AMMS® NK Cell Culture Kit (Plus)

Product Name

English Name: AMMS® NK Cell Culture Kit 3.0 (Plus)

Product Performance

Catalog Number: AS-45

Composition:

AMMS® NK Cell Culture Kit 3.0 (Plus)-A/E (Catalog number: AS45-1A/E)

Component Name	Cat. No.	Specification	Amount	Storage conditions	Product Characteristics	Shelf Life
NK reagent A-2.0	AS22-1A	200 μL	1 stick	-20℃	Liquid	18 months
NK reagent B-2.0	AS22-1B	500 μL	2 sticks	-20℃	Liquid	18 months
NK activation additive P1	AS45-1C	500 μL	1 stick	-20℃	Liquid	18 months
NK activation additive P2	AS45-1D	1000 μL	1 stick	-20℃	Liquid	18 months
NK amplification additive P1	AS45-1E	50 μg	1 stick	-20℃	Dry powder	18 months

AMMS® NK amplification additive P2 (Catalog number: AS45-1F)

Product Name	Cat. No.	Specification	Amount	Storage conditions	Product Characteristics	Shelf Life
NK amplification additive P2	AS45-1F	20 mL	2 sticks	2-8℃, protect from light	Liquid	18 months

AMMS® NK Activation Medium (Plus) (Catalog number: AS45-2)

Product Name	Cat. No.	Specification	Amount	Storage conditions	Product Characteristics	Shelf Life
AMMS® NK Activation Medium (Plus)	AS45-2	100 mL	1 stick	2-8℃, protect from light	Liquid	18 months

AMMS® NK Serum-free Medium (Catalog number: AS01-2)

Product Name	Cat. No.	Specification	Amount	Storage conditions	Product Characteristics	Shelf Life
AMMS® NK Serum-Free Medium	AS01-2	1000 mL	2 bottles	2-8℃, protect from light	Liquid	18 months

Complimentary

Product Name	Cat. No.	Specification	Amount	Storage conditions	Product Characteristics	Shelf Life
Recombinant Human IL-2 Protein (high-efficiency)	TL-402-0100F	1x10^6 IU	2 sticks	2-8℃	Dry powder	24 months

Product Description

AMMS® NK Cell Culture Kit 3.0 (Plus) is suitable for fresh or cryopreserved peripheral blood PBMC and umbilical cord blood CBMC, and high purity NK cells are obtained by in vitro activation and amplification. It is only for in vitro research use.

Instructions for Use

Instructions 1: AMMS® NK Activation Medium (Plus) (Catalog No.: AS45-2) Usage

This product requires supplementation with additives to prepare **NK Complete Activation Medium I** for the initial cell culture. The procedure for the standard 100 mL preparation is described below: Equilibrate one bottle of NK Activation Medium (Plus) at room temperature for approximately 1 h. Add one fully thawed vial of NK Activation Additive P1 (AS45-1C) (equilibrated to room temperature), and mix thoroughly before use. For the first medium replenishment, add NK Activation Additive P2 (AS45-1D) at a 100× ratio to the remaining NK Complete Activation Medium I to prepare **NK Complete Activation Medium II**. Mix thoroughly before use.

Instructions 2: AMMS® NK Serum-free Medium (Catalog No.: AS01-2) Usage

This product requires supplementation with additives and cytokines to prepare **NK Complete Expansion Medium** for cell expansion. The procedure for the standard 1000 mL preparation is described below: Equilibrate one bottle of NK Serum-free Medium at room temperature for 1-2 hours prior to use. Dissolve one vial of NK Amplification Additive P1 (AS45-1E) and one vial of Recombinant Human IL-2 Protein (TL-402-0100F) each in 1 mL of NK Serum-free Medium. Add 0.5 mL of the dissolved NK Amplification Additive P1 and 1 mL of the dissolved Recombinant Human IL-2 Protein to 1000 mL (one bottle) of NK Serum-free Medium. Then add one vial of NK Amplification Additive P2 (AS45-1F). Mix thoroughly before use.

Notes:

- Any leftover NK Amplification Additive P1 after preparation may be used to prepare an additional bottle of NK Serum-free Medium. Combine it with rhIL-2 and NK Amplification Additive in another bottle of NK Serum-free Medium to make NK Complete Expansion Medium. This medium must be used within 1-2 weeks to prevent reduction or loss of activity. Remaining NK Amplification Additive P1 may also be frozen for later use. Aliquot and freeze according to required usage volume; avoid repeated freeze-thaw cycles.
- To preserve the activity of NK Complete Expansion Medium, prepare it only after NK Complete Activation Medium has been exhausted.
- Allow all culture media to equilibrate at room temperature for ≥1 hour prior to each use.
- This kit does not include PBS buffer or human platelet lysate (HPL); these reagents must be prepared separately.

- For umbilical cord blood NK cell samples, HPL is recommended for cell activation and expansion.

AMMS® NK kit 3.0 (Plus) reference operating method

Coating Pretreatment of Cell Activation Flask (Day -1 or Day 0)

Mix 200 μL of NK Reagent A-2.0 with 13 mL D-PBS, add the mixture to a T175 culture flask (non-TC treated), spread evenly across the flat surface, and incubate overnight in a 4 ℃ refrigerator. Aspirate the coating solution prior to cell seeding. For urgent use, incubate at 37 ℃ for 2 hours.

Mononuclear cell inoculation

PBMC or CBMC isolation and induction in vials (Day 0)

1. **Separate plasma**. Collect a small aliquot of blood sample (approximately 300 μL) and transfer it to a petri dish for bacterial testing. Centrifuge at room temperature for 15 minutes; harvest the supernatant as plasma.
2. **Plasma inactivation.** Incubate the collected plasma at 56 ℃ for 30 minutes for inactivation. Cool it in a 4 ℃ refrigerator for 30 minutes, then take it out and centrifuge at room temperature for 10 minutes. Collect the supernatant for subsequent use.

   Umbilical cord blood plasma is not recommended (the high anticoagulant content may interfere with CBMC activation). Use 10% HPL as an alternative.
3. **Isolate PBMC or CBMC.** Mix the blood cell pellet with an equal volume of normal saline, then carefully layer the mixture onto Ficoll solution to maintain clear stratification. Centrifuge at 2000 rpm, room temperature, for 25 minutes. Prepare NK Complete Activation Medium I in parallel.
4. **Wash the cells.** Aspirate the PBMC or CBMC layer. Add 40-50 mL normal saline and mix by gentle pipetting. Centrifuge at 1800 rpm, room temperature, for 8 minutes. Repeat this wash step once. For cryopreserved PBMC or CBMC: After thawing, resuspend cells in 10 mL D-PBS or normal saline, then centrifuge and discard the supernatant.
5. **Cell counting.** Resuspend cells in 5-10 mL NK Complete Activation Medium I, and take an aliquot for cell counting.
6. **Cell inoculation.** Adjust the initial seeding density to 1.5-2.0×10^6 cells/mL. Resuspend cells in NK Complete Activation Medium I, then supplement with NK Reagent B-2.0 at 20 μL/mL plus 10% HPL (for umbilical cord blood NK culture) or 10% autologous plasma (for peripheral blood NK culture). Seed into pre-coated T175 culture flasks; aspirate and discard the coating solution beforehand. The maximum working volume for cell seeding with this kit is 50 mL.

CD3 cell depletion method (Day 0)

The NK3.0 (PLUS) can be paired with ActSep® CD3 Beads to improve the purity of NK cells after cultivation. Both fresh blood samples and frozen mononuclear cells can use CD3 sorting magnetic beads. Please refer to the attached instruction manual for ActSep® CD3 Beads. The separated cells were seeded into culture bottles at a seeding density of 1.0-2.0×10^6 cells/mL.

Cell Culture

First medium replenishment. (Day 3)

Preparation of NK Complete Activation Medium II. Add NK Activation Additive P2 to the remaining NK Complete Activation Medium I at a 100× ratio. Mix thoroughly to prepare NK Complete Activation Medium II

1. Medium replenishment. Observe under the microscope that the cell mass at the bottom of the flask reaches more than 30% before operation. Add equal volume of NK Complete Activation Medium II and add 10% HPL (or 10% autologous plasma).

Notes:

- Do not pipette up and down the cells!!! Hold the culture flask horizontally, and add fresh medium gently.
- If sparse cell aggregation is observed under the microscope and the medium is reddish, do not proceed with the operation. Extend incubation for 1 day. Perform the operation only when obvious cell aggregates are formed.
- If no cell aggregates are observed on Day 3 or Day 4, check each step of the procedure, as well as the storage and usage of reagents one by one.

Second medium replenishment. (Day 5)

2. Medium replenishment. When obvious cell proliferation and increased size and number of cell aggregates are observed under the microscope, add an equal volume of Complete Activation Medium II, supplemented with 10% HPL (or 10% autologous plasma).

Notes:

- Do not pipette up and down the cells!!! Hold the culture flask horizontally and add fresh medium gently.
- After the Activation Medium is exhausted, Complete Expansion Medium can be used.

Preparation of NK Complete Expansion Medium. To prepare NK Complete Expansion Medium: Add half a vial of NK amplification additive P1 (final concentration: 25 ng/mL), one vial of NK amplification additive P2, and one vial of rhIL-2 (final concentration: 1000 IU/mL) to each bottle of NK Serum-free Medium (1000 mL). Mix thoroughly before use.

Notes:

- As stated above, unused NK amplification additive P1 may be frozen for later use. Avoid repeated freeze-thaw cycles.
- Do not change culture flasks during days 5-7!!! If the culture volume exceeds the rated capacity of the culture flask, place the flask at an incline

Third medium replenishment. (Day 7 or Day 8)

3. Medium replenishment or bag transfer with medium addition. When microscopic observation shows that cell aggregates become larger and more numerous compared with the first 5 days, and the medium color changes from orange-red to orange-yellow, collect a sample for cell counting. Add fresh NK Complete Activation Medium II or NK Complete Expansion Medium. The cell density after medium replenishment must be no less than 1.0×10^6 cells/mL. Add approximately an equal volume of NK

Complete Activation Medium II or NK Complete Expansion Medium (NK Complete Expansion Medium may be used once NK Complete Activation Medium II is exhausted), supplemented with 10% HPL or remaining autologous plasma. When the cell suspension volume exceeds 200 mL, transfer the cell suspension from the culture flask to a culture bag. If minor adherent cell colonies are present, add a small amount of medium and tap the flask or gently pipette the bottom of the flask to fully detach all cells and transfer them into the culture bag.

Notes:

- Perform cell counting before transferring the cell suspension into the culture bag. This is a critical step. Cells require sufficient activation during expansion. The cell density after medium replenishment must not fall below 1.0×10^6 cells/mL; otherwise, cells may decline before entering the exponential growth phase.
- When the cell suspension volume is less than 800 mL or less than half the effective volume of the culture bag, fold the culture bag to maintain a high cell density.
- Expel excess air from the culture bag as much as possible to avoid large air bubbles above the cell suspension.

Fourth Medium Replenishment (Day 9 or Day 10)

4. Medium replenishment. Collect a sample for cell counting prior to medium addition to ensure the cell density after replenishment is no less than 1.0×10^6 cells/mL. Assess cell growth status: obvious cell aggregates should be observed, and the medium should appear orange-yellow before replenishment. Add approximately 1-1.5 volumes of fresh NK Complete Expansion Medium.

Day 11-Day 15

5. Medium replenishment every other day. Collect a sample for cell counting prior to replenishment to ensure the cell density after medium addition is no less than 1.0×10^6 cells/mL. Assess cell growth status: obvious cell aggregates should be observed, and the medium appears orange-yellow before replenishment. Add approximately an equal volume of fresh NK Complete Expansion Medium every other day.

Cell counting. Remove the culture bag from the incubator and place it on the workbench. If large cell aggregates are present, gently massage the bag with both hands to disperse aggregates and homogenize the cell suspension. Remove the rubber stopper from the sampling port, then use a 2 mL or 5 mL sterile syringe to collect a sample for counting.

6. Cell Harvest (Day 15). Transfer all or part of the cell suspension into centrifuge tubes as required. Centrifuge and discard the supernatant. Wash the cells 2-3 times with normal saline containing 1% human serum albumin. Collect samples for cell counting and flow cytometry analysis. If residual cells remain for further expansion, add approximately an equal volume of fresh NK Complete Expansion Medium.

Note: An initial volume of 25 mL can yield a final system volume of approximately 2 L.

Day 16-Day 20

7. Medium Replenishment Every 2-3 Days. Collect a sample for cell counting prior to replenishment to ensure the cell density after medium addition is no less than 1.0×10^6 cells/mL. Assess cell growth status: obvious cell aggregates should be observed, and the medium appears orange-yellow before replenishment. Add approximately an equal volume of fresh NK Complete Expansion Medium every 2–3 days.

Cell Harvest (Day 21-Day 23)

8. Transfer all or part of the cell suspension into centrifuge tubes as required. Centrifuge and discard the supernatant. Wash the cells 2-3 times with normal saline containing 1% human serum albumin. Collect samples for cell counting and flow cytometry analysis.

Precautions

1. Sample Requirements.

1.1 For umbilical cord blood (with anticoagulant): the collected volume must be > 80 mL. To reduce the impact of anticoagulants on cells, umbilical cord blood plasma is not recommended; use 10% HPL instead.

1.2 For peripheral blood samples (anticoagulant: sodium heparin), autologous plasma is recommended.

1.3 Complete the procedure within 12 hours after blood collection. Shorter processing time yields better cell quality.

1.4 For cryopreserved samples, the recommended freezing density is 2-4×10^7 cells/mL, and the post-thaw viability must be no less than 80%.

2. Seeding Density.

The recommended initial seeding density for flask culture is 1.5-2×10^6 cells/mL. When red blood cell contamination is high, perform cell counting via fluorescence counting or lyse red blood cells prior to counting to avoid compromising counting accuracy. For cryopreserved umbilical cord blood cells, increase the seeding density to 2×10^6 cells/mL.

3. Medium Replenishment Density.

Adjust the post-replenishment cell density according to the operating instructions. Generally, it shall be no less than 1.0×10^6 cells/mL.

4. Medium Handling.

4.1 Equilibrate the medium naturally at room temperature before each medium replenishment.

4.2 Do not place the entire bottle of medium in a 37 ℃ incubator for prolonged equilibration; otherwise, cytokine inactivation in the medium will be accelerated.

4.3 Prepared complete medium should be used within 1-2 weeks.

5. Proper handling and storage of plasma.

Follow the operating instructions. Ensure the plasma obtained after centrifugation is clear.

6. Culture bag usage.

Fold the culture bag (2-4 folds) when the culture volume is less than 800 mL or less than half of the culture bag's effective volume.

7. Coating duration.

After coating with Factor A, keep the flask flat and incubate overnight at 4 ℃. For urgent cases, coating at 37 ℃ for 2 hours may be used as an alternative.

8. Coated Flasks.

Use non-TC-treated culture flasks. For an initial seeding volume of 25-50 mL, use T175 culture flasks. When the initial volume is less than 12.5 mL, non-TC-treated T75 culture flasks may be used. Do not change culture flasks during the first 5-7 days of culture. If the culture volume exceeds the flask's capacity, place the flask at an incline

9. Static culture required in the early culture phase (1-7 days).

During the first two medium replenishments and cell observation, do not shake the culture flask vigorously in any direction. Add fresh medium gently along the sidewall. Do not pipette cells directly, as this will impair effective cell activation.

10. Factor handling.

To reduce factor loss due to wall adsorption, centrifuge the vial before use. Place the factor-containing vial inside a 50 mL centrifuge tube and centrifuge at 1000 rpm for 1-2 minutes.

11. Equipment maintenance.

Routinely check the temperature and CO_2 concentration of the CO_2 incubator, and replace filters in a timely manner. Perform regular maintenance and cleaning of the biosafety cabinet.

12. Environmental monitoring.

Replace primary, medium-efficiency and high-efficiency filters periodically to maintain the environmental standards of the clean area.

13. Standardized types and models of experimental consumables.

Evaluate the impact of any changes in model or specification on culture performance in advance. Examples include T75 culture flasks and cell culture bags.

Conferences

1. Umbilical Cord Blood Transplantation: Connecting Its Origin to Its Future.2023 Mar 3;12(2):55-71.
2. Natural Killer Cell Expansion and Cytotoxicity Differ Depending on the Culture Medium Used.2022 Nov 1;42(6):638-649.