INSTRUCTIONS FOR USE

GENETICALLY MODIFIED K562 FEEDER CELL

Contents

1. Product Introduction

Hillgene's genetically modified K562 feeder cells are engineered K562 cells that express IL-21 and multiple other cytokines following irradiation inactivation. When combined with mainstream immune cell culture media, these feeder cells, through the synergistic signaling of various cytokines, enable the directed activation and massive expansion of NK cells derived from cord blood and peripheral blood mononuclear cells in vitro. Moreover, the obtained NK cells exhibit high purity.

2. Application

Suitable for the expansion of NK cells derived from sources such as PBMCs, CBMCs, iPSCs, and genetically modified NK92 cell lines.

Suitable for the activation and massive expansion of CAR-NK immune cells.

3. Product Specifications

Product name	Product No.	Specifications	Volume	Shelf Life	Storage Conditions
Genetically Modified K562 feeder cell	HG-FEC002-GMP-1	1 E7 Cells/Vial	~0.5 mL	36 months	⩽-130°C
	HG-FEC002-GMP-2	2 E7 Cells/Vial	~1 mL	36 months	⩽-130°C
	HG-FEC002-GMP-3	8 E7 Cells/Vial	~4 mL	36 months	⩽-130°C
	HG-FEC002-GMP-4	1 E9 Cells/Bag	~50 mL	36 months	⩽-130°C

△ **Note: Please read the instructions carefully and check the product information before starting the experiment.**

4. Storage Conditions and Shelf Life

Please store the product according to the storage conditions specified in the manual. The shelf life is indicated by the expiration date shown on the product label. Genetically modified K562 feeder cells are shipped on dry ice or in liquid nitrogen. Upon receipt, they should be used immediately or stored in liquid nitrogen. Do not use a -80°C freezer for long-term storage.

△ **Note : It is recommended to use genetically modified K562 feeder cells immediately after thawing to avoid repeated freeze-thaw cycles.**

5. Materials Required but Not Provided

Table 1: Commonly Used Equipment for NK Cell Expansion

Equipment Name	Manufacturer	Model
A2 Biological Safety Cabinet	ESCO	AC2-4S8-CN
CO2 Incubator	ESCO	CLM-170B-8-CN
Cell Counter	Chemometec	NC200
Vapor Phase Liquid Nitrogen Tank	Thermo Fisher	CE8140
Medical Refrigerator	Zhongke Duli	MPC-5V315
Medical Freezer	Haier	DW-25L262
Medical Ultra-low Temperature Freezer	Haier	DW-86L100J
Benchtop Refrigerated Centrifuge	Thermo Fisher	Sorvall ST4R Plus

Table 2: Commonly Used Reagents/Consumables for NK Cell Expansion

Reagents & Materials	Brand	Catalog Number
Corning® Lymphocyte Serum-free Medium, KBM 581	Corning	88-581-CM
Human IL-2 Recombinant Protein	Thermo Fisher	200-02-50UG
CTS™ Immune Cell SR	Thermo Fisher	A2596101
6-well plate	Thermo Fisher	140675
T25 cell culture flask	Thermo Fisher	156367
T75 cell culture flask	Thermo Fisher	156499
T175 cell culture flask	Thermo Fisher	159910
GT-T610(B) lymphocyte culture bag (0.3～1.8L)	Takara	FU0003
Viral E-hancer C(GMP)	Hillgene	HG-PTD001-C-G

6. NK Cell Preparation Procedure

6.1. Preparation of Serum-Free Complete Medium for NK Cells:

Preparation of Serum-Free Complete Medium for NK Cells: Serum-free Medium + Autologous Plasma/Platelet Lysate/Immune Cell SR (2%–5%) + IL-2 (200–500 IU/mL). Prepare immediately before use.

△ **Note : To ensure optimal NK cell expansion, pre-warm the prepared serum-free complete medium for NK cells to 37°C before each use.**

6.2. D0 : Initial Activation

Based on the cell type used and the counting results of K562 feeder cells, take the appropriate volume of cells and genetically modified K562 feeder cells (refer to Table 3 for the addition ratio). Transfer the mixed cells and genetically modified K562 feeder cells into a suitable culture vessel, add complete medium to adjust the cell density to 1.25～5E5 cells/mL, and culture at 37°C with 5% CO_2.

△ **Note : Expand cells and K562 feeder cells should be counted based on viable cell count and added in proportion.**

Table 3 :Reference for Initial Activation Ratios of Cells from Different Sources

Cell Type for Activation	Activation Ratio (Based on Viable Cell Count)	Adjusted Cell Density
PBMC	PBMC: K562 feeder=1:2	3~5E5 cells/mL
CBMC	CBMC: K562 feeder=1:2	3~5E5 cells/mL
NK cell（CD3-）	NK cell：K562 feeder=1:2	1.25~3E5 cells/mL
NK cell（CD3-&CD56+）	NK cell：K562 feeder=1:4	1.25~3E5 cells/mL
Genetically modified NK92 cell line	Genetically modified NK92 cell line：K562 feeder=1:4	1.25~3E5 cells/mL
iPSC-NK	IPSC-NK: K562 feeder=1:4	1.25~3E5 cells/mL

6.3. D4 : Observation and Medium Replenishment

Observe the cells, add an equal volume of NK Cell Complete Medium (as used on Day 0), and then place them in an incubator at 37°C with 5% CO_2 for continued culture.

6.4. D6 : Observation and Medium Replenishment

Observe the cells. After gently pipetting to mix, take a sample for cell counting. Based on the color of the cell suspension and the cell morphology observed under the microscope, add serum-free complete medium for NK cells and adjust the cell density to 3–5E5 cells/mL. Then, culture at 37°C with 5% CO_2。

6.5. D8 : Observation, Secondary Activation, and Medium Replenishment

Observe the cells. After gently pipetting to mix, take a sample for cell counting. Then, proceed with the recovery, washing, and counting of genetically modified K562 feeder cells. Based on the number of NK cells to be activated and the addition ratio for genetically modified K562 feeder cells **(recommended ratio of NK cells : K562 feeder cells = 1:1)**, take the appropriate volume of genetically modified K562 feeder cells. Add serum-free complete medium for NK cells to adjust the cell density to 2.5E5 cells/mL, and culture at 37°C with 5% CO_2.

△ **Note : K562 feeder cells should be counted and added based on viable cell count.**

6.6. D10 : Observation and Medium Change

Observe the cells, transfer the cell suspension to a centrifuge tube of appropriate volume, and centrifuge at 400 × g for 10 minutes. Discard the supernatant, resuspend the cells in serum-free complete medium for NK cells, adjust the cell density to 5E5 cells/mL, and transfer to a suitable culture vessel. Culture at 37°C with 5% CO_2.

△ **Note : During pipetting and mixing, operate gently to prevent excessive cell death caused by overly forceful pipetting.**

6.7. D10～D14 : Observation and Medium Replenishment

Every two days, observe the cells and take samples for counting. Based on the color change of the medium or the cell density, add serum-free complete medium for NK cells to **adjust the viable cell density to 5-8 E5 cells/mL**, and culture at 37°C with 5% CO_2。

6.8. D14～D17 : Cell Harvest

Observe the cells, take a sample for counting, and harvest the cells manually or using equipment as needed. Depending on experimental requirements, cell harvest may be appropriately advanced or delayed. After harvesting and washing, resuspend the cells in a suitable cryopreservation medium to a certain cell density as needed, then aliquot into cryovials or cryobags according to specified specifications. Perform controlled-rate freezing using a controlled-rate freezing container or a programmable controlled-rate freezer, and finally store in a liquid nitrogen tank.

△ **Note : The cell culture procedure is for reference only. Due to factors such as individual sample variability and adjustments in culture methods, the status of NK cell expansion culture may vary. In such cases, appropriate adjustments may be made after observation and analysis of NK cell growth conditions.**

7. Troubleshooting

Question 1 : Significant cell clumping is observed during culture. Should intervention be performed?

Answer : NK cells specifically bind to K562 cells and become activated. Macroscopically, this appears as cell clumping, which is a normal phenomenon and indicates successful activation. Typically, around Day 7, this clumping begins to diminish, and cells start growing in small clusters. Throughout the process, it is not recommended to pipette the cells; daily medium replenishment is sufficient.

Question 2 : Why is a medium change performed on Day 10?

Answer : (1) Replacing with fresh medium supports cell growth; (2) K562 feeder cells are added during the NK cell culture process, and changing the medium helps remove these additives, reducing the risk of process-related impurity residues in the final cell product.

Question 3 : How to determine the addition amount of genetically modified K562 feeder cells?

Answer : When performing NK cell activation experiments, genetically modified K562 feeder cells are added based on the viable cell count. For example, after recovery, if the K562 feeder cells have a viability of 80%, a density of 2E7 cells/mL, and a volume of 1 mL, the addition amount of K562 feeder cells would be 1.6E7 cells.

Question 4 : How to perform recovery of genetically modified K562 feeder cells?

Answer : Remove the K562 feeder cells stored in liquid nitrogen and transport them to the cell preparation laboratory on dry ice. After the 37°C constant temperature water bath has reached and stabilized at 37°C, place the K562 feeder cells in the water bath for thawing, gently shaking the cryovial. Stop the water bath when a small amount of ice crystals remains inside the cryovial. Wipe the surface of the cryovial with 75% disinfectant alcohol and transfer it to a biosafety cabinet. Use a Pasteur pipette to transfer the cells to a sterile centrifuge tube, slowly add 10 volumes of pre-warmed serum-free medium (optional: PBS or saline) to fully protect the thawed cells, and let stand at room temperature for 1 minute. Then, centrifuge at 400 × g for 10 minutes, discard the supernatant, add 5 mL of complete medium to resuspend the cells evenly, centrifuge again at 400 × g for 10 minutes, discard the remaining supernatant, add 1 mL of complete medium, and take a sample for counting.

△ **Note : K562 feeder cells should not be exposed to room temperature for extended periods. After removal from liquid nitrogen, perform the recovery procedure within 2 minutes. Operate gently during the process to avoid causing mechanical damage to the cells.**

Question 5 : What are the steps for PBMC isolation?

Answer : Isolation of PBMCs: Mix an equal volume of physiological saline with the blood cell pellet, and gently layer the mixture onto the Ficoll layer (diluted blood cells : Ficoll = 2:1), ensuring the layers remain distinct. Centrifuge at 800 × g for 30 minutes at room temperature, with acceleration set to 4 and deceleration set to 3. Carefully aspirate the buffy coat layer from the interface, add physiological saline, and mix by pipetting gently. Centrifuge at 400 × g for 10 minutes at room temperature, discard the supernatant, and repeat the washing step once more. After discarding the supernatant, resuspend the cells in an appropriate volume of complete medium and take a sample for counting

Question 6 : What are the steps for recovering cryopreserved mononuclear cells?

Answer : Recovery of mononuclear cells: Remove the cryopreserved mononuclear cells stored in liquid nitrogen and transport them to the cell preparation laboratory on dry ice. After the 37°C constant temperature water bath has reached and stabilized at 37°C, place the mononuclear cells in the water bath for thawing, gently shaking the cryovial. Stop the water bath when a small amount of ice crystals remains inside the cryovial. Wipe the surface of the cryovial with 75% disinfectant alcohol and transfer it to a biosafety cabinet. Use a Pasteur pipette to transfer the cells to a sterile centrifuge tube, slowly add 10 volumes of pre-warmed serum-free medium (optional: PBS or saline) to fully protect the thawed cells, and let stand at room temperature for 1 minute. Then, centrifuge at 400 × g for 10 minutes, discard the supernatant, add 5 mL of complete medium to resuspend the cells evenly, centrifuge again at 400 × g for 10 minutes, discard the remaining supernatant, add 1 mL of complete medium, and take a sample for counting。

Question 7 : How to perform viral transduction during NK cell culture?

Answer : Viral transduction on Day 6 :

Remove the cells after initial activation culture, take a sample for counting, centrifuge the remaining cell suspension at 400 × g for 10 minutes at room temperature, and discard the supernatant. Based on the counting results, resuspend the cell pellet in serum-free complete medium for NK cells and adjust the cell density to 1-2E6 cells/mL.

Calculate the required volume of viral vector based on the MOI, cell number, and viral titer, and add the viral vector to the cell suspension. (△Note: The recommended MOI for NK cell viral transduction is 5–20.)

Based on the total volume of the cell suspension after adding the viral vector, add Viral Enhancer C at a volume ratio of 1:100 (Viral Enhancer C : cell suspension), and mix gently by pipetting.

Place the mixture of cells, NK cell viral transduction enhancer, and virus in a 37°C, 5% CO_2 incubator and continue culturing for 24 ± 2 hours.

8. Reference

Srinivas S. Somanchi (ed.), Natural Killer Cells: Methods and Protocols, Methods in Molecular Biology, vol. 1441, DOI 10.1007/978-1-4939-3684-7_14, © Springer Science+Business Media New York 2016.

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Attachment 1 : Related Products (For more products, please contact Hillgene at https://www.hillgene.com)

Product Name	Product No.
Genomic DNA Extraction Kit for Blood Tissue Cells	HG-NA100
CAR/TCR Gene Copy Number Detection Kit (Multiplex qPCR)	HG-CA001
BaEV Gene Copy Number Detection Kit (qPCR)	HG-BA001
Human IFN-γ ELISA Detection Kit	HG-IF002
Viral E-hancer C(GMP)	HG-PTD001-C-G
Cell Cytotoxicity Assay Kit (Suspended Target Cells)	HG-CKK001
Residual K562 feeder cell Detection Kit	HG-KF001