# NKFlowEx Kit

Cat.No. ED7078

## Description

The NKFlowEx Kit is intended for the **detection of CD69 activation marker** of Natural killer (NK) cells after their activation with variety of stimuli in human heparinized whole blood using flow cytometry.

Natural killer cells represent one of the three major types of lymphocytes (T cells, B cells and NK cells). NK cells play critical role in the innate immune system providing rapid response to protect the body from both tumors and pathogens at the site of infection. They recognize and kill infected or transformed cells, but do not usually harm normal cells.

Transmembrane signal transduction generated by interactions between NK cell activating and inhibitory receptors and molecules of ligands which are expressed on the surface of other cell regulate NK cell activation. By recognizing changes or even absence of surface class I MHC molecules, NK cell can distinguish infected and/or stressed cell from normal cell and kill the target cell subsequently. In order to prevent NK cell activation and attack of normal cell, activating signals must be blocked by inhibitory signals. Generally, NK cell activation is regulated by balance between activating and inhibitory signals [1].

Five NK cell subtypes can be recognized in human peripheral blood based on the relative expression of surface markers CD16 (FcγRIIIa, the Fc receptor of immunoglobulin G) and CD56 (NCAM glycoprotein molecule with cell to cell adhesion role) [2], [3], [4]. In combination with other specific surface markers like CD45 (LCA – Leukocyte common antigen) and CD3 (TCR receptor complex component) it is possible to distinguish populations of NK cells from other lymphocytes in human peripheral blood sample.

When stimulated with variety of stimuli like interleukin-2, interferon-alpha, mitogens (PWM, PMA), cell antigens (K562, JAR) or viral infections [5], [6] NK cells ($CD56^+$, $CD16^+$, $CD45^+$, $CD3^-$) express CD69 activation marker (C-type lectin-like receptor) on their surface. Analysis of expression of CD69 marker thus can answer the question what influence particular stimulus has on activation of human natural killer cells.

This test is based on the detection of CD69 activation marker on the surface of NK cells after stimulation of human heparinized whole blood with Stimulation Control (PWM) and/or other stimulus. Negative control blood sample, where no stimulating reagent is used, shows no expression of CD69 marker on NK cells.

After 24 – 48 h incubation with stimulation reagents the tested blood samples are subjected to the staining with cocktail of monoclonal antibodies labeled with different fluorochromes (anti-CD45 FITC / anti-CD16+CD56 PE / anti-CD3 PE-Cy™5 / anti-CD69 PE-Cy™7) which specifically bind to the antigenic determinants expressed on the surface of leukocytes. The specific staining of blood cells is followed by a lysis of red blood cells. Afterwards, unaffected leukocytes are subjected to analysis by a flow cytometer. The fluorochromes attached to the monoclonal antibodies are excited via laser beam and subsequent emissions of light from the fluorochromes of each cell are collected and analyzed by a flow cytometer. The fluorescence intensity differences enable the separation of cell subsets based on the expression of analyzed antigens.

## Specification

**Staining Reagent** (Ready-to-Use) contains premixed antibody cocktail.
**Stimulation Control** (Lyophilized) contains lyophilized lectin PWM (Pokeweed mitogen).
**Lysing Solution** (10x concentrated solution) contains solution of fixation-lysing reagent.

## Reagents provided

| | |
|---|---|
| ED7078-1 | Staining reagent, anti-CD45 (MEM-28), FITC labeled / anti-CD16 (3G8) + CD56 (LT56), PE labeled / anti-CD3 (UCHT-1), PE-Cy™5 labeled / anti-CD69 (FN50), PE-Cy™7 labeled, 1 x 0.5 ml, intended for 50 tests. |
| ED7078-2 | Stimulation Control, 2 vials, intended for 13 tests each. |
| ED7078-3 | Lysing Solution, 1 x 10 ml, intended to prepare 100 ml of 1x Lysing Solution = 222 tests. |

## Materials required but not provided

Deionized water ($dH_2O$)
Culture medium suitable for cultivation of blood samples (X-VIVO 10, Lonza, cat. no. BE04-380Q recommended)
5ml test tubes (12 × 75 mm)
96 well tissue culture test plates

## Storage and handling

Store the NKFlowEx Kit at 2-8 °C. Expiration date is stated on reagent labels and on the NKFlowEx Kit box.

## Warnings and precautions

- Intended for research use only.
- Do not use reagents after expiration date stated on vial labels.
- Avoid contamination of the reagents.
- Avoid prolonged exposure of the Staining Reagent (ED7078-1) to light.
- Any non-performance of assay procedure may produce false results.
- Blood samples are considered as potentially infectious and must be handled with care.
- **Stimulation Control** (ED7078-2) contains lectin of Phytolacca americana (pokeweed mitogen).
  H-phrases
  H317: May cause an allergic skin reaction.
  H334: May cause allergy or asthma symptoms or breathing difficulties if inhaled.
  P-phrases
  P201: Obtain special instructions before use
  P261: Avoid breathing dust.
  P280: Wear protective gloves.
  P342 + P311: If experiencing respiratory symptoms: Call a POISON CENTER or doctor/physician.
  P501: Dispose of contents/container to authorized facility for dangerous wastes.
- **Lysing Solution** (ED7078-3) contains formaldehyde, methanol, and diethylene glycol.
  H phrases
  H302+312+332: Harmful if swallowed, in contact with skin or if inhaled.
  H315: Causes skin irritation.
  H317: May cause an allergic skin reaction
  H319: Causes serious eye irritation.
  H335: May cause respiratory irritation.
  H351: Suspected of causing cancer
  H371: May cause damage to organs.
  H373: May cause damage to organs (kidney) through prolonged or repeated exposure if swallowed.
  P phrases
  P270: Do not eat, drink or smoke when using this product.
  P280: Wear protective gloves / protective clothing / eye protection / face protection.
  P301+P312: IF SWALLOWED: Call a POISON Center or doctor/physician if you feel unwell.
  P302+P352: IF ON SKIN: Wash with plenty of soap and water.
  P305+P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
  P501: Dispose of contents/container to authorized facility for dangerous wastes.
- See product Safety Data Sheet for full information on the potential hazards and how to work safely with the product.
- Concentrations of labeled antibodies in Staining Reagent were optimized to offer the best specific signal/non-specific signal ratio. Therefore, it is important to adhere to the reagent volume/sample volume ratio in every test.
- **Blood must be collected into a tube containing heparin.**
- Blood samples should be processed within 6 hours after collection.
- Blood samples from abnormal patients may exhibit abnormal values of positive cells.
- In case of a hyperleukocytose sample, it is recommended to dilute the blood sample with PBS before staining to obtain leukocyte density approximately $5 \times 10^6$ leukocytes/ml.
- Red blood cells from abnormal patients may be resistant to lysis using lysing solutions.
- Data may be incorrectly interpreted if fluorescent signals were compensated wrongly or if gates were positioned inaccurately.
- The flow cytometer should be calibrated on a routine basis using fluorescent microbeads to ensure stable sensitivity of detectors.
- Flow cytometer may produce false results if the device has not been regularly calibrated and maintained appropriately.

## Application

Measurement of Natural killer (NK) cells activation with variety of stimuli in human heparinized whole blood using flow cytometry.

### Reagent preparation

**Stimulation Control**
Aseptically reconstitute lyophilized Stimulation Control using 150 µl of recommended culture medium (X-VIVO 10). Aliquot unused reagent in appropriate volumes, freeze and store for later use at ≤ -20 °C. Avoid repeated freeze/thaw cycles.

**Lysing Solution**
Lysing Solution is a 10× concentrated and must be diluted 10× with deionized water prior to use (1 volume of concentrated solution with 9 volumes of deionized water). The prepared solution (1× concentrated) is stable for 1 month when stored at room temperature.

### Required for handling

Vortex mixer
Racks for the test tubes
Automatic pipettes with disposable tips
Cell/tissue culture incubator ($CO_2$, 37 °C)
Laminar flow tissue culture cabinet
Flow cytometer - blue laser excitation 488 nm and proper emission filters.

### Procedure

For the stimulation of blood samples prepare standard tissue culture test plate and add tested blood into individual wells together with appropriate reagents for **negative control sample**, **positive control sample** and for the **sample to be stimulated with stimulus of your choice**.

1. Add aseptically into individual wells of tissue culture test plate the following reagents for:
   - **negative control sample:** 100 µl of culture medium suitable for cultivation of blood samples + 100 µl of blood sample,
   - **positive control sample:** 10 µl of reconstituted Stimulation Control + 90 µl of culture medium suitable for cultivation of blood samples + 100 of µl blood sample.
   - **stimulated sample:** 10 µl of **stimulus of your choice** + 90 µl of culture medium suitable for cultivation of blood samples + 100 of µl blood sample.

   Cultivate the tissue culture test plate with prepared blood samples for 24 – 48 h at 37 °C and 5 % $CO_2$ atmosphere in cell/tissue culture incubator.

For the examination of blood samples after stimulating step prepare test tubes for **negative control sample**, **positive control sample** and for the **sample stimulated with stimulus of your choice**.

2. Add 10 µl of Staining Reagent into the tubes destined for:
   - **negative control sample**
   - **positive control sample**
   - **stimulated sample**
3. Add 50 µl of blood samples from tissue culture test plate wells into test tubes according to corresponding designation. Mix the tubes with a vortex mixer.
4. Incubate the tubes for 15-20 minutes at room temperature in the dark.
5. Add 450 µl of diluted lysing solution per test tube. Mix the tube with a vortex mixer.
6. Incubate for about 5-10 minutes, until the blurry blood sample solution becomes clear.
7. Analyze the samples immediately using flow cytometer or store the samples at 2-8 °C in the dark and analyze within 24 hours. No further cell fixation is required. See example data in chapter Analysis of samples.

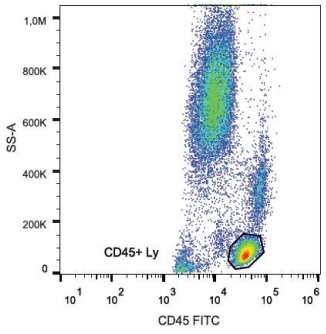
### Flow Cytometric Analysis

Analyze stained samples using flow cytometer equipped with excitation laser 488 nm and proper filters. In order to analyze sufficient number of NK cells (~ 2,000) acquire at least 10,000 –20,000 leukocytes (CD45+ gating region) per sample. Compensate fluorescent signals prior to or after data acquisition. Single reagents necessary to set up compensation matrix are available from EXBIO Praha, a.s.

### Analysis of samples

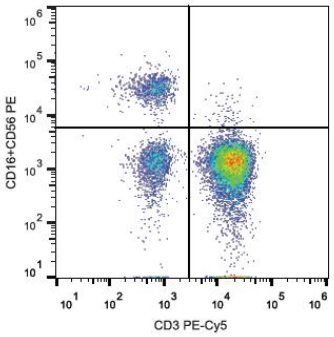
Visualize compensated data of negative control sample, positive control sample and stimulated sample in the side-scatter (SSC) versus CD45 FITC dot-plot. Set the gate around lymphocyte population as shown in figure 1.

*Fig. 1 Delimitation of $CD45^+$ lymphocyte population.*



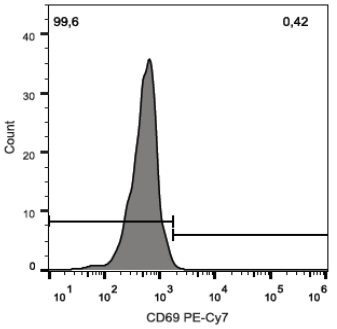

Visualize $CD45^+$ lymphocytes in a dot-plot CD16+56 PE versus CD3 PE-Cy™5 and separate populations using appropriate gate of natural killer (NK) lymphocytes situated in upper-left quadrant ($CD16+56^+$, $CD3^-$) and T lymphocytes situated in right quadrants (CD3+) on the dot-plot as shown in figure 2.

*Fig. 2 $CD45^+$ lymphocytes in dot-plot CD16+ CD56 PE vs. CD3 PE-Cy™5.*




Finally, plot population of NK cells ($CD16+56^+$, $CD3^-$) as cell count (number of events) versus CD69 PE-Cy™7 in histogram. The data in the histogram for negative control sample are used to generate the discrimination gates defined as negative and positive in the first histogram as shown in figure 3.

*Fig. 3 Negative control sample - population of NK cells ($CD16+56^+$, $CD3^-$) as cell count vs. CD69 PE-Cy™7.*




It is essential in order to set appropriate gates and calculate the percentage of CD69 positive NK cells (activated cells) in positive control sample as shown in figure 4 and stimulated sample as shown in figures 5 and 6.

*Fig. 4 Positive control sample - NK cells stimulated with Stimulation Control.*

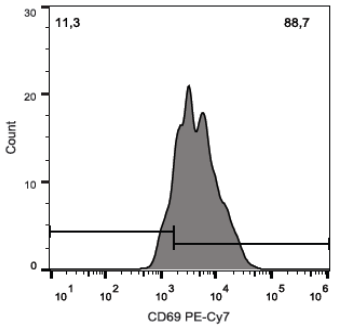



*Fig. 5 NK cells stimulated with trophoblast cell.*

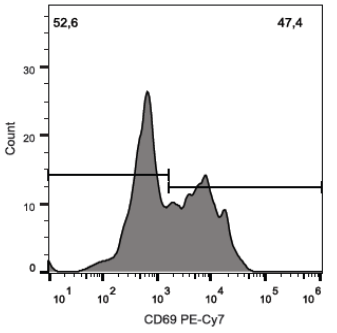
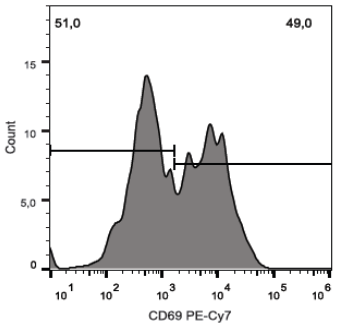


*Fig. 6 NK cells stimulated with sperm proteins.*





### References

**1.** Abbas A.K., Lichtman A.H., Pillai S. Cellular and Molecular Immunology, 6th Edition, 2007.
**2.** Caligiuri M. Human natural killer cells. Blood 112, 461-469 (2008).
**3.** Poli A. et al. CD56bright natural killer (NK) cells: an important NK cell subset. Immunology 126, 458-465 (2009).
**4.** Béziat V. et al. CD56brightCD16+ NK Cells: A Functional Intermediate Stage of NK Cell Differentiation. J. Immunol. 186, 6753-6761 (2001).
**5.** Werfel T. et al. Rapid expression of the CD69 antigen on T cells and natural killer cells upon antigenic stimulation of peripheral blood mononuclear cell suspensions. Allergy 52, 465-469 (1997).
**6.** Dons'koi B. V. et al. Measurement of NK activity in whole blood by the CD69 up-regulation after co-incubation with K562, comparison with NK cytotoxicity assays and CD107a degranulation assay. J. Immunol. Methods 372, 187-195 (2011).


### Manufacturer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### Trademarks



### Revision History

• Version 1, ED7078_TDS_v1
Initial Release
• Version 2, ED7078_TDS_v2
Volume of ED7078-1 Staining Reagent kit component changed from 1.0 ml to 0.5 ml.
Volume of added ED7078-1 Staining Reagent into the testing tubes changed from 20 µl to 10 µl.
• Version 3, ED7078_TDS_v3
Manufacturer postal code changed from 25242 to 25250.
• Version 4, ED7078_TDS_v4
The company logo changed. TDS layout changed. “Keep away from sunlight” and “For Research use only” symbols were added to Symbols.
• Version 5, ED7078_TDS_v5
In the Trademarks section was changed from GE Healthcare to Cytiva.

### Symbols

| | |
|---|---|
| REF | Catalog number |
| LOT | Batch code |
| | Use-by date |
| | Temperature limits |
| | Consult instructions for use |
| | Keep away from sunlight |
| | Manufacturer |
| RUO | For Research use only. Not for use in diagnostic or therapeutic procedures. |

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# NKFlowEx Kit

## **50 tests |** Cat.No. **ED7078**

**For Research use only.**
**Not for use in diagnostic or therapeutic procedures.**

Technical Data Sheet

Version ED7078_TDS_v5_EN
Date of Issue: 11-01-2021

EN