INSTRUCTION SHEET

HyStem-4D™ Chondrogenesis Differentiation Kit

Catalog Number: EM-2002

Kit Constituents:	Part number
PureStem HyGlu Basal DM, 242 mL	EM-2101
Pen/Strep, 2.5 mL	EM-1414
L-alanine-L-glutamine, 2.5 mL	EM-1415
Pyruvate, 2.5 mL	EM-1416
Proline plus ITS, 0.5 mL	EM-1417
2-phospho-L-ascorbic acid, 250 µL	EM-1408
Dexamethasone, 250 µL	EM-1409
TGFβ-3, 130 µL	EM-1410
BMP-4, 130 µL	EM-1411
HyStem-C 7.5 mL Kit	GS313

FOR RESEARCH USE ONLY

The HyStem-4D™ Chondrogenesis Differentiation Kit and protocol is guaranteed to induce differentiation of specific PureStem® embryonic progenitor cells using either using HyStem hydrogels (HyStem-C cultures). Always handle human sourced materials at biological safety level two.

PREPARATION OF PURESTEM DIFFERENTIATION MEDIUM

Preliminary PureStem Differentiation Medium - Working in a sterile field, supplement the PureStem HyGlu Basal medium (EM-2101) with the components listed below. Decontaminate the outside surface of each container with ethanol or isopropanol before supplementation. Rinse vials with medium to ensure the total content is used. Filter the supplemented medium through 0.22 µm unit filter and record the supplementation date. Supplemented medium can be stored at 2-8°C for up to four weeks.

2.5 mL EM-1414 (Pen/Strep)
2.5 mL EM-1415 (L-alanine-L-glutamine)
2.5 mL EM-1416 (Pyruvate)
0.5 mL EM-1417 (Proline plus ITS)
250 µL EM-1408 (2-phospho-L-ascorbic acid)
250 µL EM-1409 (Dexamethasone)

Final Differentiation Medium - Immediately before use and in a sterile field, add EM-1410 (TGFβ-3) and EM-1411 (BMP-4) to the desired volume needed to feed cells according to the instructions below. Always add supplements in a sterile field and always decontaminate the outside of the vials using ethanol or isopropanol before opening.

Add 1µL of EM-1410 (TGFβ-3) and 1µL of EM-1411 (BMP-4) to each 2 mL of Preliminary PureStem Differentiation Medium to prepare Final Differentiation Medium. We recommend that you use approximately 4 mL for each well of a 6-well plate or 15 mL for feeding cells in a 10 cm dish (assuming cell numbers are approximately $1x10^6$ and $6x10^6$ cells respectively).

NOTE: The first time EM-1410 (TGFβ-3) and EM-1411 (BMP-4) are thawed, aliquot into separate labeled sterile microfuge tubes (or equivalent) and freeze at ≤-65°C for future or near-term use. Prepare aliquots on the basis of predicted quantities of Final Differentiation Medium that will be used during each feed.

HARVESTING CELLS FOR DIFFERENTIATION:

NOTE: See Product Information Sheet provided with specific PureStem progenitor for recommended culture conditions and growth medium.

Steps 1-6 should be performed in a sterile field. Decontaminate the outside of containers before opening using ethanol or isopropanol.

1. Expand cells in appropriate growth medium according to the instructions provided to provide sufficient cell numbers for the preparation of 20 x 10^6 cells per mL of Hystem-C Hydrogel (or as desired based on the objectives of your experiment).
2. When cell number is sufficient, remove the growth medium from culture ware by aspiration. Wash cells with PBS (Ca & Mg free), THIS IS A CRITICAL STEP because growth medium contains proteins and Ca that can inactivate trypsin. Detach cells by covering with trypsin/EDTA (0.25% trypsin diluted 1/3 with PBS i.e. 1 part trypsin in 2 parts PBS). Place in incubator and check for cell detachment every few minutes. Allow trypsin to work until approximately 90% of the cells are rounded up. DO NOT OVERTRYPSINIZE as it can damage the cells. Look for cell detachment – it may be necessary to lightly rap the flask to detach the cells.
3. Following cell detachment (i.e. under 10 minutes), inactivate the trypsin by adding serum-containing growth media (at least 5X the volume of diluted trypsin used). Place the cell suspension into a sterile container or polypropylene tube.
4. Determine the cell number and concentration using a Coulter counter (Beckman Coulter Z2 Cell and Particle Counter or equivalent), hemocytometer or equivalent device.
5. Place the appropriate volume containing the desired cell number (i.e. 20 mL with 20 x 10^6 cells), into a separate sterile polypropylene 50 mL tube(s).
6. Centrifuge at 150 x g at room temperature for 5 min to pellet cells. See "Encapsulating Cells in HyStem-C Hydrogels" below for further instructions.

NOTE: A common cell seeding density to study chondrogenic differentiation is 20 x 10^6 cells per mL of Hystem-C Hydrogel, but this may vary based on the objectives of your experimentation. For more robust cartilage like constructs, it is recommended to use higher densities, even upwards of 60 x 10^6 cells per mL if possible.

ENCAPSULATING CELLS IN HYSTEM-C HYDROGELS:

Decontaminate the outside of containers containing materials that will be exposed to cells before opening using ethanol or isopropanol. Work in a sterile field when appropriate.

1. Prepare HyStem-C hydrogel according to the provided protocol (requires ~30min advanced preparation).
2. Thoroughly remove the supernatant (medium) by aspiration and gently resuspend cell pellet in the desired volume of HyStem-Gelin-S mixture.
3. Add appropriate volume of Extralink to begin crosslinking. Be sure to maintain 4:1 ratio of HyStem Gelin-S mixture (4) to Extralink solution (1). If you want to use the entire vial volume of Extralink, be sure the entire contents are used, including any that may have adhered to the inside of the cap.
4. Mix well with pipet and maintain homogeneous cell suspension for 10 minutes before final use.
5. Depending on your study objectives and methods, cell laden HyStem-C may be dispensed into non-tissue culture treated multi-well plates. If unattached constructs are desired, a less adherent mold such as agarose can be used to form constructs that can be released to float in differentiation medium.
6. Gelation should be completed within 20-30 minutes of initiation. Prevent inadvertent damage to newly formed hydrogel constructs by waiting an additional 30 minutes before covering with medium or attempting to transfer.
7. Culture hydrogel constructs in Final Differentiation Medium within a humidified incubator (37°C, 10% CO_2).
8. Feed cells 3X/week (i.e. Monday, Wednesday, and Friday) by aspirating old medium and gently adding freshly prepared differentiation medium until cell laden hydrogel constructs are harvested at your desired timepoints.

Variations on the initial HyStem-C Hydrogel stiffness may be made based on your experimental objectives. Reconstituting HyStem-C at higher concentrations (i.e. 0.5 mL HyStem; 0.5 mL Gelin-S; 0.25 mL Extralink) can increase the initial hydrogel stiffness and improve handling characteristics. More concentrated HyStem-C can lead to more mature cartilage like tissue after extended culture durations.

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