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Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold

DEFINITION

Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold is a living, bilayered skin substitute derived from neonatal foreskins manufactured under Class 100 sterile conditions. The upper, epidermal layer is formed by human keratinocytes and has a well-differentiated stratum corneum. The inner, dermal layer is composed of human fibroblasts in a bovine Type I collagen lattice. Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold does not contain Langerhans cells, melanocytes, macrophages, lymphocytes, blood vessels, hair follicles, or any other epidermally derived components. The fibroblast and keratinocyte cell banks from which Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold is derived test negative for human and animal viruses, retroviruses, bacteria, fungi, yeast, mycoplasma, and tumorigenicity. The cell banks are also tested for normal human karyology and isoenzymes. The final product is tested for morphology, cell viability, and physical container integrity. Used tissue culture media are tested for mycoplasma and sterility. All materials derived from bovine sources originate from countries free of bovine spongiform encephalopathy.

SPECIFIC TESTS

• HISTOLOGICAL CHARACTERIZATION

2.0 M Monobasic potassium phosphate: Dissolve 13.61 g of anhydrous monobasic potassium phosphate in 50 mL of water.

2.0 M Dibasic potassium phosphate: Dissolve 17.42 g of anhydrous dibasic potassium phosphate in 50 mL of water.

Phosphate-buffered saline solution (pH 7.1–7.5): Combine 3.6 mL of 2.0 M Monobasic potassium phosphate, 16.4 mL of 2.0 M Dibasic potassium phosphate, 8 g of sodium chloride, and 1 L of water. Mix thoroughly.

0.3% Acid alcohol: To 100 mL of 70% alcohol, add 0.3 mL of hydrochloric acid and mix.

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

Bluing agent: Dissolve 200 mg of sodium bicarbonate and 40 mg of lithium carbonate in 63 mL of water and 37 mL of methanol and mix.

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

Analysis: Remove three 2-cm diameter circular tissues from every 30-cm² section of Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold (NLT 30% of the total unit area), using the appropriate size biopsy punch. Cut with a circular rocking motion to prevent crushing the tissue. Immerse the sections in 3.7% dimethoxymethane for 30 min, using a gentle rocking motion. Remove the sections, and lay on a cutting surface, dermal side (glossy side) down. Cut an approximately 3-mm-wide strip through the center of the specimen, using a new, single-edged razor blade. Place the strips in a histological microwave cassette, using suitable biopsy pads premoistened with Phosphate-buffered saline solution (pH 7.1–7.5) to hold the strips in place. Insert the cassette into a histological microwave processing rack, place the rack inside a suitable microwave container, and add sufficient Phosphate-buffered saline solution (pH 7.1–7.5) to completely cover the rack. Place the container in a microwave oven suitable for histological work,¹ and heat for 4 min at 55°. Remove the Phosphate-buffered saline solution (pH 7.1–7.5), and add enough dehydrated alcohol to completely cover the rack. Return the container to the microwave oven, and heat for 4 min at 67°. Remove the alcohol, and add enough dehydrated isopropyl alcohol to completely cover the rack. Return the container to the microwave oven, and heat for 4 min at 74°.

Remove the isopropyl alcohol, and add enough suitable grade paraffin² that has been melted and held at 84° prior to use, to completely cover the rack. Return the container to the microwave oven, and heat for 7 min at 84°. Remove the histological microwave cassette from the container and rack while the paraffin is still melted, and disassemble, discarding the biopsy pads. Fill preheated embedding molds with molten paraffin³ heated to 60°, and place on top of a preheated warming platform that is designed for histological work. Using forceps, remove the Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold specimens from the cassette and place the specimens in individual molds. Orient the specimens in the molds to enable cutting of a cross-or longitudinal section. Cool the

paraffin by sliding the mold down the platform to its cool side until the paraffin has solidified. Maintain the 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 to 30 min, and clamp the paraffin 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,⁴ and heat to a temperature 5° lower than the melting point of the paraffin. Properly mount the paraffin block into a microtome, adjusting as necessary. Set the microtome to make 5-µm thick cuts with a blade angle of $5 \pm 2^\circ$. Insert into the knife holder a sharp stainless steel microtome knife that has been properly honed or a new disposable microtome knife, and cut a ribbon that contains 6–10 sections of Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold. Pick up the ribbon with forceps, and stretch it across the tissue-flotation water bath. Separate 2 to 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. Allow the mounted sections to air-dry completely, or dry the slide in a 60° oven for 1 h. The microscope slide with affixed tissue is sequentially immersed in 3 changes of a suitable histological, aliphatic xylene substitute,⁵ 5 min/step, followed by two changes of dehydrated alcohol, 3 min/step. Sequentially immerse the slide in alcohol (for 3 min), running water rinse (3 min), *Hematoxylin staining solution* (6 min), running water rinse (7 min), 0.3% *Acid alcohol* (6 s), running water rinse (5 min), *Blueing agent* (1 s), running water rinse (5 min), *Eosin solution* (2 min), two changes of alcohol (3 min each step), four changes of dehydrated alcohol (3 min each step), and four changes of a suitable histological xylene substitute (3 min each 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 suitable coverslip mountant.

Microscopic specifications: A light microscope with 4x, 10x, 20x, and 40x objectives installed in a revolving nosepiece; a 10x widefield ocular with 10 to 19 mm per 100 microdisk reticle installed; and a 10x widefield ocular with grid reticle installed

Microscopic and morphological characteristics: Score the three Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold sections for epidermal and dermal aspects, using the light microscope. Evaluate the slides from each of the sections taken. Average the aspect values for each section ($n = 3$) to determine the overall aspect score for the Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold unit. When examined microscopically, Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold shows a bilayered construct resembling the epidermal and dermal layers of human skin. Using USP Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold Reference Photomicrographs of passing and failing articles for comparison, Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold meets the requirements for epidermal aspects, including epidermal coverage, epidermal development, and keratinocyte aspect, and meets the requirements for dermal aspects, including dermal matrix thickness, fibroblast density, and matrix aspect, as described below.

Epidermal aspects

[NOTE—See USP Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold Reference Photomicrograph 1 for an example of a passing unit.]

Acceptance criteria for Epidermal coverage: NLT 95% of the dermal matrix present on the slide is covered with epidermal keratinocytes.

Acceptance criteria for Epidermal development: NLT 70% of the Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold epithelium is composed of three distinct cell layers.

[NOTE—See USP Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold Reference Photomicrograph 2 for an example of a failing unit.]

The basal cell layer of the epithelium is at least 1 cell thick, consisting of keratinocytes with a cuboidal-columnar shape.

[NOTE—See USP Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold Reference Photomicrograph 3 for an example of a failing unit.]

The suprabasal layer is composed of stratified cells and is at least 5 cells thick. Suprabasal cells closest to the basal layer are cuboidal in shape; cells become progressively stratified the closer they are to the uppermost, squamous cell layer. The squamous cell layer on the apical surface is cornified and at least 1 cell thick.

[NOTE—See USP Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold Reference Photomicrograph 4 for an example of a failing unit.]

The uppermost cell layer of the epithelium is analogous to the stratum corneum of human skin and is composed of one or more rows of flat, scaly cells that are nonliving and keratinized.

[NOTE—See USP Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold Reference Photomicrograph 5 for an example of a failing unit.]

Acceptance criteria for Keratinocyte aspects: NLT 95% of the basal keratinocytes have basophilic cytoplasm that neither has distinct vacuoles nor is necrotic. See USP Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold Reference Photomicrograph 6 for an example of a failing unit. NLT 80% of suprabasal cells (excluding those in the upper 20% of the cell layer closest to the squamous layer) have basophilic cytoplasm. Furthermore, these basophilic suprabasal cells do not have distinct vacuoles and are neither necrotic nor keratinized.

[NOTE—See USP Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold Reference Photomicrographs 7 and 8 for examples of failing units.]

Dermal aspects

Five randomly selected fields/slide will be evaluated for dermal matrix thickness and fibroblast density. The five fields will be averaged to obtain the final value for each section.

[NOTE—See USP Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold Reference Photomicrograph 1 for an example of a passing unit.]

Acceptance criteria for Dermal aspects

Dermal matrix thickness: The Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold dermal layer is NLT 40 μm thick and is composed of several rows of flat dermal cells.

Fibroblast density: The dermal matrix contains an average of at least four nonpyknotic nuclei present per microscopic field (field = 20 grid squares of reticle when using the 10 $\times$ widefield ocular and 40 $\times$ objective).

Matrix aspect: NLT 95% of the dermal matrix collagen stains uniformly with no large holes or inclusions present.

[NOTE—See USP Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold Reference Photomicrographs 9 and 10 for examples of failing units.]

• GENE EXPRESSION PROFILE

RNA extraction solution: Use an aqueous phenol and guanidine isothiocyanate solution suitable for RNA extraction.⁶

DEPC-treated water: Add 0.2 mL of diethylpyrocarbonate (DEPC) to 100 mL of sterile Purified Water, shake vigorously, and allow to stand for at least 12 h. Autoclave the resulting solution for 15 min, using the liquid cycle, to inactivate residual DEPC. Prepare fresh as needed.

5X Reaction buffer: Prepare a solution of 375 mM potassium chloride, 15 mM magnesium chloride, and 250 mM tris(hydroxymethyl) aminomethane hydrochloride. Adjust to a pH of 8.3.

10X Reaction buffer: Prepare a solution of 500 mM potassium chloride and 100 mM tris(hydroxymethyl) aminomethane hydrochloride. Adjust to a pH of 8.3.

Oligo-deoxythymidine solution: Prepare a 20-mM oligo-deoxythymidine (primer length: 18) solution, using a suitable buffer.⁷

dNTP solution I: Using a suitable buffer,⁷ prepare a solution of deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, and deoxythymidine triphosphate in which the concentration of each component is 10 mM.

dNTP solution II: Prepare a solution, in water, of deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, and deoxythymidine triphosphate, in which the concentration of each component is 10 mM.

Ribonuclease inhibitor solution: Prepare a solution containing 40 units of ribonuclease inhibitor/mL of a suitable buffer.⁷

Reverse transcriptase buffer: Prepare a solution of 0.1 M sodium chloride, 0.1 mM edetate disodium, 1.0 mM dithiothreitol, 0.01% nonylphenol polyoxyethylene ether, 50% glycerin, and 200 mM tris(hydroxymethyl) aminomethane hydrochloride. Adjust to a pH of 7.5.

Reverse transcriptase solution: Prepare a solution containing 200 units of Moloney-Murine Leukemia Virus reverse transcriptase/ μL in Reverse transcriptase solution.

DNA primer pairs: Prepare individual 20- μM solutions of the following DNA primer pairs, using deoxyribonuclease- and ribonuclease-free water.

Table 1

Transforming growth factor β 1 (TGFβ 1):	
TGF β 1-3'	agg ctc caa atg tag ggg cag g
TGF β 1-5'	gcc ctg gac acc aac tat tgc t
Interleukin-1α (IL1α):	
IL1 α -3'	tag tgc cgt gag ttt ccc aga aga aga gga gg
IL1 α -5'	caa gga gag cat ggt ggt agt agc aac caa cg
Interleukin-4 (IL4):	
IL4-3'	acg tac tct ggt tgg ctt cct tca cag gac ag



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Transforming growth factor β 1 (TGFβ 1):	
IL4-5'	cgg caa ctt tga cca cgg aca caa gtg cga ta
Platelet-derived growth factor A:	
PDGF-A-3'	ctg ctt cac cga gtg cta caa tac ttg ct
PDGF-A-5'	aga agt cca ggt gag gtt aga gga gcat
Glyceraldehyde-3-phosphate dehydrogenase:	
G3PDH-3'	cat gtg ggc cat gag gtc cac cac
G3PDH-5'	tga agg tcg gag tca acg gat ttg gt

DNA polymerase solution: Prepare a solution containing five units of deoxyribonucleic acid polymerase/mL of a solution of 100 mM potassium chloride, 0.1 mM edetate disodium, 1 mM dithiothreitol, 0.5% polyoxyethylene (20) sorbitan monolaurate, 0.5% nonylphenol polyoxyethylene ether, 50% glycerin, and 20 mM tris(hydroxymethyl) aminomethane hydrochloride. Adjust to a pH of 8.0.

Analysis

RNA extraction: Remove three 2-cm diameter circular sections from every 30 cm² of Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold (NLT 30% of the total unit area), using the appropriate size biopsy punch. Transfer each piece of tissue to individual polypropylene microcentrifuge tubes. Add 1.0 mL of *RNA extraction solution* to each tube, homogenize by repetitive pipetting, and incubate the samples for 5 min at room temperature. To each tube, add 0.2 mL of chloroform, mix on a vortex mixer, and centrifuge at 12,000 $\times g$ for 15 min at 2°–8°. Transfer the upper, aqueous phase to a second tube, add 0.5 mL of isopropanol, and incubate for 30 min to overnight at –20°. Centrifuge at 12,000 $\times g$ for 15 min, discard the supernatants by aspiration, and add 75% alcohol to each pellet. Mix the sample on a vortex mixer, centrifuge at 12,000 $\times g$ for 2 min, and discard the supernatants by aspiration without disturbing the RNA pellets. Recentrifuge at 12,000 $\times g$ for 2 min, and remove the remaining supernatants with a small-volume (20 μ L or smaller capacity) micropipet. Air-dry the pellets for 5 min at room temperature by keeping the microcentrifuge cap off, and resuspend each pellet in 50 μ L of *DEPC-treated water*. Bring absorbance into linear range by diluting 5 μ L of each suspension with 195 μ L of *DEPC-treated water*. Transfer the samples to suitable quartz microplates or cuvettes and determine the absorbance of the RNA solution at wavelengths of 260 and 280 nm, using a spectrophotometer and *DEPC-treated water* as the blank. The ratio of the absorbance at 260 versus 280 nm should be NLT 1.65. If this ratio is less than 1.65, mix the resuspended pellet by repetitive pipetting, and repeat the dilution step and absorbance measurement. If this fails to raise the absorbance ratio, repeat the RNA extraction for that sample by adding 1 mL of *RNA extraction solution*, and proceed as above, beginning with “incubate the sample for 5 min at room temperature”.

Determine the concentration of RNA, in μ g/mL:

$$\text{Result} = F \times A \times D$$

F = conversion factor, 40

A = absorbance at 260 nm

D = dilution factor

Adjust the volume of the RNA solutions with additional *DEPC-treated water* to bring the concentration of RNA to about 80 μ g/mL. If the absorbance at 260 nm is less than 0.05, discard the sample, and repeat the RNA extraction on a fresh sample.

Synthesis of cDNA: To separate, individual thin-walled polymerase chain reaction (PCR) tubes, add 12.5 μ L of the RNA solution from samples 1, 2, and 3 (3 reaction tubes total). Add 1 μ L of *Oligo-deoxythymidine solution* to each tube, and incubate at 72° for 2 min to anneal the oligo-deoxythymidine to the mRNA. Place the tubes in an ice bath, and to each tube, add 4 μ L of *5X Reaction buffer*, 1 μ L of *dNTP solution I*, 0.5 μ L of *Ribonuclease inhibitor solution*, and 1 μ L of *Reverse transcriptase solution*. Incubate at 42° for 1 h to synthesize cDNA, and then incubate at 94° for 5 min to inactivate the reverse transcriptase. To each tube, add 80 μ L of *DEPC-treated water* and mix.

cDNA positive control⁸

Polymerase chain reaction amplification of cDNA: For each of the five *DNA primer pairs*, label five individual centrifuge tubes (5 tubes total). Add the following to each centrifuge tube: 135.8 μ L of *DEPC-treated water*; 10.5 μ L of *dNTP solution II*; 21 μ L of *10X Reaction buffer*; 3.5 μ L of the appropriate 5' primer; 3.5 μ L of the appropriate 3' primer; and 12.6 μ L of 25 mM magnesium chloride. Close, mix on a vortex mixer, and pulse spin in a microcentrifuge. Add 2.1 μ L of *DNA polymerase solution* to each centrifuge tube, and mix by repetitive pipetting. For