Lonza Walkersville, Inc.
Walkersville, MD 21793-0127 USA
U.S. Scientific Support: 800 521 0390
scientific.support@lonza.com
EU/ROW Scientific Support: +32 87 321 611
scientific.support.eu@lonza.com
Document # TS-CC-112-7 02/20
www.bioscience.lonza.com

Normal Human Articular Chondrocyte Cell System

NHAC-Kn – technical information & instructions

Contents:

I. Introduction

Lonza's chondrocyte cell system offers normal human articular chondrocytes from the Hyaline cartilage in the knee and optimized medium for their growth and re-differentiation. The system can quickly generate chondrocyte cultures for experimental applications in joint research, physiology, cellular function and differentiation. Chondrocytes are specialized cells that produce and maintain the extracellular matrix of cartilage and are the only cellular component of cartilage. Although cartilage is not confined to articulating joints, articular cartilage, especially from weight bearing joints, is an area on intense research because arthritis, joint injuries and joint abnormalities cause wide spread disability.

Chondrocytes are differentiated cells when isolated, however, they lose their differentiated phenotype after serial expansion in monolayer culture and become fibroblast-like, losing their ability to produce cartilage specific markers such as type II collagen and aggrecan. By the 2nd or 3rd passage, the cells are almost completely de-differentiated. Chondrocytes can be expanded in culture and then induced to re-differentiate. The system is designed to generate NHAC-kn cultures for experimental applications including studies on the formation, breakdown, and regeneration of hyaline cartilage, osteoarthritis research and the proliferation and differentiation of chondrocytes.

Lonza's chondrocyte cell system is convenient and easy to use, allowing the researcher to focus on results. Lonza's cells, medium and reagents are quality tested together and guaranteed to give optimum performance as a complete cell system.

II. General cell information

Cat. no.	Description	Recommended growth media	Cryopreserved passage number	Proliferating passage number*	Seeding density upon thaw**	Time to subculture
CC-2550	Articular Chondrocytes-Knee (NHAC-Kn)	CGM™ BulletKit™ Medium	Passage 2	Passage 3	10,000 viable cells/cm^2	4-9 days

*Proliferating cultures are generated using Lonza's cryopreserved cell stock. Proliferating cultures are delivered Tuesday-Thursday the week after initial plating. Confluence at the time of shipment varies by cell type. Proliferating cultures are available in a variety of culture vessels including flasks and well plates. For more information regarding proliferating cultures, including catalog numbers, please contact Lonza Scientific Support.

**Please note that alternative seeding densities may be required for subculture and/or differentiation.

III. Quality control

Cat. no.	Description	Cells/vial	Viability	Maximum population doublings	Doubling time	Properties
CC-2550	Articular Chondrocytes-Knee (NHAC-Kn)	≥750,000 viable cells	≥70%	≥15	15-48 hrs	Collagen Type II^+ *(after re-differentiation)* Alcian $Blue^+$ *(after re-differentiation)*

All cells are performance assayed and test negative for HIV-1, mycoplasma, Hepatitis-B, Hepatitis-C, bacteria, yeast and fungi. Cell viability, morphology, cell number, and proliferative capacity are measured after recovery from cryopreservation. Lonza's media are formulated for optimal growth of specific types of human cells. COAs for all media products are available upon request. Please see Section XVIII (product warranty, page 11) for more information on quality control claims and guarantees.

IV. Quality control: media & reagents

Basal media

Description:	CBM™ Basal Medium	CDM™ Basal Medium
Catalog no.	CC-3217	CC-3226
Test	**Specification**	
Sterility	Negative	Negative
pH	7.6 – 8.0	7.6 – 8.0
Osmolality (mOsm/kg H_2O)	260-290	280-296
Endotoxin	FIO*	FIO*

*for information only

SingleQuots™ Kits

Description:	CGM™ SingleQuots™ Kit	CDM™ SingleQuots™ Kit
Catalog no.	CC-4409	CC-4408
Test	**Specification**	
Sterility	Negative	Negative
Performance Test	Pass	Pass

Additional differentiation reagents

Description:	TGF-β3	Ascorbic Acid
Catalog no.	PT-4124*	CC-4398
Test	**Specification**	
Sterility	N/A	Negative

*no testing required

Subculture reagents

Description:	Trypsin/ EDTA	Trypsin Neutralizing Solution	HEPES Buffered Saline Solution
Catalog no.	CC-3232	CC-5002	CC-5022
Test	**Specification**		
Sterility	Negative	Negative	Negative
Performance Test	Pass	Pass	Pass
pH	N/A	N/A	FIO* (Target: 7.15-7.55)
Osmolality (mOsm/kg H_2O)	N/A	N/A	FIO* (Target: 287-317)
Endotoxin	N/A	N/A	FIO* (Target: ≤0.050 EU/mL)

*for information only

V. Cell growth system components

(sold separately)

- One normal human chondrocyte cell product – (cryopreserved or proliferating)
- One Chondrocyte Growth Medium BulletKit™ Medium - 500 mL

  CGM™ BulletKit™ (Lonza Catalog No. CC-3216) contains 500 mL of Chondrocyte Basal Medium (CBM™ Medium) and the following growth supplements: R3-Insulin-like Growth Factor-1 (R3-IGF-1), 1.0 mL; human recombinant Fibroblast Growth Factor-Beta (hrFGF-β), 2.5 mL; Transferrin, 0.5 mL; Insulin, 1.0 mL; Fetal Bovine Serum (FBS), 25.0 mL; Gentamicin/Amphotericin-B (GA), 0.5 mL
- One Chondrocyte ReagentPack™ Subculture Reagents (CC-3233), Containing:

Trypsin/EDTA	100 mL
Trypsin Neutralizing Solution	100 mL
HEPES Buffered Saline Solution	100 mL

NOTE: Additional components are necessary for the cryopreservation and re-differentiation of these cells. Please see the corresponding selection below for more information.

VI. Unpacking and storage instructions

1. Check all containers for leakage or breakage.
2. For cryopreserved cells: Remove cryovials from the dry ice packaging and immediately place into liquid nitrogen storage. Alternatively, thaw and use the cells immediately. If no dry ice remains, please contact Customer Service.
3. For proliferating cells: Swab down the flask of proliferating cells with 70% ethanol or isopropanol, then place the flask in 37°C, 5% CO_2, humidified incubator and allow to equilibrate for three to four hours. After cells have equilibrated, remove shipping medium from the flask and replace with fresh medium.
4. BulletKit™ Medium instructions: store basal medium at 2° - 8°C and SingleQuots™ Kit at ≤-20°C in a freezer that is not self-defrosting. Once thawed, SingleQuots™ Kit should be stored at 2° - 8°C and added to basal medium within 72 hours. After SingleQuots™ Kit is added to basal medium, use within 1 month. Do not re-freeze.
5. Chondrocyte ReagentPack™ Subculture Reagents are sterile-filtered and then stored at –20°C until shipment. Subculture reagents may thaw during transport. They may be refrozen once. If you plan to use within 3 days, store at 4°C. Trypsin/EDTA Solution has a limited shelf life or activation at 4°C. If, upon arrival, Trypsin/EDTA is thawed, immediately aliquot and refreeze at –20°C. We recommend that the HEPES-BSS and the Trypsin Neutralizing Solution be stored at 4°C for no more than one month.
6. TGF-β3 is provided lyophilized. TGF-β3 is stable at ≤-50°C until the expiration date indicated on the label while lyophilized or at ≤-50°C for three months after reconstitution. Reconstituted TGF-β3 should be aliquoted upon reconstitution. Avoid repeated freeze/thaw cycles of the reconstituted TGF-β3.
7. Ascorbic acid is sterile-filtered. Ascorbic acid is stable at ≤-20°C until the expiration date indicated on the label. Ascorbic Acid should be aliquoted upon thaw. Avoid repeated freeze/thaw cycles of the Ascorbic acid.

NOTE: To keep Trypsin/EDTA fresh and active after thawing, you may aliquot it into sterile centrifuge tubes and re-freeze at –20°C.

Using media or reagents other than what is recommended will void the cell warranty. Please contact Scientific Support if you need help selecting media and/or reagents.

VII. Preparation of culture media

1. Decontaminate external surfaces of all vials, including the medium bottle, with ethanol or isopropanol.
2. To formulate Chondrocyte Growth Medium (CGM™ Medium), transfer the contents of the CGM™ SingleQuots™ Kit (Catalog No. CC-4409 containing R3-Insulin-like Growth Factor-1 [R3-IGF-1], human recombinant Fibroblast Growth Factor-Beta [hrFGF-β], Transferrin, Insulin, Fetal Bovine Serum [FBS], and Gentamicin/Amphotericin-B [GA]) to CBM™ Basal Medium with a pipette, and rinse each vial with medium.
3. When preparing these BulletKit™ Media, it may not be possible to recover the entire volume

listed for each vial. Small losses (up to 10%) should not affect the cell growth characteristics of the supplemented medium.

4. Transfer the label provided with each kit to the basal medium bottle(s) being supplemented (avoid covering the basal medium lot # and expiration date). Use it to record the date and amount of each supplement added. After SingleQuots™ Kit is added to basal medium, store at 2° - 8°C and use within 1 month. Do not freeze medium.

NOTE: If there is concern that sterility was compromised during the supplementation process, the entire newly prepared growth medium may be re-filtered with a 0.2 µm filter to assure sterility. Routine re-filtration is not recommended.

VIII. Thawing of cells / initiation of culture process

NOTE: For proliferation of these cells, cells must be cultured at 37°C±1°C, 5% CO_2, 90%±2% humidity.

1. The recommended seeding density when initially thawing NHAC-Kn from cryopreservation is 10,000 viable cells/cm^2.
2. To set up culture vessels, calculate the number of vessels needed based on the recommended seeding density as well as the surface area of the vessels being used.
3. Add the appropriate amount of medium to the vessels (1 mL/5 cm^2) and allow the vessels to equilibrate in a 37°C±1°C, 5% CO_2, 90%±2% humidity incubator for at least 30 minutes.
4. Wipe cryovial with ethanol or isopropanol before opening. In a sterile field, briefly twist the cap a quarter turn to relieve pressure and then retighten. Quickly thaw the cryovial in a 37°C water bath being careful not to submerge the entire vial. Watch your cryovial closely; when the last sliver of ice melts, remove it. Do not submerge it completely. Thawing the cells for longer than 2 minutes results in less than optimal results.

NOTE: Centrifugation should **not** be performed to remove cells from cryoprotectant cocktail. This action is more damaging than the effects of DMSO residue in the culture

5. Carefully mix the cell suspension using a micropipette. Dispense cells into the culture vessels set up in previous steps. Gently rock the culture vessel to evenly distribute the cells and return to the 37°C±1°C, 5% CO_2, 90%±2% humidity incubator.
6. Change the growth medium the day after seeding.

IX. Maintenance

1. Change the growth medium the day after seeding and every other day thereafter.
2. When cell confluence is 25 - 45%, increase the media volume to 1.5 mL/5 cm^2.
3. When cell confluence is greater than 45%, increase the media volume to 2 mL/5 cm^2.
4. Warm an appropriate amount of medium to 37°C in a sterile container. Remove the medium and replace it with the warmed, fresh medium and return the flask to the incubator.
5. Avoid repeated warming and cooling of the medium. If the entire contents are not needed for a single procedure, transfer and warm only the required volume to a sterile secondary container.

X. Subculturing

NOTE: Lonza warrants its primary cells only if Lonza subculturing reagents are used. The recommended subculturing reagents for these cells are trypsin/EDTA (CC-3232), trypsin neutralizing solution (CC-5002), and HEPES buffered saline solution (CC-5022). These reagents can be purchased individually or together as part of the Chondrocyte ReagentPack™ Subculture Reagents (CC-3233).

The following instructions are for a 25 cm^2 flask. Adjust all volumes accordingly for other size flasks.

1. Subculture the cells when they are 80% - 90% confluent.
2. For each 25 cm^2 of cells to be subcultured:
   a. Thaw 3 mL of Trypsin/EDTA and allow to come to room temperature.
   b. Allow 7 mL of HEPES Buffered Saline Solution (HEPES-BSS) to come to room temperature.
   c. Allow 6 mL of Trypsin Neutralizing Solution (TNS) to come to room temperature.
   d. Remove growth medium from 4°C storage and allow warming to room temperature.
   e. Prepare new culture vessels.
3. Subculture one flask at a time. All flasks following the first flask will be subcultured

following an optimization of this protocol (explained later in this procedure), based on calculated cell count, cell viability, and seeding density.

NOTE: The following steps must be performed in a sterile field.

4. Aspirate the medium from one culture vessel.
5. Rinse the cells with 5 mL of room temperature HEPES-BSS. DO NOT forget this step. The medium contains complex proteins and calcium that neutralize the trypsin.
6. Aspirate the HEPES-BSS from the flask.
7. Cover the cells with 3 mL of Trypsin/EDTA solution.
8. Periodically examine the cell layer microscopically and check for cell detachment.
9. Allow the trypsinization to continue until approximately 90% of the cells are rounded up. This entire process takes about two to six minutes, depending on cell type.
10. At this point, tap the flask against the palm of your hand to release the majority of cells from the culture surface. If only a few cells detach, you may not have let them trypsinize long enough. Wait 30 seconds and tap again. If cells still do not detach, wait and tap every 30 seconds thereafter.
11. After cells are released, neutralize the trypsin in the flask with 6 mL of Trypsin Neutralizing Solution at room temperature. If the majority of cells do not detach within seven minutes, the trypsin is either not warm enough or not active enough to release the cells. Harvest the culture vessel as described above, and either re-trypsinize with fresh, warm Trypsin/EDTA solution or rinse with Trypsin Neutralizing Solution and then add fresh, warm medium to the culture vessel. Return to an incubator until fresh trypsinization reagents are available.
12. Quickly transfer the detached cells to a sterile 15 mL centrifuge tube.
13. Rinse the flask with a final 2 mL of HEPES-BSS to collect residual cells, and add this rinse to the centrifuge tube.
14. Examine the harvested flask under the microscope to make sure the harvest was successful by looking at the number of cells left behind. This should be less than 5%.
15. Centrifuge the harvested cells at 210 x g for five minutes to pellet the cells.
    a. Aspirate most of the supernatant, except for 100-200 µL.
    b. Flick the cryovial with your finger to loosen the pellet.
16. If seeding into flasks for further proliferation or for re-differentiation using the monolayer method, dilute the cells to a final volume of 2 to 3 mL of growth medium and note the total volume of the diluted cell suspension. If preparing cells for re-differentiation using the alginate bead method, proceed to Step 2 of Section XVI (Re-Differentiation [Alginate Bead Method], Page 9).
17. Determine cell count and viability using a hemacytometer and Trypan Blue or cell counter. Make a note of your cell yield for later use.
18. If necessary, dilute the suspension with growth medium to achieve the desired “cells/mL” and re-count the cells.
19. Use the following equation to determine the total number of viable cells.

$$\text{Total \# of Viable Cells} = \frac{\text{Total cell count} \times \text{percent viability}}{100}$$

20. The number of flasks needed depends upon cell yield and seeding density. If seeding into flasks for further proliferation or for re-differentiation using the monolayer method, the recommended density is 10,000 cells/cm^2. Determine the total number of flasks to inoculate by using the following equation.

$$\text{Total \# of Flasks to innoculate} = \frac{\text{Total \# of viable cells}}{\text{Growth area} \times \text{Rec. Seeding Density}}$$

21. Use the following equation to calculate the volume of cell suspension to seed into your flasks.

$$\text{Seeding Volume} = \frac{\text{Total volume of diluted cell suspension}}{\text{\# of flasks as determined in step 18}}$$

22. Prepare flasks by labeling each flask with the passage number, cell type, and date.
23. Carefully transfer growth medium to new culture vessels by adding 1 mL growth medium for every 5 cm^2 surface area of the flask (1 mL/5 cm^2).
24. After mixing the diluted cells with a 5 mL pipet to ensure a uniform suspension, dispense the

calculated volume into the prepared subculture flasks.

25. If not using vented caps, loosen caps of flasks. Place the new culture vessels into a 37°C±1°C, 5% CO_2, 90%±2% humidity incubator.

XI. Cryopreservation

NOTE: Cryopreservation may compromise cell quality and performance. **Lonza CANNOT guarantee performance of primary cells that have been cryopreserved outside of Lonza.** To avoid loss of cells and forfeiture of your warranty, we recommend keeping cells in continuous culture without cryopreservation.

Cryopreservation media

Description	Base media	DMSO	FBS
NHAC-Kn	80% CGM™ without FBS	10% DMSO	10% FBS

1. Prepare cryopreservation media according to the chart listed above and chill to 4°C.
2. Prepare freezing vials or ampoules by labeling each with the passage number, cell type and date.
3. Sterile filter cryopreservation media using a 0.2 micron filter
4. Harvest and centrifuge cells according to Steps 1 to 15 of Section X (Subculturing, Page 4).
5. Resuspend cells in cold cryopreservation media at 500,000 to 2,000,000 cells per mL.

NOTE: Work Quickly! Once exposed to the DMSO, cells become very fragile.

6. Pipet aliquots (1 mL each) into freezing vials or ampoules and seal.
7. Insulate aliquots with Styrofoam or propanol freezing canister.
8. Store cells at -80°C overnight.
9. Within 12 to 24 hours, place cells in liquid nitrogen (-200°C) for long-term storage. Cells will be compromised by storage in -80°C.

XII. Re-differentiation

Chondrocytes are differentiated cells when isolated, however, they lose their differentiated phenotype (de-differentiate) after serial expansion in a proliferation medium in monolayer culture and become fibroblast-like, losing their ability to produce cartilage specific markers such as type II collagen and aggrecan. By the 2nd or 3rd passage, the cells are almost completely de-differentiated. Chondrocytes can be expanded in culture and then induced to re-differentiate in a variety of formats including monolayer culture in a differentiation medium or 3D culture formats such as alginate bead suspension culture. The level of re-differentiation of the culture decreases with increasing numbers of passages in a proliferation medium in monolayer culture.

XIII. Preparation of re-differentiation medium (monolayer method)

Differentiation medium components

(sold separately)

- Lyophilized Transforming Growth Factor Beta 3 (TGF-β3) – 2 µg (Lonza Catalog No. PT-4124, or similar)
- 2N Hydrochloric Acid (Sigma Catalog No. 653799, or similar)
- 7.5% Bovine Albumin Fraction (Life Technologies Catalog No. 15260-037, or similar)
- 70 mM Ascorbic Acid – 0.5 mL (Lonza Catalog No. CC-4398, or similar)
- One Chondrocyte Differentiation Medium BulletKit™ Medium - 250 mL

  CDM™ BulletKit™ (Lonza Catalog No. CC-3225) contains 250 mL of Chondrocyte Differentiation Basal Medium (CDM™ Basal Medium) and the following growth supplements: R3-Insulin-like Growth Factor-1 (R3-IGF-1), 0.5 mL; Transforming Growth Factor Beta 1 (TGF-β1), 1.25 mL; Transferrin, 0.5 mL; Insulin, 0.5 mL; Fetal Bovine Serum (FBS), 12.5 mL; Gentamicin/Amphotericin-B (GA), 0.25 mL

1. Decontaminate external surfaces of all vials and the medium bottle with ethanol or isopropanol.
2. To formulate Chondrocyte Differentiation Medium (CDM™ Medium), transfer the contents of the CDM™ SingleQuots™ Kit (Catalog No. CC-4408, containing R3-Insulin-like Growth Factor-1 [R3-IGF-1], Transforming Growth Factor Beta 1 [TGF-β1], Transferrin, Insulin, Fetal Bovine Serum [FBS], and Gentamicin/Amphotericin-B [GA]) to CDM™ Basal Medium with a pipette, and rinse each vial with medium.

3. When preparing these media, it may not be possible to recover the entire volume listed for each vial. Small losses (up to 10%) should not affect the cell growth/differentiation characteristics of the supplemented medium.
4. Thaw vial of 70 mM Ascorbic Acid and add 250 µL of 70 mM Ascorbic Acid to 250 mL bottle of Chondrocyte Differentiation Medium (CDM™ Medium) for a final Ascorbic Acid concentration of 70 µM. Re-freeze remaining 70 mM Ascorbic Acid and store at ≤-20°C.

NOTE: Ascorbic acid is necessary for maturation and deposition of all types of collagen. Analysis of the cells by immunocytochemistry or collagen type II requires the addition of ascorbic acid to the complete differentiation medium for a minimum period of 24 to 72 hours. Ascorbic acid may be required in the differentiation medium for other methods especially those involving the maturation and deposition of collagen(s). Ascorbic acid may be added at the recommended concentration of 70 µM, it may be titrated to determine the optimal level for each experimental condition, or it may be omitted entirely depending on experimental application.

5. Transfer the label provided with each kit to the basal medium bottle(s) being supplemented (avoid covering the basal medium lot # and expiration date). Use it to record the date and amount of each supplement added. After SingleQuots™ Kit and Ascorbic Acid are added to basal medium, store at 2° - 8°C and use within 1 month. Do not freeze medium.

NOTE: If there is concern that sterility was compromised during the supplementation process, the entire newly prepared growth medium may be re-filtered with a 0.2 µm filter to assure sterility. Routine re-filtration is not recommended.

6. Prepare a 20 µg/mL TGF-β3 Stock Solution by adding 2 µL of 2N Hydrochloric Acid, 13 µL of 7.5% Bovine Albumin Fraction and 85 µL of cell culture water to the vial containing 2 µg of lyophilized Transforming Growth Factor Beta 3 (TGF-β3). Gently pipet the solution to mix. Aliquot 20 µg/mL TGF-β3 Stock Solution in 4 µL aliquots and store at ≤-50°C for up to three months.
7. Immediately prior to plating cells for re-differentiation or changing medium during re-differentiation process, prepare Complete Chondrocyte Differentiation Medium (CDM™ Medium) by adding aliquots of TGF-β3 to aliquots of Chondrocyte Differentiation Medium containing Ascorbic Acid at a final TGF-β3 concentration of 10 ng/mL. **Do not complete the differentiation medium with TGF-β3 until ready to start the re-differentiation and feed cultures (the TGF-β3 must be used fresh).**
    a. The first batch of complete differentiation medium will be prepared day 0 of the re-differentiation process. Add fresh TGF-β3 for each subsequent media change.
    b. Thaw and add 4 µL aliquot of 20 µg/mL TGF-β3 Stock Solution to 8 mL of the differentiation medium containing ascorbic acid in a clean 15 mL polypropylene tube for a final TGF-β3 concentration of 10 ng/mL. Rinse TGF-β3 vial with complete differentiation medium. Use complete differentiation medium immediately.

XIV. Re-differentiation (monolayer method)

NOTE: All lots of Lonza's NHAC-Kn are currently tested for re-differentiation using the monolayer method. As such, the monolayer method is the preferred and guaranteed method for NHAC-Kn re-differentiation.

Differentiation components (sold separately)

- One normal human chondrocyte cell product – (cryopreserved or proliferating)
- One Chondrocyte Growth Medium BulletKit™ Medium – 500 (prepared as described in Section VII [Preparation of Culture Medium, Page 3)
- One Chondrocyte Differentiation Medium containing Ascorbic Acid - 250 mL (prepared as described in Section XIII [Preparation of Re-Differentiation Medium (Monolayer Method)], Page 6, Steps 1-5)
- 20 µg/mL TGF-β3 Stock Solution Aliquots (prepared as described in Section XIII [Preparation of Re-Differentiation Medium (Monolayer Method)], Page 7, Step 6)

1. The recommended seeding density when plating NHAC-Kn for re-differentiation is 10,000 cells/cm^2.
2. To set up culture vessels, calculate the number of vessels needed based on the recommended seeding density and the surface area of the

vessels being used. The suggested culture vessel for re-differentiation of NHAC-Kn is a 24-well plate, however, alternative culture vessels may be used.

3. Add the appropriate amount of growth medium (CGM™ Medium) to the vessels (1 mL/4 cm^2) and allow the vessels to equilibrate in a 37°C±1°C, 5% CO_2, 90%±2% humidity incubator for at least 30 minutes
4. Subculture cells according to Section X (Subculturing, Page 4).
5. Culture cells under standard culturing conditions in a 37°C±1°C, 5% CO_2, 90%±2% humidity incubator in growth medium (CGM™ Medium) for 18 - 24 hours.
6. After 18 - 24 hours. warm an appropriate amount of Chondrocyte Differentiation Medium containing Ascorbic Acid to 37°C and add TGF-β3 to create Complete Chondrocyte Differentiation Medium (prepared as described in Section XII [Preparation of Re-Differentiation Medium (Monolayer Method)], Page 7, Step 7). Remove the medium and replace it with of the warmed, fresh Complete Chondrocyte Differentiation Medium at 1 mL/4 cm^2 and return the vessel to the incubator.
7. Change the differentiation medium every 3-4 days with fresh Complete Chondrocyte Differentiation Medium containing freshly added aliquot of TGF-β3. When changing medium, use caution as to not dislodge the monolayer of cells or the loosely attached cell aggregates which are often observed at later stages of re-differentiation.
8. Re-differentiation of the culture can be analyzed on Days 7-21 or later depending on the desired biomarkers or lot. Standard analysis of re-differentiated chondrocytes includes staining with Alcian blue for sulfated proteoglycans or staining for Collagen Type II production.

XV. Preparation of re-differentiation medium (alginate bead method)

Differentiation medium components
(sold separately)

- Sodium Chloride (NaCl) (Sigma Catalog No. S5886, or similar)
- Calcium Chloride Dihydrate ($CaCl_2$-$2H_2O$) (Sigma Catalog No. C7902, or similar)
- Alginic Acid Sodium Salt (Sigma Catalog No. A1112, or similar)
- Sodium Citrate Dihydrate (Sigma Catalog No. W302600, or similar)
- 70 mM Ascorbic Acid – 0.5 mL (Lonza Catalog No. CC-4398, or similar)
- One Chondrocyte Differentiation Medium BulletKit™ Medium - 250 mL

  CDM™ BulletKit™ (Lonza Catalog No. CC-3225) contains 250 mL of Chondrocyte Differentiation Basal Medium (CDM™ Basal Medium) and the following growth supplements: R3-Insulin-like Growth Factor-1 (R3-IGF-1), 0.5 mL; Transforming Growth Factor Beta 1 (TGF-β1), 1.25 mL; Transferrin, 0.5 mL; Insulin, 0.5 mL; Fetal Bovine Serum (FBS), 12.5 mL; Gentamicin/Amphotericin-B (GA), 0.25 mL

1. Decontaminate external surfaces of all vials and the medium bottle with ethanol or isopropanol.
2. To formulate Chondrocyte Differentiation Medium (CDM™ Medium), transfer the contents of the CDM™ SingleQuots™ Kit (Catalog No. CC-4408, containing R3-Insulin-like Growth Factor-1 [R3-IGF-1], Transforming Growth Factor Beta 1 [TGF-β1], Transferrin, Insulin, Fetal Bovine Serum [FBS], and Gentamicin/Amphotericin-B [GA]) to CDM™ Basal Medium with a pipette, and rinse each vial with medium.
3. When preparing these media, it may not be possible to recover the entire volume listed for each vial. Small losses (up to 10%) should not affect the cell growth/differentiation characteristics of the supplemented medium.
4. Thaw vial of 70 mM Ascorbic Acid and add 250 µL of 70 mM Ascorbic Acid to 250 mL bottle of Chondrocyte Differentiation Medium (CDM™ Medium) for a final Ascorbic Acid concentration of 70 µM. Re-freeze remaining 70 mM Ascorbic Acid and store at ≤-20°C.

NOTE: Ascorbic acid is necessary for maturation and deposition of all types of collagen. Analysis of the cells by immunocytochemistry or collagen type II requires the addition of ascorbic acid to the complete differentiation medium for a minimum period of 24 to 72 hours. Ascorbic acid may be required in the differentiation medium for other methods especially those involving the maturation and deposition of collagen(s). Ascorbic acid may be added at the recommended concentration of 70 µM, it may be titrated to determine the optimal level for each experimental condition, or it may be omitted entirely depending on experimental application.

5. Transfer the label provided with each kit to the basal medium bottle(s) being supplemented (avoid covering the basal medium lot # and expiration date). Use it to record the date and amount of each supplement added. After SingleQuots™ Kit and Ascorbic Acid are added to basal medium, store at 2°-8°C and use within 1 month. Do not freeze medium.

NOTE: If there is concern that sterility was compromised during the supplementation process, the entire newly prepared growth medium may be re-filtered with a 0.2 µm filter to assure sterility. Routine re-filtration is not recommended.

6. Prepare a 155 mM Sodium Chloride (NaCl) Solution by adding 906 mg of Sodium Chloride (NaCl) into a sterile container. Fill the container with cell culture water to a final volume of 100 mL. Swirl solution until Sodium Chloride (NaCl) is completely dissolved. Filter solution through a 0.2 µm filter into a new, labeled, sterile container and store at room temperature (15°C - 30°C) for up to twelve months.
7. Prepare a 102 mM Calcium Chloride Dihydrate ($CaCl_2$-$2H_2O$) Solution by adding 1,499 mg of Calcium Chloride Dihydrate ($CaCl_2$-$2H_2O$) into a sterile container. Fill the container with cell culture water to a final volume of 100 mL. Swirl solution until Calcium Chloride Dihydrate ($CaCl_2$-$2H_2O$) is completely dissolved. Filter solution through a 0.2 µm filter into a new, labeled, sterile container and store at room temperature (15°C-30°C) for up to twelve months.
8. Prepare a 1.2% Sodium Alginate Solution by adding 906 mg of Sodium Chloride (NaCl into a sterile beaker. Fill the beaker with cell culture water to a final volume of 100 mL. Place the solution onto a magnetic stir plate with a heating element and mix solution for at least five minutes. Turn on heating element and continue mixing until solution is warm to the touch. Add 1,200 mg of Alginic Acid Sodium Salt and continue to mix with heat until Alginic Acid Sodium Salt is completely dissolved (this may take 3-4 hours). Filter solution through a 0.45 µm filter into a new, labeled, sterile container and store at 2° - 8°C for up to nine months.
9. Prepare a 55 mM Sodium Citrate Solution by adding 1618 mg of Sodium Citrate Dihydrate and 526 mg of Sodium Chloride (NaCl) into a sterile container. Fill the container with cell culture water to a final volume of 100 mL. Swirl solution until Sodium Citrate Dihydrate and Sodium Chloride (NaCl) are completely dissolved. Filter solution through a 0.2 µm filter into a new, labeled, sterile container and store at 2° - 8°C for up to twelve months.

XVI. Re-differentiation (alginate bead method)

NOTE: All lots of Lonza's NHAC-Kn are currently tested for re-differentiation using the monolayer method. As such, the monolayer method is the preferred and guaranteed method for NHAC-Kn re-differentiation. While the alginate bead method has been used historically by Lonza for re-differentiation of NHAC-Kn and the provided protocol has been validated, Lonza does not currently use the alginate bead method for re-differentiation of NHAC-Kn cells.

Differentiation components (sold separately)

- One normal human chondrocyte cell product – (cryopreserved or proliferating)
- One Chondrocyte Differentiation Medium containing Ascorbic Acid - 250 mL (prepared as described in Section XV [Preparation of Re-Differentiation Medium (Alginate Bead Method)], Page 8, Steps 1-5)
- 155 mM Sodium Chloride (NaCl) Solution (prepared as described in Section XV [Preparation of Re-Differentiation Medium (Alginate Bead Method)], Page 9, Step 6)
- 102 mM Calcium Chloride Dihydrate ($CaCl_2$-$2H_2O$) Solution (prepared as described in Section XV [Preparation of Re-Differentiation Medium (Alginate Bead Method)], Page 9, Step 7)
- 1.2% Sodium Alginate Solution (prepared as described in Section XV [Preparation of Re-Differentiation Medium (Alginate Bead Method)], Page 9, Step 8)
- 55 mM Sodium Citrate Solution (prepared as described in Section XV [Preparation of Re-Differentiation Medium (Alginate Bead Method)], Page 9, Step 9)

1. Subculture cells according to Section X (Subculturing, Page 4, Steps 1-15).
2. Dilute the cells to a final volume of 8 to 10 mL of 155 mM Sodium Chloride (NaCl) Solution and note the total volume of the diluted cell suspension.

3. Determine cell count and viability using a hemacytometer and Trypan Blue or cell counter. Make a note of your cell yield.
4. Centrifuge the cells re-suspended in 155 mM Sodium Chloride (NaCl) Solution at 210 x g for five minutes to pellet the cells.
    a. Aspirate most of the supernatant, except for 100-200 µL
    a. Flick the cryovial with your finger to loosen the pellet
5. Dilute the cells in 1.2% Sodium Alginate Solution to a final density of 350,000 to 400,000 viable cells/mL.
6. Suck up the alginate suspension into a 20 mL syringe and then attach a 22 gauge needle to the syringe.
7. Holding the syringe so that the needle is approximately at a 45 degree angle to a bottle containing 4 to 5 volumes of 102 mM Calcium Chloride Dihydrate ($CaCl_2$-$2H_2O$) Solution, express the cell suspension in drop-wise fashion into the 102 mM Calcium Chloride Dihydrate ($CaCl_2$-$2H_2O$) Solution while gently shaking bottle with the other hand or use a plate shaker.
8. After all of the suspension has been expressed, let set for 10 minutes, shaking the bottle gently every 2-3 minutes.
9. Carefully aspirate most of the 102 mM Calcium Chloride Dihydrate ($CaCl_2$-$2H_2O$) Solution without disrupting the beads. Pour the remaining mixture (with the beads) into a sterile 50 mL conical tube. When beads have settled, aspirate most of the remaining supernatant, except for 100-200 µL being careful not to disrupt beads.
10. Wash the beads in 155 mM Sodium Chloride (NaCl) Solution by adding 10 mL of 155 mM Sodium Chloride (NaCl) Solution to the beads, allowing the beads to settle, and aspirate most of the remaining supernatant except for 100-200 µL being careful not to disrupt beads.
11. Repeat Step 10 (washing the beads with 155 mM Sodium Chloride (NaCl) Solution) 3-4 times.
12. After removing 155 mM Sodium Chloride (NaCl) Solution in final wash, wash the beads in Chondrocyte Differentiation Medium containing Ascorbic Acid by adding 10 mL of Chondrocyte Differentiation Medium containing Ascorbic Acid to the beads, allowing the beads to settled, and aspirate most of the remaining supernatant except for 100-200 µL, being careful not to disrupt beads.
13. After removing Chondrocyte Differentiation Medium containing Ascorbic Acid in differentiation medium wash, add 1 to 2 volumes of Chondrocyte Differentiation Medium containing Ascorbic Acid to the beads.
14. Place beads in appropriate vessel, such as a culture dish or a multi-well culture plate, in approximately 1 mL of Chondrocyte Differentiation Medium containing Ascorbic Acid per mL of the original cell/alginate suspension. Culture cells in a 37°C±1°C, 5% CO_2, 90%±2% humidity incubator for 22-28 days.
15. Change the differentiation medium every other day. When changing medium, replace medium with an equal volume of fresh, pre-warmed Chondrocyte Differentiation Medium containing Ascorbic Acid using a 5 mL pipet or an 100 µL pipette tip (do not use an aspirator).
16. At the end of incubation, remove the medium and depolymerise the beads by adding 3 volumes of 55 mM Sodium Citrate Solution. Let stand at room temperature for 20-30 minutes until the beads have completely dissolved. Pellet cells at 500 x g for 10 minutes. Wash cells twice with 155 mM Sodium Chloride (NaCl) Solution.
17. Re-differentiation of the culture can be analyzed on Days 22-28 or later depending on the desired biomarkers or lot. Standard analyzation of re-differentiated chondrocytes includes staining with Alcian blue for sulfated proteoglycans or staining for Collagen Type II production.

XVII. Ordering information

Cryopreserved chondrocyte cells:

Cat. no.	Product	Description
CC-2550	NHAC-Kn	≥750,000 viable cells

Proliferating cultures are also available in a variety of culture vessels including flasks and well plates. For more information regarding proliferating cultures, including for catalog numbers, please contact Lonza Scientific Support.

Chondrocyte growth media (sold separately):

Cat. no.	Product	Description
CC-3216	CGM™ BulletKit™ Medium	500 mL CBM™ Basal Medium plus CC-4409 SingleQuots™ Kit to formulate CGM™ Medium (growth medium)
CC-3217	CBM™ Basal Medium	Chondrocyte basal medium (500 mL)
CC-4409	CGM™ SingleQuots™ Kit	Formulates 500 mL of CGM™ Basal Medium to CGM™ Growth Medium; contains R3-IGF-1, 1.0 mL; hrFGF-β, 2.5 mL; Transferrin, 0.5 mL; Insulin, 1.0 mL; FBS, 25.0 mL; GA, 0.5 mL

Chondrocyte differentiation media & reagents (sold separately):

CC-3225	CDM™ BulletKit™ Medium	250 mL CDM™ Basal Medium plus CC-4408 SingleQuots™ Kit to formulate CDM™ Medium (differentiation medium)
CC-3226	CDM™ Basal Medium	Chondrocyte differentiation basal medium (250 mL)
CC-4408	CDM™ SingleQuots™ Kit	Formulates 250 mL of CDM™ Basal Medium to CDM™ Differentiation Medium; contains R3-IGF-1, 0.5 mL; TGF-β1, 1.25 mL; Transferrin, 0.5 mL; Insulin, 0.5 mL; FBS, 12.5 mL; GA, 0.25 mL
CC-4398	Ascorbic Acid	70 mM Ascorbic Acid (0.5 mL)
PT-4124	TGF-β3	Lyophilized Transforming Growth Factor Beta 3 (TGF-β3) (2 µg)

Additional components are required for re-differentiation using the alginate bead method. Please see Section XV (preparation of re-differentiation medium [alginate bead method], page 8) for a complete listing of required components.

Subculturing reagents (sold separately):

Cat. no.	Product	Description
CC-3233	Chondrocyte ReagentPack™	Provides necessary components for subculture of NHAC-Kn; contains Trypsin/EDTA Solution, 100 mL; Trypsin Neutralizing Solution (TNS), 100 mL; HEPES Buffered Saline Solution, 100 mL

XVIII. Product Warranty

Cultures have a finite lifespan *in vitro.*

Lonza guarantees the performance of its cells in the following manner only if Lonza's Media and Reagents are used exclusively and the recommend protocols are followed. The performance of cells is not guaranteed if any modifications are made to the complete cell system.

1. NHAC-Kn Cryopreserved Cultures are assured for experimental use for **fifteen** population doublings.
2. NHAC-Kn Proliferating Cultures are assured for experimental use for **ten** population doublings.
3. Additional population doublings and subcultures are possible, but growth rate, biological responsiveness and function deteriorate with subsequent passage.
4. NHAC-Kn can become irreversibly contact-inhibited if allowed to reach confluence. To avoid the loss of your cells and forfeiture of your warranty, subculture cells before they reach 90% confluence.

When placing an order or for Scientific Support, please refer to the product numbers and descriptions listed above. For a complete listing of all Lonza's Cell Culture Products, refer to the Lonza website or the current Lonza catalog. To obtain a catalog, additional information or want to speak with Scientific Support, you may contact Lonza by web, e-mail, telephone, fax or mail (See Page 1 for details).

THESE PRODUCTS ARE FOR RESEARCH USE ONLY. Not approved for human or veterinary use, for application to humans or animals, or for use in clinical or *in vitro* diagnostic procedures.

WARNING: LONZA'S PRIMARY CELLS CONTAIN HUMAN SOURCE MATERIAL, TREAT AS POTENTIALLY INFECTIOUS. Each donor is tested and found non-reactive by an FDA-approved method for the presence of HIV-I, hepatitis B virus and hepatitis C virus. Where donor testing is not possible, cell products are tested for the presence of viral nucleic acid from HIV, hepatitis B virus, and hepatitis C virus. Testing cannot offer complete assurance that HIV-1, hepatitis B virus, and hepatitis C virus are absent. All human-sourced products should be handled at the biological safety level 2 to minimize exposure of potentially infectious products, as recommended in the CDC-NIH manual, Biosafety in Microbiological and Biomedical Laboratories, 5thed. If you require further information, please contact your site safety officer or Scientific Support.