., 614 McKinley Place N.E., Minneapolis, MN; product number DFB50.

[2] A suitable buffered solution containing 10,000 USP Penicillin Units of penicillin/mL and 10 mg of streptomycin/mL can be obtained from Sigma-Aldrich Corp., St. Louis, MO.

[3] A suitable horse serum can be obtained from American Type Culture Collection, P.O. Box 1549, Manassas, VA (www.atcc.org).

[4] A suitable fetal bovine serum can be obtained from American Type Culture Collection, P.O. Box 1549, Manassas, VA (www.atcc.org).

[5] A suitable solution of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide can be obtained as part of the MTT Cell Proliferation Assay (Catalog # 30-1010K) from American Type Culture Collection, P.O. Box 1549, Manassas, VA (www.atcc.org) or equivalent.

[6] A suitable sodium dodecyl sulfate detergent reagent can be obtained as part of the MTT Cell Proliferation Assay (Catalog #30-1010K) from American Type Culture Collection, P.O. Box 1549, Manassas, VA (www.atcc.org) or equivalent.

Auxiliary Information - Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
SCAFFOLD PORCINE SMALL INTESTINAL SUBMUCOSA	Rebecca C. Potts Associate Scientific Liaison	BIO52020 Biologics Monographs 5 - Advanced Therapies
REFERENCE STANDARD SUPPORT	RS Technical Services RSTECH@usp.org	BIO52020 Biologics Monographs 5 - Advanced Therapies

Chromatographic Database Information: Chromatographic Database

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