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KnockOut™ SR XenoFree CTS™

Description

KnockOut™ SR XenoFree CTS™ enables the growth and expansion of human embryonic stem cells (hESC) in a cell culture medium containing only human-derived or human recombinant proteins, to facilitate the transition of hESC research from the bench to the clinic. KnockOut™ SR XenoFree CTS™ does not contain bovine or other non-human, animal-derived components. Besides hESC culture expansion and maintenance, KnockOut™ SR XenoFree CTS™ can be used for hESC cryopreservation, hESC and induced pluripotent stem cell (iPSC) derivation, and hESC differentiation studies. Each container is sterile filtered.

Product	Catalog No.*	Part No.*	Amount	Storage	Shelf Life**
KnockOut™ SR XenoFree CTS™	A10992-01 A10992-02	12618-012 12618-013	100 mL 500 mL	–20°C to –5°C; Protect from light	18 months

* Catalog nos. include KnockOut™ SR XenoFree CTS™and this instruction booklet. Part nos. cannot be ordered separately.
** Shelf Life duration is determined from Date of Manufacture.

Intended Use

For human *ex-vivo* tissue and cell culture processing applications. CAUTION: When used as a medical device, Federal Law restricts this device to sale by or on the order of a physician.

Important Information

To thaw KnockOut™ SR XenoFree CTS™, place at 2°C to 8°C overnight. Alternatively, KnockOut™ SR XenoFree CTS™ can be thawed in a 37°C water bath with frequent gentle swirling to expedite thawing. **Do not heat-inactivate.**

- Occasionally flocculent material may be observed while thawing. This material will go into solution with gentle swirling at 37°C. Minimize dwell time in waterbath.
- KnockOut™ SR XenoFree CTS™ is stable for up to 4 weeks at 2°C to 8°C protected from light.
- Working volumes can be aliquoted and stored at –20°C to –5°C. Thaw aliquots as needed. Avoid additional freeze-thaw cycles.

Refer to **www.lifetechnologies.com/stemcells** for detailed protocols and new applications using KnockOut™ products.

KnockOut™ SR XenoFree CTS™ **cannot** be used as a replacement for FBS in the plating of feeder cells.

KnockOut™ SR XenoFree CTS™ does not contain trypsin inhibitors. Therefore, trypsin must be removed or inactivated when culturing ESCs in KnockOut™ SR XenoFree CTS™-containing medium.

Safety Information

Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Human origin materials are non-reactive (donor level) for anti-HIV 1 & 2, anti-HCV, and HB_sAg. Handle in accordance with established bio-safety practices.

Culture Conditions

Media: KnockOut™ SR XenoFree CTS™ Complete Medium

Cell Type: hESC or iPSC

Culture Type: Adherent

Recommended Culture Vessels: T-Flasks

Temperature Range: 36°C to 38°C

Incubator Atmosphere: Humidified atmosphere of 4–6% CO_2 in air. For best results, pre-equilibrate complete medium to temperature (37°C) and gases (5% CO_2 in humidified air) before use. Ensure proper gas exchange and avoid overexposure of cultures to light.

Prepare Media

Prepare complete media for human ESCs and iPSCs as outlined in table 1. Complete Medium is stable for at least 10 days when stored in the dark at 2°C to 8°C. Avoid repeated warming and chilling of the complete medium. Warm only the volume required for that day's use.

- Reconstitute basic Fibroblast Growth Factor (bFGF) to a stock concentration of 10 µg/mL in Dulbecco's Phosphate Buffered Saline (DPBS).

Table 1 Media for human ESCs/iPSCs

Reagents	Stock Conc.	Catalog No.	Final Conc.	For 100 mL
KnockOut™ DMEM CTS™	—	A12861	1X	82.75 mL
KnockOut™ SR XenoFree CTS™		12618	15%	15 mL
GlutaMAX™-I CTS™	200 mM	A12860	2 mM	1 mL
NEAA*	10 mM	11140	0.1 mM	1 mL
bFGF	10 µg/mL	13256	8 ng/mL	80 µL
2-Mercaptoethanol**	55 mM	21985	0.1 mM	182 µL

* Non-Essential Amino Acids
** It is recommended to add fresh 2-Mercaptoethanol to KnockOut™ SR XenoFree CTS™ Complete Medium (only to the volume required for that day's use) immediately prior to use.

Wash Medium

Dilute 2.5 mL KnockOut™ SR XenoFree CTS™ in 97.5 mL KnockOut™ DMEM CTS™. Wash medium is stable at 2°C to 8°C for up to 2 weeks.

Use

Cultures may be grown in KnockOut™ SR XenoFree CTS™ Complete Medium using either feeder cells or feeder-free conditions. For XenoFree culture using human foreskin fibroblast (HFF) feeder cells, tissue-culture treated vessels can be coated with CELLstart™ CTS™ humanized substrate prior to plating HFF in complete medium. Once HFF feeder cells have attached and spread (generally 8 hours to overnight), hESCs can be plated on the HFF vessels as desired (see Figure 1). Research indicates that KnockOut™ SR XenoFree CTS™ and CELLstart™ CTS™ will also support feeder-free growth of hESCs when supplemented with bFGF and additional growth factors[1]. For more information contact our Technical Support Team at **celltherapyresearchsupport@lifetech.com**.

Recovery of Cryopreserved hESCs Using KnockOut™ SR XenoFree CTS™

1. Rapidly thaw (<1 minute) frozen vial of cells in a 37°C water bath, until a small frozen piece remains in the vial.
2. Decontaminate vial with 70% isopropyl alcohol.
3. Aseptically transfer the entire contents of the vial into a 15-mL conical tube.
4. Dropwise, add 3 mL pre-warmed KnockOut™ SR XenoFree CTS™ Complete Medium to the conical tube containing thawed hESC.
5. Rinse the vial with 1–2 mL fresh, pre-warmed KnockOut™ SR XenoFree CTS™ Complete Medium and add to the same conical tube.
6. Pellet cells by centrifuging at 200 × *g* at room temperature for 2 minutes. Aspirate and discard the supernatant without disturbing the cell pellet.
7. Gently "flick" the tube to fully dislodge the cell pellet from the tube bottom.
8. Add the desired volume of pre-equilibrated KnockOut™ SR XenoFree CTS™ Complete Medium to the hESC pellet. Do ***not*** triturate cells.
9. Gently invert the conical tube containing hESCs to mix cells. Using a pipet, transfer the cells to a prepared feeder-containing or CELLstart™ CTS-coated cell culture vessel (see **CELLstart™ CTS™ Coating of Culture Vessels**).
10. Place vessel in a 37°C incubator with a humidified atmosphere of 5% CO_2 in air. Carefully swirl vessel in a back and forth and then a left and right pattern to evenly distribute hESC.
11. Exchange spent media with fresh KnockOut™ SR XenoFree CTS™ Complete Medium 24 hours post-thaw and daily thereafter, until approximately 70–80% confluent.

Tips for hESC Adaptation to KnockOut™ SR XenoFree CTS™

Different hESC lines will behave differently in KnockOut™ SR XenoFree CTS™ Medium and optimal growth conditions must be determined for each application.

- Starter cultures should be of high quality, be 70–80% confluent, and contain no differentiated hESCs.
- **Feeder cultures***:* The best adaptation results will be obtained when the parent hESC culture has been maintained in traditional KnockOut™ SR on either murine embryonic fibroblast (MEF) or HFF feeder cells prior to adapting to KnockOut™ SR XenoFree CTS™.
- **Feeder-free cultures:** The best adaptation results will be obtained when the parent hESC culture has been maintained in traditional MEF-conditioned medium (MEF-CM) prior to adapting to KnockOut™ SR XenoFree CTS™.
- Make a frozen bank of cells in control medium prior to adaptation.
- Maintain a "backup" culture in control medium throughout hESC adaptation to KnockOut™ SR XenoFree CTS™.

"Direct" adaptation: If hESCs are passaged directly into KnockOut™ SR XenoFree CTS™ Complete Medium, a 1:2 split ratio is suggested for the first 3 passages.

- To increase the chances of successful adaptation, seed one plate directly in KnockOut™ SR XenoFree CTS™ Complete Medium and two in MEF-CM control medium. Fluid-change the KnockOut™ SR XenoFree CTS™ plate and one control plate with KnockOut™ SR XenoFree CTS™ ™ Complete Medium that day and daily thereafter. At the second passage, seed both plates directly in KnockOut™ SR XenoFree CTS™ Complete Medium at 1:2.

"Sequential" adaptation: Best results may be obtained by gradually adapting hESCs to KnockOut™ SR XenoFree CTS™.

- Passage 1: 75% control medium + 25% KnockOut™ SR XenoFree CTS™ Complete Medium.
- Passage 2: 50% control medium + 50% KnockOut™ SR XenoFree CTS™ Complete Medium.
- Passage 3: 25% control medium + 75% KnockOut™ SR XenoFree CTS™ Complete Medium.
- Passage 4 and thereafter: 100% KnockOut™ SR XenoFree CTS™ Complete Medium.
- If the hESC line is difficult to adapt, a further level of caution can be taken by maintaining a culture in each prior passage medium while starting the next level of adaptation. For example, when passaging the 25/75 control medium/ KnockOut™ SR XenoFree CTS™ culture (Passage 3, above), hESCs can be passaged into both 100% KnockOut™ SR XenoFree CTS™ medium AND 25/75 medium. If the 100% culture does poorly, adaptation can be resumed using the backup 25/75 culture.

Timing of passage is critical. For best results, hESCs should be nearing confluence (70–80%) at the time of passage. If hESCs are passaged at low confluency or when overgrown, hESCs will differentiate.

Seeding density at passage is also critical. If seeded too low, hESCs will differentiate. hESC cultures must be fluid-changed daily for optimal performance.

CELLstart™ CTS™ Coating of Culture Vessels

Detailed CELLstart™ CTS™ information and protocols can be found at **www.lifetechnologies.com/cellstartCTS**.

1. Dilute CELLstart™ CTS™ 1:50 in Dulbecco's Phosphate Buffered Saline CTS™ (DPBS CTS™) with calcium and magnesium. Pipet gently to mix. **DO NOT VORTEX**.
2. Add diluted CELLstart™ CTS™ to culture plates at a final volume per surface area of 78 µL/cm^2. Refer to table for respective culture container:

Culture Vessel	Surface Area (cm^2)	Volume of Diluted CELLstart™
60-mm plate	28.25	2.25 mL per plate
6-well plate	9.6	750 µL per well
12-well plate	3.2	250 µL per well
24-well plate	2.0	160 µL per well

3. Incubate at 37°C in a humidified atmosphere containing 5% CO_2 in air, for 1–2 hours.
4. After incubation, remove coated vessels from the incubator. For *immediate* use, place vessels at room temperature. For use the next day, carefully wrap vessels containing diluted CELLstart™ CTS with Parafilm® laboratory film, and store at 2°C to 8°C.

Plating of Feeder Cells

1. Immediately before use, remove all CELLstart™ CTS™ diluent from the vessel. It is not necessary to rinse vessels following removal of CELLstart™ CTS™.
2. If growing cells in a feeder-free system proceed with plating hESCs on the CELLstart-coated vessel at the desired density (see **Passaging hESCs Using KnockOut™ SR XenoFree CTS™**).
3. If growing hESCs on feeder cells, harvest inactive fibroblast feeder cells (e.g., HFF) as normal, resuspending feeders in KnockOut™ SR XenoFree Complete Medium.
   Note: Addition of bFGF is **not** essential for plating feeders.
4. Plate inactive feeders on CELLstart™ CTS™-coated vessels at the desired density.
5. Place feeder-seeded vessels in a 37°C incubator, with a humidified atmosphere of 5% CO_2 in air. Carefully swirl vessel in a back and forth and then a left and right pattern to evenly distribute fibroblast feeder cells.
6. Incubate overnight to enable feeder attachment and spreading. Vessels are now ready to receive hESCs and can be used for up to one week.

Passaging hESCs Using KnockOut™ SR XenoFree CTS™

KnockOut™ SR XenoFree CTS™ can be used in place of KnockOut™SR for the maintenance of hESCs in feeder-containing or feeder-free culture systems. To maintain a xeno-free culture system, TrypLE™ Select CTS™ is recommended for cell dissociation.

1. Observe stock culture vessel (hESCs growing in current medium formulation or in KnockOut™ SR XenoFree CTS™ Complete Medium) under the microscope and confirm that the cells are ready to be subcultured (70–80% confluent).
2. Cut out and remove any differentiated hESC colonies prior to passaging the culture. A 22 gauge 1½″ needle attached to a syringe works well for removing differentiated hESCs.
3. Pre-warm the required volumes of TrypLE™ Select CTS™ and Wash Medium to 37°C, and pre-equilibrate the required volume of KnockOut™ SR XenoFree CTS™ Complete Medium to temperature and gases before use. Minimize dwell time.
4. Aspirate and discard the spent medium.
5. Rinse hESCs twice with DPBS CTS™ without Ca/Mg.
6. Add warm TrypLE™ Select CTS™ to the culture vessel (1 mL/60-mm dish). Swirl vessel to coat the entire cell surface.
7. Place in 37°C incubator for 2–3 minutes (less time may be required for feeder-free hESC dissociation).
8. Remove vessel from the incubator. Gently tap the sides of the dish to dislodge cells.
9. Transfer cells to a sterile 15-mL conical tube.
10. Rinse dish twice with pre-warmed Wash Medium, gently "spraying off" any cells that haven't detached, and pool with cells in tube. **DO NOT TRITURATE!**
11. Pellet cells by centrifugation at 200 × *g* for 2 minutes at room temperature. Aspirate and discard the supernatant without disturbing the cell pellet.
12. Gently "flick" the tube to fully dislodge the cell pellet from the tube bottom.
13. Gently resuspend the cells in pre-equilibrated complete medium using a 2-mL or 5-mL serological pipette. **DO NOT TRITURATE!**
14. Transfer cells to a fresh feeder-plated or CELLstart™ CTS™-coated vessel at the desired cell ratio or seeding density. A 1:2 split is recommended during adaptation, or 8×10^4 hESC/cm^2. For routine maintenance, cells can be split at 1:4 – 1:8, or 4×10^4 hESC/cm^2 using KnockOut™ SR XenoFree CTS™ Complete Medium. Adjust densities as needed to suit your particular hESC line.
15. Incubate at 37°C in a humidified atmosphere containing 5% CO_2 in air. Carefully swirl vessel in a back and forth and then a left and right pattern to evenly distribute hESCs.
16. Gently fluid-change culture the next day to remove cell debris and to provide fresh nutrients, and daily thereafter.
17. Observe cells daily and passage by the above protocol whenever required (approximately every 3–5 days).

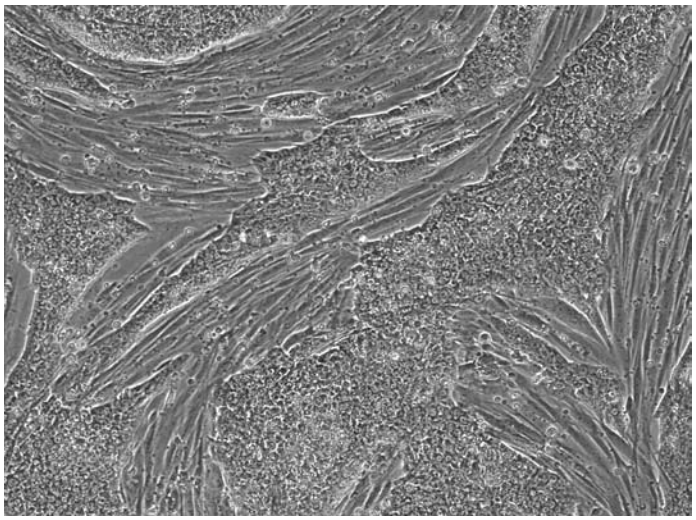

Figure 1a Xeno-Free Growth of hESCs on Feeders.
BG01v morphology when cultured in 15% KnockOut™ SR XenoFree CTS™ on human foreskin fibroblasts (HFF) attached with CELLstart™ CTS™ substrate; passage 4.

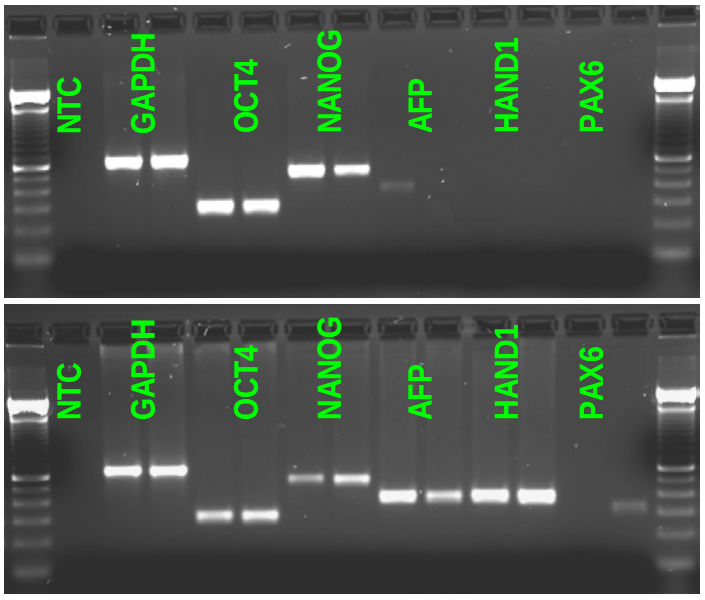

Figure 1b Maintenance of Pluripotency using KnockOut™ SR XenoFree CTS™. Following 10 passages in either KnockOut™ SR (left lane) or KnockOut™ SR XenoFree CTS™ (right lane) on HFF attached with CELLstart™ CTS™ substrate, BG01v gene expression was examined (top). Gene expression of embryoid bodies generated from the same P10 BG01v/HFF cultures (bottom).

Cryopreservation of hESCs Using KnockOut™ SR XenoFree CTS™

Prepare cryopreservation medium by supplementing KnockOut™SR XenoFree CTS™ Complete Medium with an additional 10% KnockOut™ SR XenoFree CTS™ (to yield a final concentration of 25%) and 10% Dimethyl Sulfoxide (DMSO) cryoprotectant.

Expect some cell death at recovery, and freeze hESCs at a higher density than would normally be passaged (if cells are routinely passaged at a 1:5 dilution, a 1:3 or 1:4 dilution is recommended). Following the protocol for **Passaging hESCs Using KnockOut™SR XenoFree CTS™** through step 12, gently resuspend the cell pellet with cryopreservation medium without triturating. While vialing, invert the capped hESC tube routinely to mix the cells. For best results, hESC vials should be cryopreserved using a controlled rate freezing device (e.g., CryoMed® Freezer or Mr. Frosty Nalgene® Cryo 1°C Freezing Container). Transfer frozen cells to liquid nitrogen (vapor phase); storage at –200°C to –125°C is recommended.

GIBCO® XenoFree Media and reagents include products that may contain discrete proteins, bulk protein fractions or recombinant proteins of human or **non-animal origin.** They may also contain proteins, hydrolysates, or components of unknown composition of human or **non-animal origin**.

Related Products

Product	Catalog No.
CELLStart™ CTS™	A10142
Dulbecco's Phosphate Buffered Saline CTS™ (DPBS) with calcium, with magnesium (1X), liquid	A12858
Dulbecco's Phosphate Buffered Saline CTS™ (DPBS) without calcium, without magnesium (1X), liquid	A12856
KnockOut™ DMEM CTS™ (1X), liquid	A12861
GlutaMAX™-I CTS™, 200 mM (100X), liquid	A12860
TrypLE™ Select CTS™ (1X), liquid	A12859
KnockOut™ SR, liquid	10828
KnockOut™ DMEM/F-12 (1X), liquid	12660
L-Glutamine, 200 mM (100X), liquid	25030
FGF-basic, Recombinant Human, lyophilized powder	13256
MEM Non-Essential Amino Acids (100X), liquid	11140
2-Mercaptoethanol (1000X), liquid	21985 & 31350*
TrypLE™ Express (1X), without Phenol Red	12604
0.05% Trypsin-EDTA (1X)	25300
Gentamicin Reagent Solution (50 mg/mL), liquid	15750
Collagenase Type IV powder	17104
Penicillin-Streptomycin, (100X) liquid	15070
Human Mammary Epithelial Cells (HMEC)	A10565
Trypan Blue Stain	15250
Countess® Automated Cell Counter	C10227

* European customers only

Limited Product Warranty

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References

1) Wang, L., T. Schulz, E. Sherrer, D. Dauphin, S. Shin, A. Nelson, C. Ware, M. Zhan, C-Z. Song, X. Chen, S. Brimble, A. McLean, M. Galeano, E. Uhl, K. D'Amour, J. Chesnut, M. Rao, C. Blau and A. Robins. 2007. Self-renewal of human embryonic stem cells requires insulin-like growth factor-1 receptor and ERBB2 receptor signaling. Blood **110**:4111–4119.

2) Rodríguez-Pizà I, et al., 2010. Reprogramming of Human Fibroblasts To Induced Pluripotent Stem Cells Under XenoFree Conditions. Stem Cells **28**:36–44.

3) Swistowski A, Peng J, Han Y, Swistowska AM, Rao MS, et al. (2009) Xeno-Free Defined Conditions for Culture of Human Embryonic Stem Cells, Neural Stem Cells and Dopaminergic Neurons Derived from Them. PLoS ONE 4: e6233.

For additional technical information such as Safety Data Sheets (SDS), Certificates of Analysis, visit **www.lifetechnologies.com/celltherapyresearchsupport**
For further assistance, email **techsupport@lifetech.com**