

NKTEST®

TEST KIT FOR THE QUANTIFICATION OF THE CYTOTOXIC ACTIVITY OF NATURAL KILLER (NK) CELLS

For *in vitro* diagnostic use.

**ORPEGEN Pharma
Gesellschaft für biotechnologische Forschung, Entwicklung und Produktion m.b.H.
Czerny-Ring 22, D-69115 Heidelberg, F.R.G.
Tel. 06221/9105-0, Fax 06221/910510, E-mail: info@orpegen.com**

Cryopreserved prestained K562 target cells and reagents for 100 tests. Cryopreserved K562 target cells in 5 aliquots, each for 20 assays/single day.

Please read the instructions carefully before use!

SUMMARY AND EXPLANATION

This test kit allows the quantitative determination of the cytotoxic activity of human natural killer (NK) cells. It contains cryopreserved prestained K562 target cells, complete medium and a DNA-staining reagent. The K562 target cells are labelled with a lipophilic green fluorescent membrane dye in order to discriminate effector and target cells. After the incubation period in the cytotoxicity assay, killed target cells are identified by a DNA-stain, which penetrates the dead cells and specifically stains their nuclei. By this way, the percentage of target cells killed by effector NK cells can be determined.

The evaluation of NK activity should be performed by flow cytometry. The detailed instructions result from specific experience and precise validation assays. Critical steps are in bold letters.

APPLICATIONS

This test is intended to investigate altered NK function found in various disorders and to evaluate the effects of drugs on NK activity.

Natural killer (NK) cells were originally described as "null" lymphocytes mediating cytotoxicity against certain tumors and virus-infected target cells, without requiring the presence of class I or II major histocompatibility complex (MHC) molecules on the target cell (1, 2). The majority (70 - 95%) of cells responsible for NK activity are morphologically large granular lymphocytes (LGLs) (3). NK cells express the CD56 NCAM antigen and the IgG Fc III receptor (CD16 antigen) and are therefore immunophenotypically identified as CD3⁻ and CD16⁺ and/or CD56⁺ (4). A small proportion of T cells (CD3⁺CD56⁺) also shows a substantial degree of NK activity (5).

Depressed NK-cell mediated cytotoxicity is one of the many immunological defects observed in patients with AIDS and AIDS-related complex (6). NK activity is also impaired in multiple sclerosis patients (7), in patients with systemic lupus erythematosus and with Sjögren's syndrome (8). Decreased NK-cell mediated cytotoxicity is observed patients with chronique fatigue syndrome (CFS) (9) and in Typ I diabetes patients (10).

A depression of NK cell activity is usually observed in patients with advanced cancer and with metastatic disease (11). Depression of NK cell activity is observed in patients with preleukemia, acute or chronic leukemia (12, 13). NK cell cytotoxic activity is of prognostic value for the probability of developing metastasis in patients with primary tumors (14).

NK cells play also a central role in the defence against virus infection. An enhanced NK cell activity has been observed during several acute virus infections (15).

Various immunomodulators (e.g., cytokines (interleukin-2, interferons) seem to augment cytotoxic activity *in vitro* (16, 17) and *in vivo* (18). This augmentation of NK cell activity can now be proven in an objective and reproducible manner with this testkit.

TEST PRINCIPLES

The NKTEST® kit contains cryopreserved K562 TARGET CELLS. This cell line derived from a patient with chronic myeloid leukemia in terminal blastic crisis (19) is the most sensitive and widely used target cell for human NK cells. K562 cells lack MHC class I and II antigens.

The basic requirement in any cytotoxicity assay is the ability to discriminate effector and target cell populations. This test kit contains cryopreserved K562 target cells prestained with a green fluorescent membrane dye. This dye remains in the membrane of the target cell thereby permitting a clear separation between effector and target cells.

The lytic process is characterized by the following main stages: recognition of target cells by effector cells, binding of effectors to targets, activation of the lytic machinery of effector cells and lytic effects on target cells. NK cells kill the target cells either by a perforin-granzyme-based mechanism, which results in a perforin-mediated loss of membrane integrity, or by the direct or indirect induction of apoptosis in target cells.

After incubation of effector and target cells a red fluorescent DNA dye is added to label the target cells permeabilized by NK activity. This dye labels only cells with compromised plasma membranes. In this way, a clear separation between four cell populations can be obtained: live target cells, dead target cells, live effector cells and dead effector cells. Thus, the actual ratio between effector and target cells can be confirmed. Routinely, only FL1 positive events (dead and live target cells) have to be collected for the determination of cytotoxic activity.

MATERIAL and REAGENTS

The test kit contains:

1. 5 vials of cryopreserved **prestained K562 TARGET CELLS**, store at -70°C.
2. 5 vials (300 µl) containing **INTERLEUKIN-2** (200 U/ml), store at -20°C or -70°C.
3. 5 bottles (100 ml) **COMPLETE MEDIUM** (ready to use), store at 4°C.
4. 1 bottle (5 ml) **DNA STAINING SOLUTION** (ready to use), store at 4°C.

The test kit does not contain the following material:

1. Blood collection tubes containing **heparin anticoagulant**.
2. Isopaque-Ficoll (Histopaque®, Sigma, #1077-1; Ficoll-Paque®, Pharmacia, #17-0840-02) or preparation of effector cells.
3. 12 x 75 mm disposable test tubes (Falcon, Becton Dickinson # 352052).
4. 15 ml disposable test tubes (e.g. Costar #3215 or Falcon #2095).
5. 50 ml disposable test tubes (e.g. Costar #3250 or Falcon #2070).
6. Sterile PBS (Phosphate-buffered saline).
7. Ice bath with cover.
8. Serological pipets (1, 5, 10 ml).

Required apparatus:

1. Variable volume micropipettes 20 - 200 µl (e.g., Eppendorf) and pipette tips.
2. Waterbath.
3. CO₂ incubator at 37°C.
4. Digital thermometer.
5. Vortex mixer.
6. Hemacytometer chamber and coverslip or electronic particle counter.
7. Centrifuge with swinging buckets and 12 x 75 mm tube carriers.
8. Flow cytometer (e.g., FACScan™, Becton Dickinson) with 488 nm excitation wavelength (argon-ion laser).

STORAGE AND STABILITY

The components have to be stored separately at different temperatures.

The **COMPLETE MEDIUM** and the **DNA STAINING SOLUTION** has to be stored at **4°C** (in refrigerator). Store **INTERLEUKIN-2** at **-20°C** or at **-70°C**.

The **K562 TARGET CELLS** have to be stored at least at **-70°C**. The cells are stable for 12 weeks showing a viability > 90%.

ASSAY PROCEDURE

1. Preparations:

- 1.1. Prewarm water bath to 37°C (**precise temperature control!**).
- 1.2. Prewarm COMPLETE MEDIUM in water bath to 37°C.
- 1.3. Prepare ice bath for holding of samples prior measurement.
- 1.4. Switch on and calibrate the flow cytometer. The same amplifier adjustments as for immunofluorescence analysis with directly conjugated monoclonal antibodies are possible.

2. Isolation of effector cells

Collect **5 ml** of **heparinized blood from each patient or control person** by venous puncture. Obtain blood with standard aseptic techniques.

Prepare effector cells by a standard "Ficoll-Hypaque" (density 1.077) - procedure.

- 2.1 Fill a 15 ml test tube with 5 ml Ficoll solution. Dilute blood 1 : 2 with saline or PBS. Carefully layer the diluted blood sample on top of the Ficoll cushion.
- 2.2 Centrifuge for 20 min at 700 x g at room temperature (**without brake!**).
- 2.3 Remove the upper layer and collect the mononuclear cell layer from the interface using a clean serological pipette. Transfer the cells to a 15 ml test tube. Add 12 ml of PBS, vortex.
- 2.4 Centrifuge for 10 min at 250 x g at room temperature. Aspirate the supernatant without disturbing the cell pellet.
- 2.5 Resuspend the cell pellet in 1 ml of COMPLETE MEDIUM, determine the cell number in a hemacytometer and adjust the **cell concentration to 5 x 10⁶ cells per ml** in COMPLETE MEDIUM.

3. Thawing of K562 TARGET CELLS

Rapid thawing of K562 TARGET CELLS is essential for optimal recovery of viable cells.

- 3.1 Fill a 50 ml test tube with 50 ml prewarmed (37°C) COMPLETE MEDIUM.
- 3.2 Thaw 1 vial of cryopreserved target cells by rapid agitation in 37°C water bath. Thawing should be rapid (within 20 - 60 sec).
- 3.3 As soon as the ice is melted, remove the vial from the water bath. Transfer the cell suspension to the 50 ml centrifuge tube containing 50 ml of COMPLETE MEDIUM. Vortex or mix the tube gently.
- 3.4 Centrifuge the cell suspension at 120 x g for 5 min at room temperature. Discard the supernatant.

Resuspend the cell pellet in 1 ml of COMPLETE MEDIUM. Determine the cell number and adjust **cell concentration to 1 x 10⁵/ml** in COMPLETE MEDIUM.

4. Flow cytometric cytotoxicity assay

Effector cells (E) were mixed with K562 TARGET CELLS (T) at the desired E : T ratios ranging from 50:1 to 12.5:1 (**Table 1**). Effector cells and target cells were incubated in a final volume of 200 µl (**effector-target cell mixture**).

- 4.1 Pipette effector cells and target cell suspension (**test samples**) into 12 x 75 mm tubes according to the following scheme (Table 1). Add target cells alone to another tube. This target control tube has to be incubated and analysed in parallel to monitor the spontaneous cell death (**control sample**).

Table 1: Pipette scheme for control and test samples

Ratio E:T	Effector cell suspension (µl)	Target cell suspension (µl)	COMPLETE MEDIUM (µl)
50:1	100	100	0
25:1	50	100	50
12.5:1	25	100	75
Control	0	100	100

- 4.2 Add 30 µl Interleukin-2 (200 U/ml) to some cell aliquots ("**high control**" samples). Interleukin-2 should be added to the effector cell suspension prior to the addition of the target cell suspension.
- 4.3 Vortex all tubes. Centrifuge tubes for 2 - 3 min at 120 x g.
- 4.4 **Incubate** tubes for **120 min** or **240 min** in a humidified CO₂ incubator (in the water reservoir at the bottom of the incubator). At the end of the incubation, tubes were placed on ice until flow cytometric analysis.
- 4.5 Add **50 µl DNA STAINING SOLUTION per tube**, vortex and incubate 5 min at 0°C (light protected in ice bath). Measure the cell suspensions within 30 min after addition of the DNA STAINING SOLUTION!

5. Flow cytometric analysis

Cells were analysed with the flow cytometer using the blue-green excitation light (488 nm argon-ion laser, e.g., FACScan[™], LYSIS[™] II software or FACSCalibur[™], CELLQuest[™] software).

Measurement:

During data acquisition a "**live**" gate is set in the **FL1 histogram** on the **green fluorescent target cells** in order to discriminate effector and target cells. Use a target control tube for this purpose. Since the FL1 fluorescence intensity of dead target cells decreases to some extent, set the lower channel boundary as seen in **Fig. 1A**. Collect at least **2,500 target events per sample**.

Alternatively, effector cells can be excluded by using **fluorescence triggering in the FL1 channel** to gate on target cells.

Data evaluation:

The percentage of dead (= FL2 or FL3 positive) target cells is analysed. The red fluorescence of the DNA stain can either be detected by the FL2 or FL3 channel. For that purpose an **analysis gate** is set in the **FL3/FL1 dot plot** around all live and dead target cells (see **Fig. 1B**).

Percent specific cytotoxicity is determined by subtracting the percentage of dead cells in the incubated targets alone (target control tube, see **Fig. 2A**) from the percentage of killed target cells in the test samples (see **Fig. 2B, C**).

REMARKS

- Heparinized blood** should be processed **within 24 h of sampling**. **Blood samples** should **remain at room temperature** prior to processing.
- Duplicates or triplicates are useful in establishing the assay.
- Monocytes** can suppress NK cell cytotoxic activity. Therefore, it may be advantageous to **remove** monocytes by **plastic adherence** (1 h, 37°C) or by adherence to **nylon wool**. NK activity of peripheral blood mononuclear cells (PBMCs) is therefore lower than that of monocyte-depleted PBMCs.
- Flow cytometric NK assays identify lytic events before cell lysis. Therefore, the proposed test protocol recommends a target/effector cell **incubation time of 2 h**, but a 4h effector/target cell incubation is also possible. **Fig. 5** shows the **kinetics of the flow cytometric NK assay**.
- When evaluating NK activity at **different E : T ratios** it was found that **linearity in percent specific lysis** is usually observed at E : T ratios below 25:1 (see **Fig. 6**).

EXPECTED VALUES

The normal range of the NK cytotoxic activity was determined on peripheral blood mononuclear cells (PBMC) from healthy subjects. The results are shown in **Table 2** (incubation time of 2 h) and in **Table 3** (incubation time of 4 h).

Table 2: Normal range of NK activity after 2 h of incubation

E:T Ratio	% specific activity	% specific activity after addition of IL-2 (6 U/sample)
12.5:1	5.6 - 14.3 (Mean 9.9)	8.4 - 20.6 (Mean 14.5)
25:1	9.9 - 26.1 (Mean 18.0)	14.8 - 33.4 (Mean 24.1)
50:1	13.7 - 33.5 (Mean 23.6)	23.2 - 48.0 (Mean 35.6)

Table 3: Normal range of NK activity after 4 h of incubation

E:T Ratio	% specific activity	% specific activity after addition of IL-2 (6 U/sample)
12.5:1	8.6 - 21.3 (Mean 15.0)	13.2 - 37.7 (Mean 25.5)
25:1	13.8 - 34.8 (Mean 24.3)	24.0 - 54.2 (Mean 39.1)
50:1	18.4 - 43.8 (Mean 31.1)	33.6 - 67.6 (Mean 50.6)

PRECISION OF THE METHOD

The intra-assay precision of this flow cytometric NK assay was determined on triplicate samples of Ficoll-Paque separated effector cells from healthy subjects (n = 6).

	% Specific Lysis (E:T = 12.5:1)	% Specific Lysis (E:T = 25:1)	% Specific Lysis (E:T = 50:1)
Range of values	7.79 - 33.48	15.76 - 54.09	20.42 - 63.69
Average CV (%)	6.92	7.63	5.9
n	6	6	6

LIMITATIONS OF THE METHOD

- Each laboratory should establish its own range of normal values using its own test conditions.
- Samples ready for measurement without DNA STAINING SOLUTION are stable for 1 hour on ice. **After addition of the DNA STAINING SOLUTION, the samples should be measured within 30 min.**
- The viability of the K562 target cells is > 90 % for 12 weeks when stored at -70°C or below.

WARNINGS

- Blood samples must always be regarded as potentially infectious (hepatitis, HIV etc.)! Wear suitable gloves and protective clothing.
- The dye of the DNA STAINING SOLUTION might be carcinogenic!
- The DNA-dye contaminates pipettes and the sample delivery system of the flow cytometer as well. It might disturb future immunofluorescence analyses esp. in the case of phycoerythrin labelled antibodies. Diluted sodium hypochlorite (0.5 - 1.5 %) eliminates the DNA-dye contamination.

REFERENCES

- Reynolds, C.W. & Ortaldo, J.R. 1987. Natural killer activity: The definition of a function rather than a cell type. Immunol. Today 8: 172.
- Trinchieri, G. 1989. Biology of natural killer cells. Adv. Immunol. 47: 187-376.
- Grossi, C.E., Cadoni, A., Zicca, A., Leprini, A., Ferrarini, M. 1982. Large granular lymphocytes in human peripheral blood: Ultrastructural and cytochemical characterization of the granules. Blood 59: 277.

- (4) Hercend, t., Griffin, J.D., Bensussan, A., Schmidt, R.E., Edson, M.A., Brennan, A., Murray, C., Daley, J.F., Schlossman, S.F. & Ritz, J. 1985. Generation of monoclonal antibodies to a human NK clone: Characterization of two NK associated antigens NKH1 and NKH2, expressed on subsets of large granular lymphocytes. *J. Clin. Invest.* 75: 932-943.
- (5) Lanier, L., Le A., Civin, C., Loken, M. & Philipps, J. 1986. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J. Immunol.* 136: 4480-4486.
- (6) Rosenberg, Z.F. & Fauci, A.S. 1989. The immunopathogenesis of HIV infection. *Adv. Immunol.* 47: 377-431.
- (7) Neighbour, P.A. 1984. Studies of interferon production and natural killing by lymphocytes from multiple sclerosis patients. *Ann. N.Y. Acad. Sci.* 436: 181.
- (8) Sibbitt, W.L. & Bankhurst, A.D. 1985. Natural killer cells in connective tissue disorders. *Clin. Rheum. Dis.* 11: 507.
- (9) Caligiuri, M., Murray, C., Buchwald, D., Levine, H., Cheney, P., Peterson, D., Komaroff, A.L. & Ritz, J. 1987. Phenotypic and functional deficiency of natural killer cells in patients with chronic fatigue syndrome. *J. Immunol.* 139: 3306-3313.
- (10) Negishi, K., Gupta, S., Chandy, K.G., Waldeck, N., Kershner, A., Buckingham, B. & Charles, M.A. 1988. Interferon responsiveness of natural killer cells in type I human diabetes. *Diabetes Res.* 7: 49.
- (11) Kadish, A.S., Doyle, A.T., Steinhauer, E.H. & Ghossein, N.A. 1981. Natural cytotoxicity and interferon production in human cancer: Deficient natural killer activity and normal interferon production in patients with advanced disease. *J. Immunol.* 127: 1817.
- (12) Kerndrup, G., Meyer, K., Ellegaard, J. & Hokland, P. 1984. Natural killer (NK)-cell activity and antibody-dependent cellular cytotoxicity (ADCC) in primary preleukemic syndrome. *Leuk. Res.* 8: 239.
- (13) Srskaar, D., Frre, O., Albrechtsen, D. & Stavem, P. 1986. Decreased natural killer cell activity versus normal natural killer cell markers in mononuclear cells from patients with smouldering leukemia. *Scand. J. Haematol.* 37: 154.
- (14) Hersey, P., Edwards, A., McCarthy, W. & Milton, G. 1982. Tumor related changes and prognostic significance of natural killer cell activity in melanoma patients. In: "NK cells and Other Natural Effector Cells" (R.B. Herberman, ed.), p.1167. Academic Press, New York.
- (15) Cauda, R., Prasthofer, E.F., Grossi, C.E., Whitley, R.J. & Pass, R.F. 1987. Congenital cytomegalovirus: Immunological alterations. *J. Med. Virol.* 23: 41.
- (16) Herberman, R.B., Ortaldo, J.R. & Bonnard, C.D. 1979. Augmentation by interferon of human natural and antibody-dependent cell mediated cytotoxicity. *Nature* 277: 221.
- (17) Henney, C.S., Kuribayashi, K., Kern, D.E. & Gillis, S. 1981. Interleukin-2 augments natural killer cell activity. *Nature*: 291: 335.
- (18) Deehan, D.J., Heys, S.D., Ashby, J. & Eremin, O. 1995. Interleukin-2 (IL-2) augments host cellular immune reactivity in the perioperative period in patients with malignant disease. *Eur. J. Surg. Oncol.* 21: 16-22.
- (19) Lozzio, C.B. & Lozzio, B.B. 1975. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. *Blood* 45: 321-334.

NKTEST® is a registered trademark of ORPEGEN Pharma, Patents pending.

FIGURES

Fig. 1: Recommended gating on green fluorescent target cells

- A) Live Gate on green fluorescent target cells during data acquisition (FL1 histogram, control sample without effector cells)
- B) Narrow analysis gate (FL3 vs FL1 dot plot, test sample with effector cells)

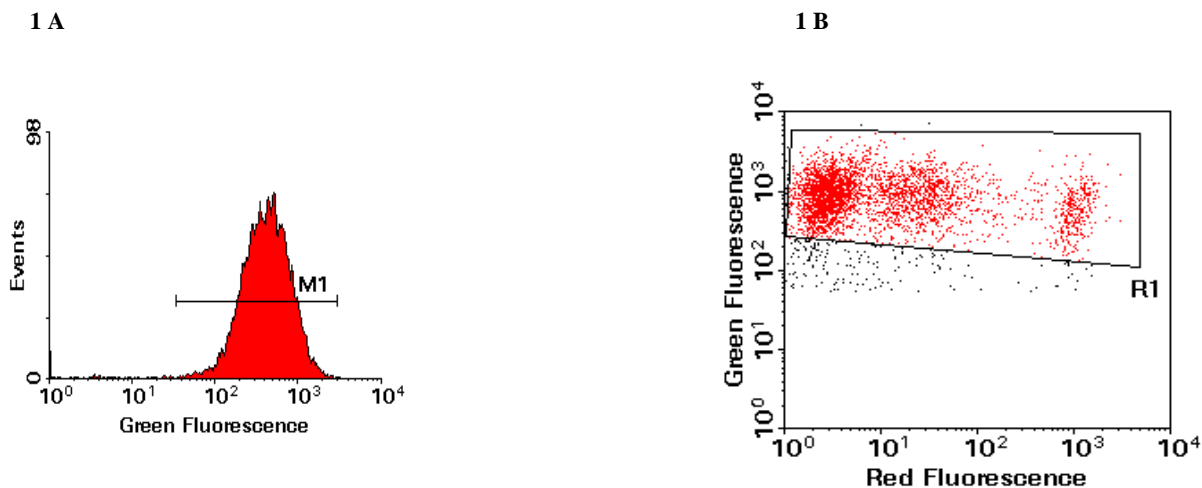


Fig. 2: Typical FL 3 histograms of the NK functional analysis (incubation time of 2 h at 37°C)

- A) Control sample, target cells alone (4.1% dead target cells)
- B) Test sample, effector target mixture (E:T ratio of 25:1, 40.7% dead target cells)
- C) "High control" sample, effector target mixture with added IL-2 (E:T ratio of 25:1, 54.6% dead target cells)

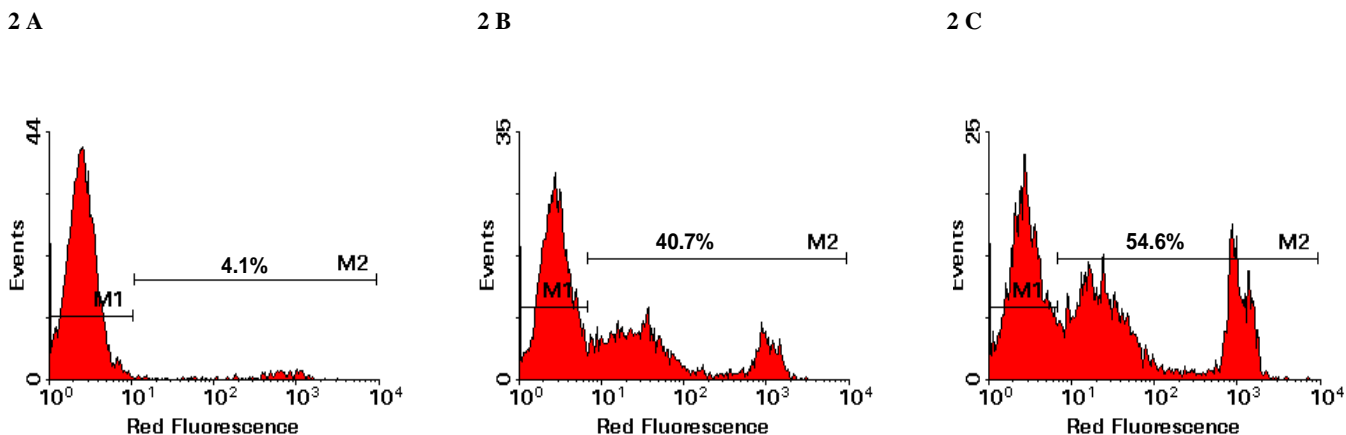


Fig. 3: Kinetics of the flow cytometric NK assay. At different time intervals percent specific lysis was determined for 4 donors at an E/T ratio of 50:1.

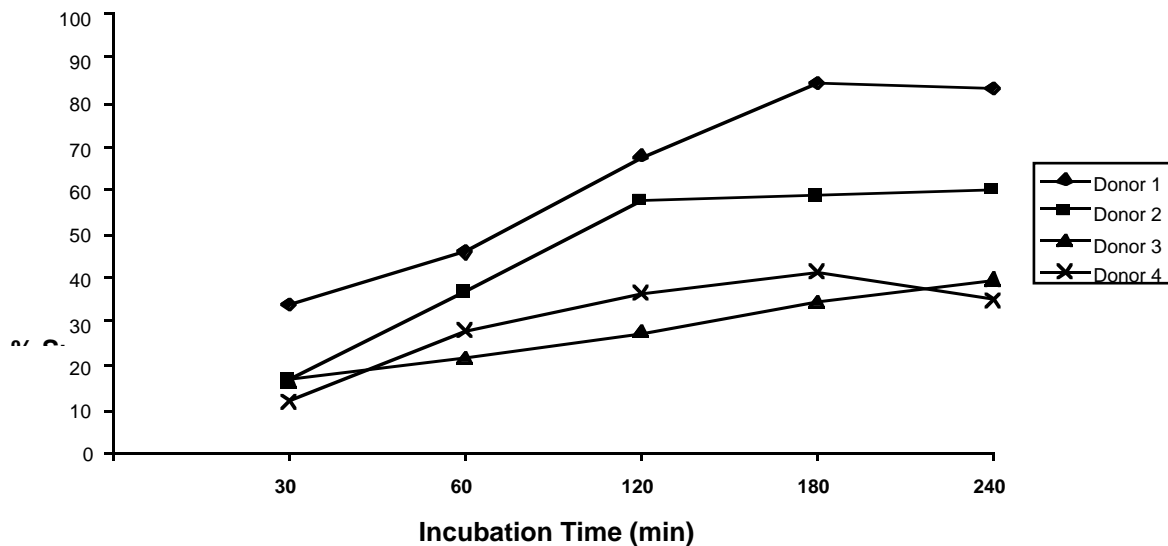


Fig. 4: Linearity of NK activity. NK activity was determined after an incubation time of 2 h at different E:T ratios (50:1, 25:1, 12:1, 6:1, 3:1).

