Frequently Asked Questions:

1. *Q: I noticed a very fine precipitate when I thawed my KnockOut™ SR XenoFree overnight at 4°C. Can I still use it?*
   A: Yes. This precipitate is normal. Upon thawing KnockOut™ SR XenoFree, warm the bottle in a 37°C water bath with occasional swirling, until the supplement is completely clear. Minimize dwell time.

2. *Q: My bottle of KnockOut™ SR XenoFree has been sitting at 4°C for over a month. Can I still use it?*
   A: We recommend using KnockOut™ SR XenoFree stored at 2 to 8°C within two weeks. For long-term use, aliquot into useful volumes, store at -5 to -20°C, and thaw aliquots as needed.

3. *Q: How long can I use my complete (1X) KnockOut™ SR XenoFree Medium?*
   A: KnockOut™ SR XenoFree Complete Medium is stable for at least 1 week when stored in the dark at 2 to 8°C.

4. *Q: My hESC seem to be growing slower in KnockOut™ SR XenoFree than KnockOut. Is this normal?*
   A: Different hESC lines will behave differently in KnockOut™ SR XenoFree Medium. Growth rates may increase once cells are fully adapted to the medium.

5. *Q: Do I need to slowly adapt my hESC to KnockOut™ SR XenoFree, or can I seed my cells in it directly?*
   A: We recommend sequential adaptation for best results. See tips below for starter culture and adaptation tips.

6. *Q: Can I freeze and recover my hESC using KnockOut™ SR XenoFree?*
   A: Yes. KnockOut™ SR XenoFree has been used successfully to cryopreserve both feeder-adapted and feeder-free hESC cultures. Increase your KnockOut™ SR XenoFree concentration to 25% (in complete medium) and use 10% Dimethly Sulfoxide as cryoprotectant. See below for additional tips.

7. *Q: I am seeing poor cell attachment using CELLstart. What am I doing wrong?*
   A: Are you using TrypLE to dissociate your hESC, as suggested in the product insert? Trypsin and Collagenase may be too harsh for use with KnockOut™ SR XenoFree Medium & CELLstart.

8. *Q: What growth factors do I need to add, to make KnockOut™ SR XenoFree work without feeders?*
   A: Invitrogen is optimizing a supplement for feeder-free growth using KnockOut™ SR XenoFree. This growth factor cocktail will soon be available for beta-site testing. Please contact Technical Services for additional information at Techsupport@Invitrogen.com. Here is a useful reference for feeder-free hESC culture: Blood, 2007 Dec 1;110(12):4111-9.

9. *Q: Does KnockOut™ SR XenoFree show better lot-to-lot consistency than traditional KnockOut? Do I need to pre-qualify it?*
A: We expect that different hESC lines will perform differently in KnockOut™ SR XenoFree. As it is a new product, we are still generating this data.

10. *Q: Can I have a copy of the KnockOut™ SR XenoFree formulation? I really want to know what is in it.*
A: No, I'm sorry. KnockOut™ SR XenoFree is a proprietary Invitrogen formulation. Some component information can be provided if there is a confidentiality agreement in place between Invitrogen and your institution. Please contact Cell Culture Technical Services for more information.

11. *Q: What hESC Systems Have Been Tested Using KnockOut XenoFree?*
A: hESC lines
- BG01v, BG02, CyT49, KhES-1 & -2, and many others

Feeder cells
- Human foreskin fibroblasts (HFF), Murine embryonic fibroblasts (MEF)

Matrices tested
- CELLstart, Matrigel, Human serum

mESC line
- D3

Tips for hESC Adaptation to KNOCKOUT SR XenoFree

- Different hESC lines will behave differently in KnockOut™ SR XenoFree 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.
- *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.
- Make a frozen bank of cells in control medium prior to adaptation.
- Maintain a stock culture in control medium throughout hESC adaptation to KnockOut™ SR XenoFree, as a "backup".
- ***"Direct" adaptation:*** If hESCs are passaged directly into KnockOut™ SR XenoFree 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 Complete Medium and two in MEF-CM control medium. Fluid-change the KnockOut™ SR XenoFree plate and one control plate with KnockOut™ SR XenoFree Complete Medium that day and daily thereafter. At the second passage, seed both plates directly in KnockOut™ SR XenoFree Complete Medium at 1:2.

- ***"Sequential" adaptation:*** Best results may be obtained by gradually adapting hESCs to KnockOut™ SR XenoFree:
    - Passage 1: 75% control medium + 25% KnockOut™ SR XenoFree Complete Medium.
    - Passage 2: 50% control medium + 50% KnockOut™ SR XenoFree Complete Medium.
    - Passage 3: 25% control medium + 75% KnockOut™ SR XenoFree Complete Medium.
    - Passage 4 & thereafter: 100% KnockOut™ SR XenoFree 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 culture (as described above), hESCs can be passaged into both 100% KnockOut™ SR XenoFree 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.

Cryopreservation of hESCs Using KnockOut™ SR XenoFree

Prepare cryopreservation medium by supplementing KnockOut™ SR XenoFree Complete Medium with an additional 10% KnockOut™ SR XenoFree (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*** through step 13, 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).