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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-cm^2 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 paraffin[2] 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 paraffin[3] 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 ± 2°. 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), *Bluing 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 4×, 10×, 20×, and 40× objectives installed in a revolving nosepiece; a 10× widefield ocular with 10 to 19 mm per 100 microdisk reticle installed; and a 10× 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× widefield ocular and 40× 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):	
TGFβ 1-3'	agg ctc caa atg tag ggg cag g
TGFβ 1-5'	gcc ctg gac acc aac tat tgc t
Interleukin-1α (IL1α):	
IL1α-3'	tag tgc cgt gag ttt ccc aga aga aga gga gg
IL1α-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

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 cm^2 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 × *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 × *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 × *g* for 2 min, and discard the supernatants by aspiration without disturbing the RNA pellets. Recentrifuge at 12,000 × *g* for 2 min, and remove the remaining supernatants with a small-volume (20 µL 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 µL of *DEPC-treated water*. Bring absorbance into linear range by diluting 5 µL of each suspension with 195 µL 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 µg/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 µg/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 µL of the RNA solution from samples 1, 2, and 3 (3 reaction tubes total). Add 1 µL 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 µL of *5X Reaction buffer*, 1 µL of *dNTP solution I*, 0.5 µL of *Ribonuclease inhibitor solution*, and 1 µL 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 µL of *DEPC-treated water* and mix.

cDNA positive control[8]

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 µL of *DEPC-treated water*; 10.5 µL of *dNTP solution II*; 21 µL of *10X Reaction buffer*; 3.5 µL of the appropriate 5' primer; 3.5 µL of the appropriate 3' primer; and 12.6 µL of 25 mM magnesium chloride. Close, mix on a vortex mixer, and pulse spin in a microcentrifuge. Add 2.1 µL of *DNA polymerase solution* to each centrifuge tube, and mix by repetitive pipetting. For

each primer pair, transfer 27 µL of the resulting solution to five thin-walled PCR tubes. There should be a total of 25 PCR tubes. Add the following to the PCR tubes of each primer set:

Table 2

PCR tube number	Sample
1	3 µL Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold sample 1 cDNA
2	3 µL Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold sample 2 cDNA
3	3 µL Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold sample 3 cDNA
4	3 µL *cDNA positive control*
5 Negative control	3 µL *DEPC-treated water*

Repeat for the remaining primer pairs. The positive control contains authentic cDNA of *Transforming growth factor β*, *Interleukin-1α*, *Interleukin-4*, *Platelet-derived growth factor A*, and *Glyceraldehyde-3-phosphate dehydrogenase*, as appropriate for each primer set. Pulse spin the PCR tubes in a microcentrifuge to mix, and place the tubes in a single PCR thermal cycler. Cycling conditions are as follows.

Table 3

Melting temperature	94°
Melting time	45 s
Anneal temperature	58°
Anneal time	45 s
Elongation temperature	72°
Elongation time	2 min
Number of cycles	30
Final elongation temperature	72°
Final elongation time	2 min

Terminate the PCR amplification by heating each tube to 72° for 7 min.

Electrophoresis identification

Tris-boric acid buffer: 89 mM of tris(hydroxymethyl) aminomethane, 89 mM of boric acid, and 2 mM of edetate disodium

6X Loading buffer: A solution containing 15% of a branched polymeric sucrose (400 kDa), 0.25% bromophenol blue, and 0.25% xylene cyanole FF

Ethidium bromide solution: 10 mg/mL Ethidium bromide in *Tris-boric acid buffer*

Agarose gel: A horizontal 2% agarose[9] gel in *Tris-boric acid buffer*

Once the gel is set, remove the comb, and place the gel into the electrophoresis chamber with the comb end of the gel situated closest to the cathode terminal. Fill the electrophoresis chamber with *Tris-boric acid buffer* until the buffer reaches 3–5 mm over the surface of the gel.

100-bp DNA ladder markers: A solution containing 10 DNA fragments covering the range of 100–1000 base pairs (bp) in 100-bp increments, with a total DNA content of approximately 100 ng/µL (15–20 ng of DNA per band) in an appropriate buffer[10]

Analysis: Dilute the 25 PCR samples prepared in the *Polymerase chain reaction amplification of cDNA* with *6X Loading buffer* so that the final concentration of the buffer is one-sixth of its original concentration. Load 5 µL of the *100-bp DNA ladder markers* in the first lane of the

agarose gel. Load 10 µL of each PCR sample into each gel well, and attach the cathode to the terminal close to the loaded wells. Attach the anode to the terminal farthest from the loaded wells, and apply 120 V to the gel. Run the gel until the bromophenol blue is two-thirds the length of the gel. Remove the gel from the electrophoresis apparatus, and place it in a tray containing enough *Ethidium bromide solution* to cover the gel. Slowly agitate the gel on a shaker table for 30 min. Completely remove the *Ethidium bromide solution* from the tray, add an equal amount of *Tris-boric acid buffer*, and slowly agitate the gel on a shaker table for 60 min. Place the gel on a 312-nm UV light source, photograph the gel, and inspect the image for bands that have migrated from each individual well. If a band appears, it is verified for size in base pairs by comparing it to the lane for the *100-bp DNA ladder markers*.

System suitability: If a band appears and it is of the appropriate size, it is considered positive. The analysis is considered valid if the positive controls show the appropriately sized cDNA–PCR products, no PCR product bands appear in the negative controls, and all bands are observed to be visually discrete.

Acceptance criteria: The lanes of the agarose gel that correspond to Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold show cDNA bands for *Interleukin-1α* (expected PCR product band size of 491 base pairs, limit of detection NLT 9.6×10^{-21} moles); *Platelet-derived growth factor A* (expected PCR product band size of 304 base pairs, limit of detection NLT 1.5×10^{-20} moles); *Transforming growth factor β1* (expected PCR product band size of 161 base pairs, limit of detection NLT 1.5×10^{-20}) moles; and *Glyceraldehyde-3-phosphate dehydrogenase* (expected PCR product band size of 983 base pairs); but not *Interleukin-4* (expected PCR product band size of 344 base pairs, limit of detection NLT 1.5×10^{-22} moles). If one of the replicates tested yields results discordant with the other two replicates, repeat the test, and accept only if all three replicates are concordant.

- **Barrier Integrity Assessment**

Ham's F-12 tissue culture medium: Prepare a solution that contains the following:

Table 4

Component	mg/mL
L-Alanine	8.91
L-Arginine hydrochloride	210.7
L-Asparagine monohydrate	15.01
L-Aspartic acid	13.30
L-Cysteine hydrochloride monohydrate	35.12
L-Glutamic acid	14.70
L-Glutamine	146.2
Aminoacetic acid	7.51
L-Histidine hydrochloride monohydrate	20.96
L-Isoleucine	3.94
L-Leucine	13.12
L-Lysine hydrochloride	36.54
L-Methionine	4.48
L-Phenylalanine	4.96
L-Proline	34.53
L-Serine	10.51
L-Threonine	11.91

Component	mg/mL
L-Tryptophan	2.04
L-Tyrosine disodium	6.71
L-Valine	11.71
Calcium chloride	44.00
Cupric sulfate, pentahydrate	0.0025
Ferric sulfate, heptahydrate	0.834
Potassium chloride	223.7
Magnesium chloride	57.22
Sodium chloride	7599.0
Sodium phosphate, dibasic	142.0
Zinc sulfate, heptahydrate	0.863
D-Biotin	0.0073
D-Calcium pantothenate	0.238
Choline chloride	13.96
Folic acid	1.30
Hypoxanthine	4.04
Inositol	18.02
Niacinamide	0.0366
Pyridoxine hydrochloride	0.0617
Riboflavin	0.0376
Thiamine hydrochloride	0.337
Thymidine	0.727
Cyanocobalamin	1.36
α-Lipoic acid	0.206
Linoleic acid	0.0841
Dextrose	1801.6
Phenol red, sodium	1.30
Sodium pyruvate	110.0
Putrescine dihydrochloride	0.161

Component	mg/mL
Sodium bicarbonate	1176.0

Tritiated water: 2.0 µCi/mL

Percutaneous absorption apparatus: Prepare the apparatus as described below.[11]

Six-well cell culture plate: The dimensions are inner diameter, 35 mm; depth, 18 mm.

Cell culture well insert: Each well is a plastic cylinder with inner length, 15 mm; inner diameter, 24 mm; outer diameter, 27 mm, with a flanged end extending 4 mm from the outer diameter. The inner diameter opposite the flanged end is covered by a taut polycarbonate membrane having a porosity of 3 µm. The flange should allow the *Cell culture well insert* to be suspended in the well of a *Six-well cell culture plate*, leaving a 3-mm space between the bottom of the *Cell culture well insert* and the inner bottom surface of the *Six-well cell culture plate*.

Percutaneous absorption insert: Use a polytetrafluoroethylene cylinder having the following dimensions: length, 20 mm; inner diameter, 20 mm; outer diameter, 23 mm with a flanged end extending 3 mm from the outer diameter. Ten mm from the flanged end of the cylinder, the inner diameter begins to funnel so that the inner diameter at 10 mm from the flanged end is 20 mm, and the inner diameter at 15 mm from the flanged end is 8 mm. From 15 to 20 mm from the flanged end, the inner diameter remains at 8 mm. The outer diameter of the cylinder remains constant at 23 mm. The flanged end is considered to be the top of the component.

Silicon grease: Use high-vacuum silicon grease suitable for glass.[12]

Analysis: Fill each well of the *Six-well cell culture* plate with 1.5 mL of *Ham's F-12 tissue culture medium*. Remove two 2-cm circular sections from every 30 cm^2 of Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold (NLT 20% of the total unit area), using the appropriate size biopsy punch. Transfer each excised section to a separate *Cell culture well insert*, dermal side down on the polycarbonate membrane. Using forceps, gently smooth out the section to remove any wrinkles. Apply a narrow ring of *Silicon grease* to the underside of the *Percutaneous absorption insert*, and place the insert into the *Cell culture well insert*, grease side down, onto the epidermal surface of the Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold biopsy, with slight pressure to form a tight seal. Do not allow any grease to enter the 8-mm diameter exposed area of the Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold surface. Place the *Cell culture well insert* containing the *Percutaneous absorption insert* into one of the wells of the *Six-well cell culture plate* containing 1.5 mL of *Ham's F-12 tissue culture medium*. Apply 1.0 mL of *Tritiated water* to the exposed surface of the Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold unit in the *Percutaneous absorption insert*, and incubate at ambient temperature for 6 h. At the end of each h, transfer the *Cell culture well insert* containing the *Percutaneous absorption insert* to a new well within the *Six-well cell culture plate* containing 1.5 mL of fresh *Ham's F-12 tissue culture medium*. After the 6-h incubation, remove the *Cell culture well insert*. Remove a 0.5-mL aliquot of *Ham's F-12 tissue culture medium* from each well of the *Six-well cell culture plate*, and transfer into individual scintillation vials. Dispense 0.5 mL of *Tritiated water* to a separate scintillation vial as a control. Add 4.5 mL of a suitable scintillation cocktail[13] to each scintillation vial and gently mix. Place the scintillation vials into a liquid scintillation counter, and count the emissions in the tritium spectrum for 60 s. Average the counts for each of the six time points (punch average) and duplicate sections (unit average).

Determine the percent penetration per h:

$$\text{Result} = F \times (C_S/C_C)$$

F = conversion factor, 150

C_S = counts/min of the 0.5-mL aliquot of the *Ham's F-12 tissue culture medium* taken at the end of the incubation period

C_C = counts/min in the 0.5-mL aliquot of *Tritiated water*

Acceptance criteria: NMT 1.97% penetration is found.

- **Metabolic Activity Assessment**

Dulbecco's modified Eagle's tissue culture medium: Prepare a solution that contains the following:

Table 5

Component	mg/L
Calcium chloride	264.9
Ferric nitrate, nonahydrate	0.10
Potassium chloride	400.0

Component	mg/L
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
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

MTT solution: Dissolve 0.33 g of (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide in 1 L of *Dulbecco's modified Eagle's tissue culture medium*, with constant stirring. Pass the solution through a suitable size filter having a 0.2-µm porosity.

0.04 N Acidified isopropyl alcohol: Add 3.45 mL of hydrochloric acid to 1 L of isopropyl alcohol, and mix thoroughly. Store at room temperature no longer than 6 months.

Analysis: Immerse the Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold in separate 40.0-mL portions of *MTT solution*, making sure that about 20 mL of *MTT solution* is under the sample article, and 20 mL of *MTT solution* is on the surface. Take care not to produce any bubbles. Incubate for 3 h at 37°, in an environment of air enriched with 10% carbon dioxide. After incubation, remove from the 37°, 10% carbon dioxide-enriched air environment. Transfer the Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold to a suitable cutting surface, and, using an appropriate biopsy punch, remove three 8-mm diameter circular sections from every 30 cm^2 of Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold (5% of unit area). Transfer each punch to individual snap-top test tubes. Add 0.9 mL of *0.04 N Acidified isopropyl alcohol* to each tube, making sure that the tissue is completely submerged. If not submerged, use forceps to place the sample into the *0.04 N Acidified isopropyl alcohol*. Cap each tube tightly, place on an orbital shaker, and shake for 1 h at a moderate setting. After 1 h, remove the tubes from the orbital shaker, and mix each tube on a vortex mixer. Inspect the tubes to make sure that the tissue samples continue to be submerged. If not, use forceps or another device to resubmerge the tissues. Return the tubes to the orbital shaker, and continue to shake for an additional 1 h. Remove the tubes from the orbital shaker, mix the tubes on a vortex mixer, and transfer a 0.2-mL aliquot to a suitable 96-well flat-bottom plate. Read the absorbance of each sample at 570 nm, using 0.2 mL of *0.04 N Acidified isopropyl alcohol* as the blank.

Acceptance criteria: The average absorbance value is NLT 0.237.

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold is aseptically packaged in a Class 100 environment in single-use containers that preserve cell viability and product integrity. Store at controlled room temperature for NMT 5 days, and do not subject to freezing temperatures. The atmosphere within the package contains air enriched with 10% carbon dioxide. The device is translucent and off-white in color. The upper, epidermal surface is dull with small irregularities resulting from the cornification of keratinocytes, while the bottom surface is smooth and shiny in appearance. The device is packaged so that the dermal layer (glossy layer) is closest to the agarose-based nutrient medium. The packaging permits easy observation of the medium and provides ready access to the Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold when needed. The medium contains all of the required nutrients for the living cell components of Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold plus an appropriate, nontoxic, pH-sensitive dye to indicate package breaches or microbial contamination. The medium should appear pink (pH 6.8–7.7) when compared to the enclosed pH color chart.

• **Labeling:** Label it to indicate the dimensions of the enclosed Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold, the expiry date, the required storage conditions, and the lot number. The label indicates that the enclosed Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold and surrounding medium are to be examined for signs of contamination or deterioration. The label also contains a pH color code to be used for determination of the acceptability of the pH of the Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold medium. The label cautions that Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold is not to be used if the package shows signs of damage or microbial contamination. Label it to indicate that sterile techniques are to be used in handling Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold and that cytotoxic agents are not to be used. Label it to indicate 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 Keratinocytes and Fibroblasts in Bovine Collagen Scaffold Reference Photomicrographs

[Note—These 10 photomicrographs represent examples of both passing and failing Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold units. They are specified to assist in ascertaining histological quality.]

[1] A microwave oven suitable for histological preparation can be obtained from Energy Beam Sciences, Inc., 11 Bowles Road, P.O. Box 14508, Agawam, MA or equivalent.

[2] A suitable paraffin for use is Accumate™ Tissue Embedding/Infiltration Medium, which can be obtained from Sigma Diagnostics, P.O. Box 468, St. Louis, MO 63178 or equivalent.

[3] A suitable paraffin for use is Paraplast® X-Tra Tissue Embedding Medium ASTM, melting point 50–54°, which can be obtained from Fisher Scientific, 300 Industry Drive, Pittsburgh, PA 15275 or equivalent.

[4] A suitable histological adhesive for use is Histoslide® Adhesive, which can be obtained from Poly Scientific R & D Corp., 70 Cleveland Ave., Bay Shore, NY 11706-1282 or equivalent.

[5] A suitable histological xylene substitute is Shandon Xylene Substitute from Thermo Scientific (http://www.thermoscientific.com) or equivalent.

[6] A suitable RNA extraction solution is Trizol® reagent, which can be obtained from Life Technologies, (https://www.lifetechnologies.com) or equivalent.

[7] A suitable buffer can be obtained from the RT-for-PCR Kit, Clontech, 1290 Terra Bella Ave., Mountain View, CA 94043 or equivalent.

[8] A suitable cDNA positive control can be obtained from Clontech, 1290 Terra Bella Ave., Mountain View, CA 94043.

[9] An agarose suitable for electrophoresis analysis of Construct Human Keratinocytes and Fibroblasts in Bovine Collagen Scaffold cytokine PCR product is SeaKem® GTG agarose and can be obtained from Lonza Inc., 90 Boroline Road, Allendale, NJ 07401 or equivalent.

[10] A suitable buffered solution of *100-bp DNA ladder markers* can be obtained from Lonza Inc., 90 Boroline Road, Allendale, NJ 07401 or equivalent.

[11] A suitable *Percutaneous absorption apparatus*, not including the *Percutaneous absorption insert*, is a Costar® 6-well culture cluster, flat bottom with lid, and a Costar® Transwell®, 24 mm in a 6-well cluster plate with lid and can be obtained from Corning Life Sciences, 836 North Street, Building 300, Suite 3401, Tewksbury, MA 01876 or equivalent.

[12] A suitable *Silicon grease* is High Vacuum Silicon Lubricant for Glass and can be obtained from Dow Corning Corporation, P.O. Box 0994, Midland, MI 48686-0994.

[13] A suitable scintillation cocktail is OptiPhase Supermix Perkin-Elmer Life Sciences, Inc., 549 Albany St., Boston, MA 02118 or equivalent.

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

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

Chromatographic Database Information: Chromatographic Database

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