# KBM NK Kit
## User Guide

KOHJIN BIO

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# 1. Product information

## 1-1. Product description

KBM NK kit is designed culture system optimized for expansion of NK cells from human peripheral blood lymphocytes.

Key features of the kit:

- OPTIMIZED formulation for expansion of NK cells
- STREAMLINED media preparation and culture method

## 1-2. Procedure overview

In this procedure, total PBMCs and NK cells included are expanded approximately minimum of 3 x 109 cells and 1.5 x 109 cells, respectively, within primary culture and cell expansion culture (depending on characteristic of primary PBMCs, final PBMCs numbers and ratio of NK cells in total PBMCs are varied).

## 1-3. Contents and storage

| No. | Component | Amount | Purpose of use | storage |
|---|---|---|---|---|
| 1 | KBM NKCC-1 | 50 mL | Primary culture | 2 ℃ to 8 ℃ |
| 2 | KBM NKCC-2 | 1,000 mL | Suspension culture | 2 ℃ to 8 ℃ |
| 3 | KBM NKCC-c | 1 mL | Coating agent | 2 ℃ to 8 ℃ |
| 4 | KBM NKCC-b | 1 bag | Culture bag | - |


- All components in KBM NK kit

# 1. Product information

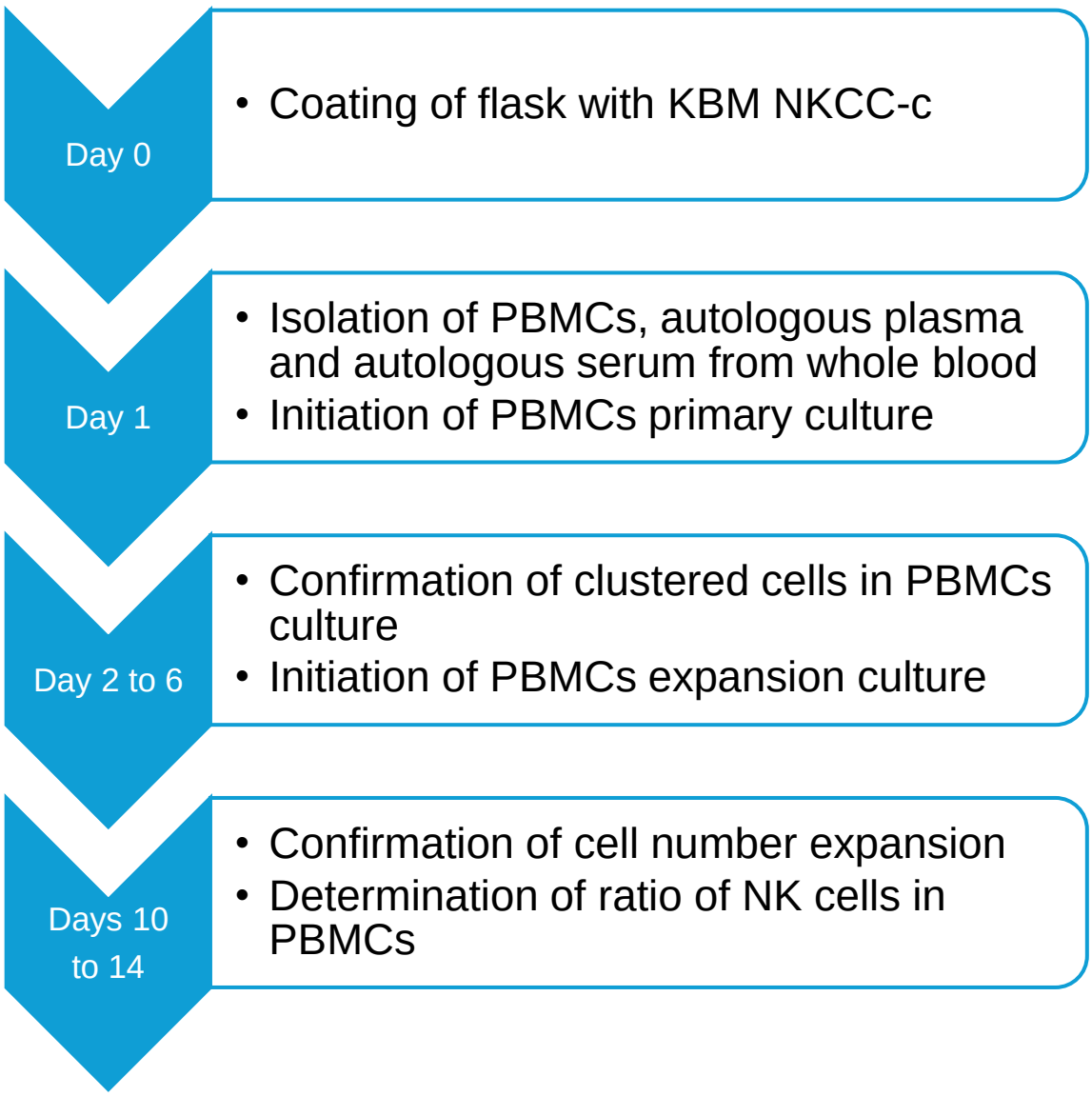
## 1-4 Required materials not supplied

- Typical equipment for cell culture also required
- Human peripheral blood as source of PBMCs, autologous plasma and autologous serum is needed for minimum of 40 mL.
- Lymphocyte separation medium (*d*=1.077) and heparin are required for isolation of PBMCs, plasma and serum.

| Item | remarks |
|---|---|
| Equipment | |
| $CO_2$ incubator | |
| Centrifuge | |
| Clamp and stand | (use in sterile conditions) |
| Tubes, flask and other consumables | |
| 15 mL Conical tube | |
| 50 mL Conical tube | |
| 75 $cm^2$ Flask | 430641U (Corning) |
| 50 mL syringe | (Terumo, etc) |
| Reagents | |
| D-PBS(-) | 16220015(KohjinBio) |
| Trypan Blue solution | |

# 1. Product information

## 1-5 Workflow



# 2. Methods

## 2-1 Coating of Flask

1. Mix 13 mL D-PBS(-) and KBM NKCC-c for preparation of coating solution. Total volume of mixture is 14 mL and add whole solution to 15 mL conical tube (Photo 1).

2. Coat a 75 $cm^2$ flask with the coating solution (Photo 2).

3. Incubate the coated flask at 2-8 ℃ for 24 hours or at 37 ℃ for 4 hours.

4. Remove the coating solution and rinse twice with each 15 mL D-PBS(-).

5. Store at refrigerator (2-8 ℃). Avoid drying out of coating surface with D-PBS(-) for long-term store.

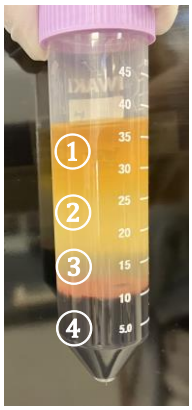
Preparation of coating agent (Photo 1)

Coat a flask with the coating agent (Photo 2)

## 2-2. Primary culture of PBMCs

1. Isolate PBMCs (Photo 3-②) and autologous plasma (Photo 3-①) from 30-50 mL human peripheral blood according to the protocol for Lymphocyte separation medium. Store plasma at 4 ℃.

2. Add D-PBS (-) to collected PBMCs and centrifuge at 250 x *g* for 5 minutes to wash and collect the cells (Photo 4). Remove the supernatant.




① : Plasma

② : PBMCs

③ : Separation medium

④ : Erythrocyte and granulocyte

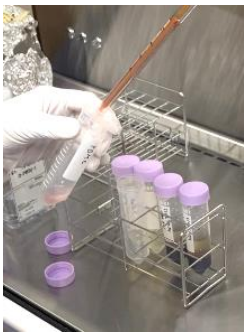
Isolation of PBMCs and plasma (Photo 3)

Collected PBMCs (Photo 4)

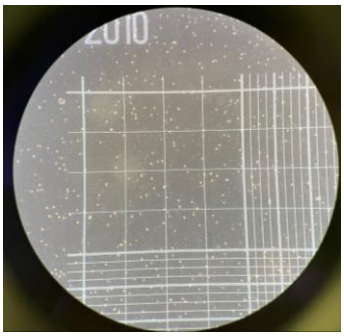
# 2. Methods

## 2-2. Primary culture of PBMCs (cont.)

3. Suspend PBMCs in 2-3 mL of NKCC-1 (Photo 5) and measure cell viability with trypan blue solution (Photo 6).



PBMCs suspended in KBM NKCC-1 (Photo 5)



Measurement of cell viability using Hemocytometer (Photo 6)

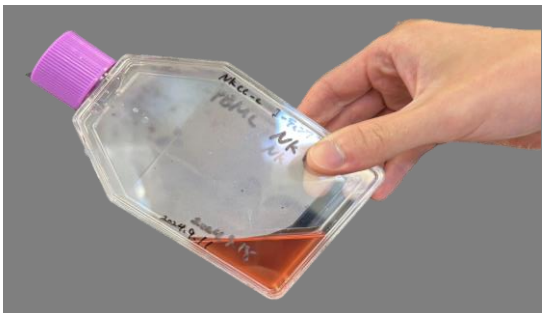
4. Isolate autologous serum from 10-20 mL human peripheral blood based on serum separation methods.
5. Inactivate autologous plasma (1) and autologous serum by incubation at 56 ℃ for 30 minutes. Store them at room temperature.
6. Add KBM NKCC-1 and inactivated serum or plasma to the PBMCs cell suspension tube to dilute it so that the calculated cell concentration is approximately 1 x $10^6$ cells/mL.
    - At this time, the concentration of serum or plasma concentration should be approximately 10%.
    - If the amount of medium exceeds the amount of culturable fluid in the flask, the cell concentration may be changed to as low as 2 x 106 cells/mL.

# 2. Methods

## 2-2. Primary culture of PBMCs (cont.)

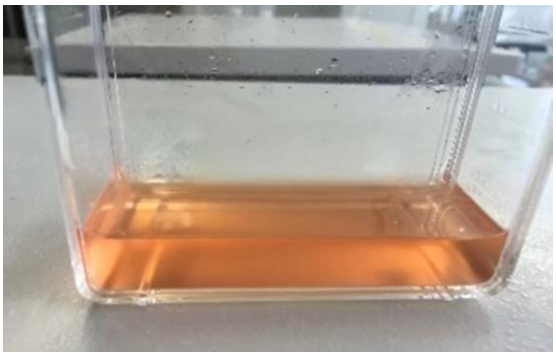
7. Put the cell suspension in the coated flask (Photo 7).
   Incubate the flask in at 37 °C with 5 % $CO_2$ for 4-6 days.
   Progress of culture should be observed (Photo 8)

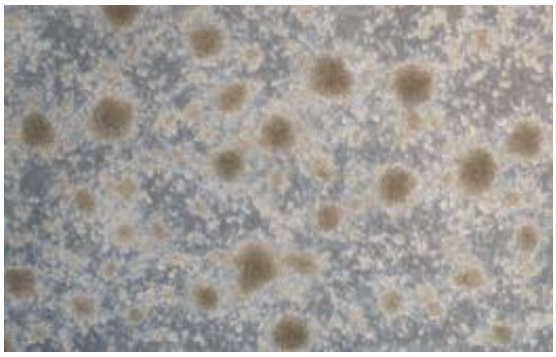
Seeding PBMCs in flask (Photo 7)



The 2nd day in culture(progress) (Photo 8)

8. When the culture of cells are confluent (briefly, color of medium turns a yellowish and aggregations of cells confirmed (Photo 9)) , transfer to expansion culture.
   Collect whole cells and centrifuge centrifugation at 250 x *g* for 5 minutes,
   Remove the supernatant and the cells are suspended in an appropriate amount of KBM NKCC-2.
   Cell viability are measured with Trypan blue solution and the cell concentration is calculated.



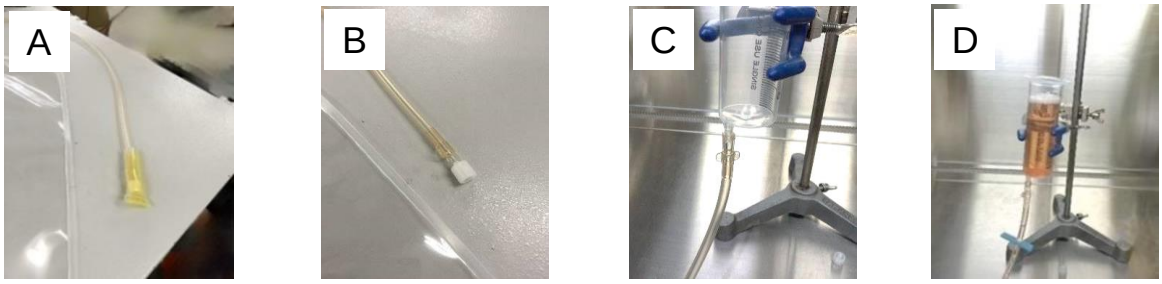


Color change (Left) and aggregations of cells (Right) (Photo 9)

# 2. Methods

## 2-3. Expansion culture of PBMCs

1. Prepare KBM NKCC-b for expansion of PBMCs culture. KBM NKCC-b has components of transfer tube with port of white cap (photo 10-B) and yellow cover (photo 10-A). Connect the port with a 50 mL syringe (Photo 10-C).
2. Transfer cell suspension to KBM NKCC-b through syringe (Photo 10-D) and add KBM NKCC-2 as a cell concentration of about 5 x $10^5$ cells/mL. Add all serum and plasma collected in 2-2 5. in optional.




Method of culture filling to KBM NKCC-b (Photo 10)
A:Syringe port with cover attached B:Syringe port at cover removal
C:Syringe connected with NKCC-b D:Syringe filling culture medium

3. Incubate culture bag with 5 % $CO_2$ at 37 °C. Check cell concentration every 2 days (Photo 11). When the cell concentration has increased to about 1 x $10^6$ to 2 x $10^6$ cells/mL, add NKCC-2 and dilute to a cell concentration of 2.5 x $10^5$ cells/mL.
4. Repeat feeding NKCC-2 until total volume reaches to 1,000 mL. With full volume of cell suspension, collect whole cells for further application.

Sampling of culture medium (Photo 11)

## 2-4. Analysis of NK cells

- To confirm that NK cells in culture suspension, analysis of Cell surface markers by flow cytometer and cytotoxic activity with K562 cells recommended.

# 2. Methods

## 2-5. Example of NK cell analysis

【Measurement of cell counting and cytotoxic activity】
At 10 days in expansion culture, cell counting and analysis of cell surface markers by flow cytometer were performed. Cytotoxic activity with K562 cells was also measured.

**Methods for experiment**

| | |
|---|---|
| Primary culture of PBMCs | NKCC-1 + 10% serum (inactivated) |
| Expansion culture of PBMCs | NKCC-2 + 5 % plasma (inactivated) |

**Table 1　Changes in cell number**

| Day of culture | Cell yield(/mL) | Viability |
|---|---|---|
| 0 | $2.0\times10^{7}$ | — |
| 5 | $8.2\times10^{7}$ | — |
| 10 | $3.7\times10^{9}$ | 94.6% |

**Table 2　Percentage of Cytotoxicity**

| Ratio of E/T | Cytotoxicity | |
|---|---|---|
| | 2 hr | 4 hr |
| 12:1 | 100 % | 100 % |
| 24:1 | 100 % | 100 % |

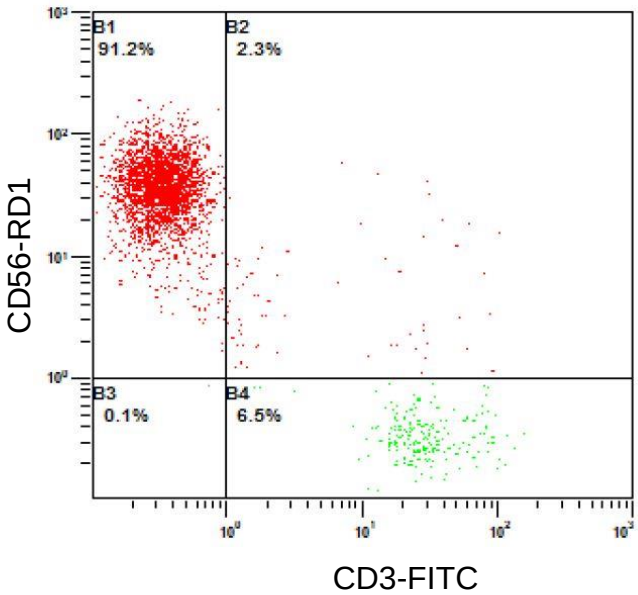



**Fig.1　Surface makers by flow cytometer**

**Table 3　Surface makers by flow cytometer**

| Marker | Rate |
|---|---|
| CD56+/CD3- | 91.2 % |
| CD56+/CD3+ | 2.3 % |
| CD56-/CD3+ | 6.5 % |
| CD56-/CD3- | 0.1 % |

# 2. Methods

## 2-6 Related products

Additional components of the KBM NK Kit are listed below.

- KBM 501 and KBM 551 are utilized for culture of PBMCs, but not exactly equivalent to KBM NKCC-1 and KBM NKCC-2, respectively, as a difference of composition.
- KBM W Bag is equivalent to KBM NKCC-b.

| Product No. | Product Name | Size | Price | |
|---|---|---|---|---|
| 16025015 | KBM 501 | 500 mL | JPY20,000- | Primary culture |
| 16025510 | KBM 551 | 1,000 mL | JPY12,000- | Suspension culture |
| 16087430 | KBM W Bag | 10 pcs. | JPY39,000- | Culture bag |
| 16087431 | KBM W Bag | 1 pcs. | JPY5,000- | Culture bag |
| 16220015 | D-PBS(-) | 500 mL | JPY1,600- | — |

*For details, please contact our sales staff.

| Product No. | Product Name | Size | Price |
|---|---|---|---|
| 16030210 | KBM NK Kit | 1 Kit | JPY57,500- |

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