This document covers operation instructions for:

1. Preparation of the medium
    a. Medium I: Complete-Medium
    b. Medium II: Starvation-Medium
2. Cultivation of suspension mammalian cells
    a. Protocol I: Multiple Passage Protocol
    b. Protocol II: Starvation Protocol (If applicable, use protocol from literature)

The following instructions are exemplary. The user may have to adapt the procedure depending on the mammalian cell line, transfection method, plasmid, cell growth parameters, antibiotic selection, etc.

General information

Content of the kit

| Product | Storage | Sterility |
|---|---|---|
| Medium Base (solution) | 2 – 8 °C | Sterile |
| Mixture A (powder) | Dry, 2 – 8 °C | Non-sterile |
| Mixture B (powder) | Dry, 2 – 8 °C | Non-sterile |
| Unlabeled L-glutamine (powder) | Dry, 2 – 8 °C | Non-sterile |

Shipping and storage
All components are shipped at room temperature.

Upon receipt, store all components at 2 - 8 °C.

After combining all components, the culture medium is stable for at least 10 weeks and must be stored in the dark at 2 - 8°C.

## 1. Preparation of the Silantes Medium Kit for *suspension* mammalian cells

Silantes has developed two protocols for the preparation of media to express stable isotope-labeled proteins in suspension mammalian cells. Medium I for a complete medium that is suitable for multiple passages and Medium II for starvation. Select the protocol according to your cultivation strategy.
The following protocols are designed for the preparation of 1 L medium. Please adjust the amounts of the components according to your desired final media volume.

Note: The following protocol involves the use of unlabeled L-glutamine and dialyzed FBS (dFBS). The isotopic enrichment in the protein can be increased by using stable isotope-labeled L-glutamine. Stable isotope-labeled L-glutamine and dFBS are not included in the kit. These can be ordered separately from Silantes.

Medium I: Complete Medium

1. Dissolving: Add 70 mL of "Medium Base" to "Mixture A". Add 60 mL of "Medium Base" to "Mixture B". Add 20 mL of the "Medium Base" to "L-glutamine" to make a stock solution of 200 mM L-glutamine. Prewarm the dissolved "Mixtures A and B" at 37°C and stir the mixtures for 15 min to ensure that all powder is completely dissolved.
2. Mixing: Combine dissolved "Mixture A" and "Mixture B". Mix thoroughly.
3. Sterilization: Filter sterilize the combined mixtures with a 0.22 µm PVDF membrane filter into the remaining "Medium Base". Sterile filter L-glutamine separately.
   *Note: The complete medium (medium base + mixture A + mixture B) can also be sterile filtered after osmolality and pH adjustment using a 0.22 µm pore Stericup sterile filter unit.*
4. Adjustment: If necessary, adjust the osmolality to 280 - 320 mOsmol/kg with sterile NaCl.
5. Supplementation: If applicable to your system, add sterile dialyzed FBS and/or antibiotics. Supplement Silantes complete-medium with 200 mM L-glutamine for 4 mM final concentration prior to use.
6. Complete medium: The media can now be used for cell growth and protein expression or can be stored in the dark at 2 – 8 °C.

Medium II: Starvation Medium

1. Dissolving: Add 70 mL of "Medium Base" to "Mixture A" and 20 mL of the "Medium Base" to "L-glutamine" to make a stock solution of 200 mM L-glutamine. Prewarm the dissolved mixture A at 37°C and stir the mixtures for 15 min to ensure that all powder is completely dissolved.
2. Mixing and Sterilization: Filter sterilize dissolved Mixture A with a 0.22 µm PVDF membrane filter into the "Medium Base" to make "Intermediate Medium A" that will be used for both "Starvation and Complete Medium" preparation.
3. Preparation of "Starvation Medium": Depending on the size of your culture, transfer appropriate amount of "Intermediate Medium A" into a separate sterile bottle and supplement with 10 % sterile dialyzed FBS and 4 mM L-glutamine. If applicable to your system, add antibiotics.
   *Note: Do not use the whole amount of "Intermediate Media A" for the preparation of "Starvation Media".*
4. Starvation-Medium: The media can be use directly or stored at 2 – 8°C.
5. Preparation of "Complete-medium": Add 60 mL of "Intermediate Medium A" to "Mixture B" to make "Intermediate Mixture B" and stir it for 15 min to ensure that all powder is completely dissolved. Filter sterilize "Intermediate Mixture B" with a 0.22 µm PVDF membrane filter. Depending on the size of your culture, transfer appropriate amount of "Intermediate Medium A" into a separate sterile bottle. Then add "Intermediate Mixture B" to "Intermediate Medium A" at a ratio of 1:25 (v/v). Adjustment and supplementation are the same as described for "Medium I".

## 2. Cultivation of mammalian cells in Silantes medium

Note: For most experimental setups, a cell seeding density of 0.5 to 1x$10^6$ cells/ml and a culture time of 2 to 4 days before cell passage are recommended. Cell passaging should be done at >80 % confluence. (HEK293sus.2 cell line)

Protocol I: Multiple Passage Protocol

Passaging of the cells can lead to an increase in isotope enrichment in the protein while maintaining the yield. Passaging is not recommended if the cells were previously cultivated in "Starvation-Medium."

1. Prior to growing the cells in stable isotope-labeled medium, culture the cells in standard medium (e.g., BalanCD HEK media) for at least 3 passages after thawing the cells.
2. Prepare Silantes Complete-Medium for suspension mammalian cells according to the instructions for "Medium I".
3. Transfer appropriate volume (15 mL) of Silantes Complete-Medium into a T75/125 mL shake flask and equilibrate in an incubator at 37 °C, 5 % $CO_2$, 95 % humidity.
4. Transfer cell suspension to pre-equilibrated Silantes Complete-Medium with a cell density of 0.5 to 1x$10^6$ cells/mL.
5. Incubate cells at 37 °C, 5 % $CO_2$, 95 % humidity for 2-4 days until cell density has reached 3x$10^6$ cells/mL
6. Passage cells every 2-4 days to keep cells in logarithmic phase and seed in fresh Silantes Complete Medium at a density of 0.5 to 1x$10^6$ cells/mL. If cells are growing slowly, increase the seeding density or spin down and re-suspend cells into fresh medium at each passage.
7. Prior to transfection, repeat step 6 depending on the number of passages planned.