REF. Number : ATGBF001

Murine NK Activity Kit

FOR RESEARCH USE ONLY

1. INTENDED USE

The Murine NK Activity Kit is an IFN-γ quantitation assay for plasma samples collected and prepared with the NK Cell activating agent. Murine NK Activity Kit is intended for in vitro research use, for the monitoring of the immune status of individuals.

2. PRINCIPLE OF THE ASSAY

Murine NK Activity Kit employs a proprietary stabilized immunomodulatory cytokine (Activator) to stimulate NK cells in mouse whole blood in vitro. After their activation, a quantitative sandwich enzyme immunoassay (ELISA) is used to determine the levels of IFN-γ secreted. To this end, an anti-IFN-γ monoclonal antibody has been pre-adsorbed on a microwell plate. Samples are pipetted into the wells and IFN-γ allowed to bind to the immobilized antibody. After washing away all unbound material, a second anti-IFN-γ monoclonal antibody conjugated to a reporter enzyme (HRP) is added to the wells. Following a final wash to remove any unbound antibody-HRP complex, the substrate solution is added to the wells and color is allowed to develop. Absorbance at 450 nm is measured, and the amount of IFN-γ released by the NK cells is finally quantitated by comparison to an IFN-γ standard curve.

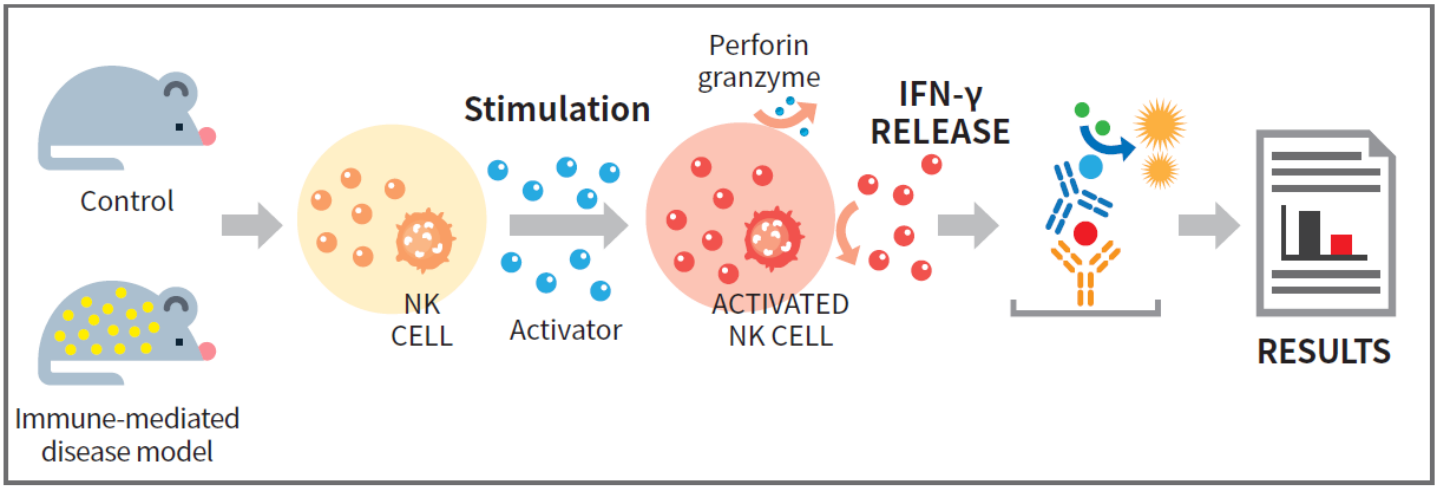

Figure 1. Principle of Murine NK Activity Kit.

3. REAGENTS AND STORAGE

3.1 Kit Components and Storage

Component	Quantity	Feature	Storage	Expiration Date
Activator (NK Cell activating agent)	3 vials (3 mL	Colorless liquid	- 20 °C Protect from light	12 months

Standard (recombinant mouse IFN-γ)	1 vial (4 ng)	Colorless liquid	- 20 °C Protect from light	12 months
Mab Coated Plate (Microwell plate coated with antibody against mouse IFN-γ)	1 plate (12 strips of 8 wells)	Colorless Polystyrene plate	2–8 °C Protect from light	12 months
Diluent (contains PBS)	1 bottle (10 mL)	Clear yellow liquid	2–8 °C Protect from light	12 months
Biotin Conjugate (100X) (biotin-conjugated murine monoclonal antibody against mouse IFN-γ)	1 vial (0.15 mL)	Clear orange liquid	2–8 °C Protect from light	12 months
Streptavidin HRP (100X)	1 vial (0.15 mL)	Clear orange liquid	2–8 °C Protect from light	12 months
Conjugate Diluent (contains bovine serum albumin)	1 bottle (13 mL)	Clear orange liquid	2–8 °C Protect from light	12 months
Washing Solution (20X) (contains polysorbate-20)	1 bottle (50 mL)	Colorless liquid	2–8 °C Protect from light	12 months
TMB Substrate (contains tetramethyl benzidine, TMB)	1 bottle (12 mL)	Colorless liquid	2–8 °C Protect from light	12 months
Stop Solution (contains 1N HCl)	1 bottle (12 mL)	Colorless liquid	2–8 °C Protect from light	12 months
Adhesive film for microwell plate	2 films	Clear film	N/A	N/A

Table 1. Kit Components and Storage Recommendations

3.2 Materials required but not provided

1. Blood collection tube
2. Blood incubation 96 well plate or tube
3. 37°C Incubator, CO_2 not required.
4. Adjustable, automatic micropipettes (P200 and P1,000, or similar)
5. 8- or 12-channel multi-pipette, able to deliver 50 µL, 100 µL (for samples and standard), and 300 µL (for washing, optional).
6. Disposable pipette tips
7. Tabletop microcentrifuge (able to deliver 11,500 x g)
8. Graduated cylinder (500 or 1000 mL); vortex mixer; microtube rack; microwell shaker (optional)
9. Double-distilled water, or equivalent or higher grade (e.g., Milli-Q grade)
10. Aspiration pump or automatic microplate washer (optional)
11. Microplate reader, set to read at 450 nm (with a minimum dynamic range of 0-3.0; 0-3.5 recommended). It is highly recommended to also read at 600-650 nm (correction wavelength)
12. Heparin

4. PRECAUTIONS

1. For in vitro research use only.
2. Use only after fully reading and understanding these guidelines.
3. The TMB Substrate contains 3,3',5,5'-tetramethyl benzidine, a suspected carcinogen which can be harmful by ingestion, inhalation and eye or skin contact. Use eye protection, wear gloves, and handle with care.

4. The Stop Solution contains 1N Hydrochloric acid (HCl) which can be harmful by ingestion, inhalation, and eye or skin contact. Use eye protection, wear gloves and normal laboratory protective clothing. If the Stop Solution contacts skin or eyes, rinse generously with water and seek medical attention.
5. Conjugate Diluent contains bovine serum albumin that can cause allergic reactions. Avoid skin contact.
6. Handle mouse blood as if potentially infectious. Observe relevant blood handling guidelines. Wear eye protection, disposable gloves, and wash hands thoroughly after use.
7. DO NOT use kit if any component shows signs of damage or leakage.
8. When opening the lid/cap of any reagent tube/bottle or blood samples, or when removing their contents, use GLP procedures, to avoid microbial contamination or spraying the surroundings.
9. DO NOT mix reagents/components from different kit lots.
10. DO NOT use expired Murine NK Activity Kit components.
11. When using equipment such as a plate washer or a plate reader, ensure it has been properly calibrated through a regular maintenance schedule.
12. When pipetting samples or reagents, use new disposable tips and regularly calibrated pipettes.
13. Discard solid waste, unused reagents and biological samples in accordance with Local, Provincial, and Federal regulations.
14. Follow general laboratory safety guidelines.
15. The researcher should optimize the precise conditions for a particular assay.
16. Drawing the blood using EDTA is not recommended.

5. ASSAY PROCEDURE

Bring all reagents and samples to room temperature (20-25℃) before use. It is recommended that all samples, standards, and control be assayed in duplicate.

5.1 Step 1: Reagent preparation

1. Washing solution (20X) must be diluted 1:19 in advance into purified water (distilled or deionized water). E.g., for a full plate, 50 mL of concentrated Washing solution(20X) must be diluted into 950ml of water to make a total of 1,000 mL of 1X solution. Diluted Washing solution is stable at room temperature (20℃~25℃) for 3 months if stored in a tightly closed bottle. If crystals appear upon storage, warm up the container at 37°C in a water bath or incubator to re-dissolve(DO NOT use a microwave).

2. Aliquot Activator in one-time use quantities, and store at -20°C.

3. Aliquot reconstituted standard in one-time use quantities, and store at -20°C (Recommended 250µl aliquots once reconstituted, but smaller aliquots are possible). DO NOT use more than once.

5.2 Step 2: Collection and culture of blood, and harvesting of induced plasma

1. Blood collection: Collect whole blood in test tube containing anticoagulant.

   • Anesthetic is not recommended during blood collection as it may affect the results.

2. The collected the blood should be mixed with anticoagulant.
3. Aliquot 30 µL of Activator into micro-centrifuge tube or 96-well plate. (not provided)
4. Add 100 µL of whole blood into wells (or tubes) containing Activator and mix gently by repeated pipetting.

   • Collected blood samples have to be treated with Activator within 1 hours after collection. If not, samples should be discarded.

   • Lipemic or hemolyzed samples should be discarded.

   • Before blood culture, please make sure blood sample is mixed and homogenized.

5. Close the lid (not provided) of plate.

   • Avoid using adhesive film for proper air supply

6. Incubate the 96-well plate containing the samples for 24 hours at 37℃.
   (Depend on strain, such as ICR, it is recommended to adjust incubation time between 8 to 24 hours.)
7. After incubation, carefully remove the adhesive film from the plate and transfer cultured samples to new micro-centrifuge tubes.

8. Centrifuge the samples for 15 minutes at 1,000g, then carefully transfer separated supernatant (plasma) to new tubes.

   • Cultured sample (plasma) can be kept refrigerated at 2~8℃ for up to 3 days, or frozen at -20~-70℃ for up to 3 months.

5.3 Step 3: Murine IFN-γ ELISA assay

General recommendations:

- Ensure in advance that all standards and samples can be promptly loaded onto the plate (within 15 minutes). This will avoid significant variations due to the time gap between loading the first and last microwell plate.
- Plasma samples, antibody-coated microwell strips and all reagents must be brought to room temperature (20-25°C) before use.
- It is very important to ensure that plasma samples are completely thawed and centrifuged at 11,500g for 1 min at room temperature (20-25°C) just before loading them into the ELISA well.
- Microwell strips that are not required must be promptly returned to the foil pouch with desiccant, resealed, and returned to the refrigerator for storage until required.
- Washing solution (20X) must be diluted 1:19 in advance into purified water (double-distilled or higher grade). E.g., for a full plate, 50 mL of concentrated (20X) washing solution must be diluted into 950 mL of water to make a total of 1,000 mL of 1X solution. Diluted washing solution (1X) is stable at room temperature (20-25℃) for 3 months if stored in a tightly closed bottle. If crystals appear upon storage, warm up the container at 37 °C in a water bath or incubator to re-dissolve (DO NOT use a microwave).
- Never leave the strip wells empty or to dry out. Always have the next solution to be pipetted prepared beforehand.

Protocol:

1. Each vial of standard must be brought to room temperature (20-25°C) at least 30 minutes prior to use. The concentration of standard in stock solution is 4000 pg/ml.

   • The remaining volumes of standard can be aliquoted for further use and stored frozen for up to three months (-20°C or colder).

2. Label eight 1.5 mL microcentrifuge tubes from #1 to #8 to generate the dilutions for the standard curve. Serial-dilute the standard solution as follows (see Figure 2): Pipet 200 µL of Diluent into tube #8 to 1, as shown in the diagram below. Make a serial dilution of standard (4,000 pg/mL) starting by transferring 200 µL of it into tube #8, and subsequently 200 µL from tube #7 to #6, as shown in the diagram, and mixing well after each transfer. DO NOT transfer any further volume into tube #1 (0 pg/mL).

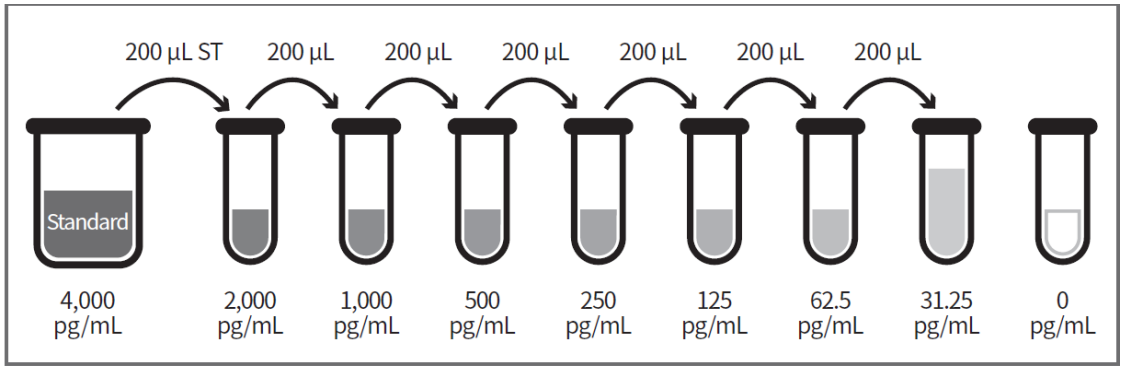

Figure 2. Preparation of standard curve dilutions

3. Prepare 2 coated wells (duplicate) for each of the eight standard curve dilutions (2,000 to 0 pg/mL) and enough wells for all specimens to be tested.
4. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal, and keep refrigerated.
5. Aliquot 50µL of Diluent into each well.

6. Add 50µL of standard and 50µL of sample to wells containing Diluent. Cover with the adhesive strip provided and lightly tap the frame to ensure proper mixing. Incubate for 1 hour at room temperature (20-25°C).
7. Aspirate each well and wash, repeating the process for a total of four (4X) washes. Wash by filling each well with Wash Buffer (300µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
8. Dilute equal parts of Biotin-Conjugate and Streptavidin-HRP in proportion of 1:99 with Conjugate Diluent. For example: 100µL Biotin-Conjugate + 100µL Streptavidin-HRP + 9.8mL Conjugate Diluent = 10mL of Detection antibody conjugate.

   • Prepare detection antibody conjugate for single use (ie. Biotin-Conjugate, Streptavidin-HRP, Conjugate Diluent) right before performing the assay; not in advance

   • DO NOT re-use or store prepared detection antibody conjugate.

9. Add 100µL of detection antibody conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature (20-25°C).
10. Repeat the aspiration/wash as in step 7.
11. Add 100µL of TMB Substrate to each well. Incubate for 30 minutes at room temperature (20-25°C). Protect from light.
12. Add 100µL of Stop Solution to each well. The color in the well should change from blue to yellow. If the color in the well is green or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
13. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. It is highly recommended to simultaneously read at 600-650 nm as correction wavelength.

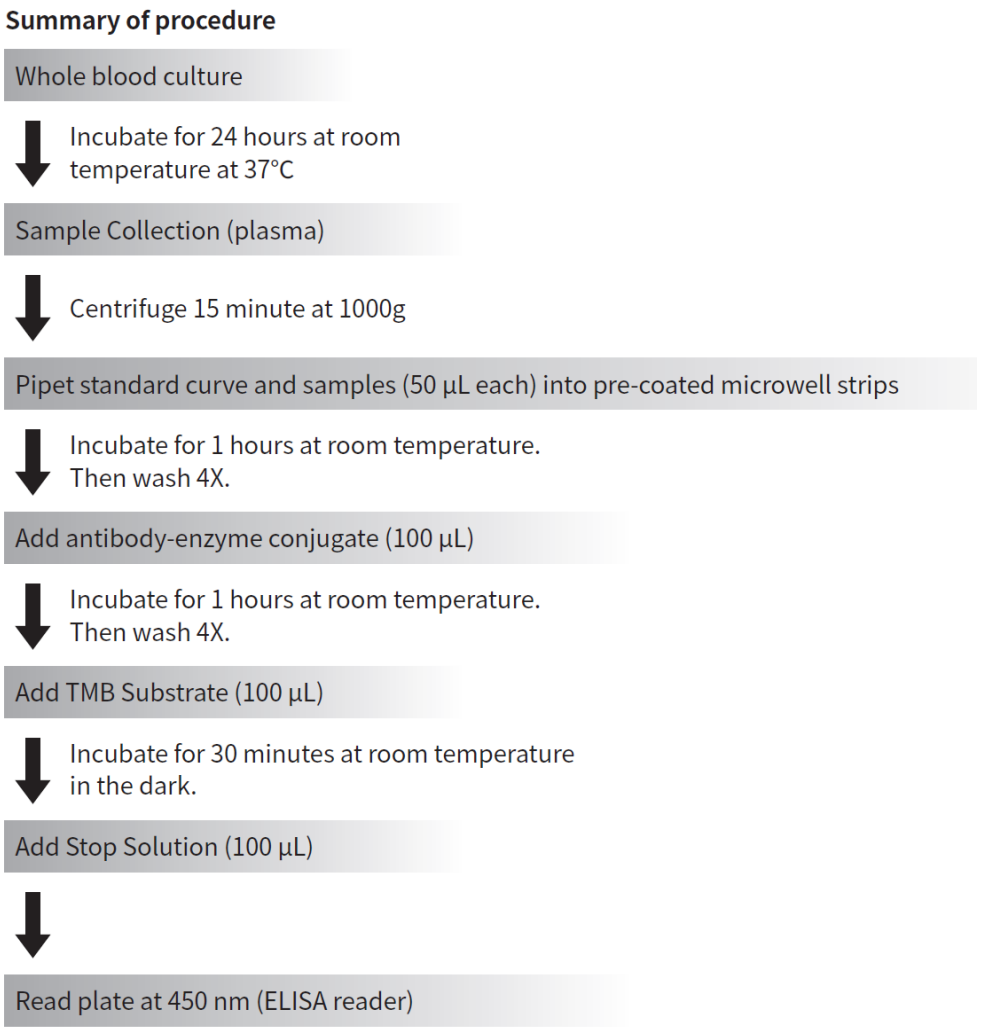

Figure 3. Summary of procedure

6. CALCULATION AND RESULT ANALYSIS

Standard curve calculation:

Plot the standard curve. The X axis shows the concentration of standard solution, in pg/mL, and the Y axis the absorbance at 450 nm. To determine the unknown IFN-γ concentrations in the samples, find the absorbance value of the unknown on the y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the corresponding IFN-γ concentration. If the samples were diluted, multiply by the appropriate dilution factor. All data processing and calculations can be carried out using software packages available with microwell plate readers, standard spreadsheets (e.g., MS Excel) or common statistical software (e.g., GraphPad, Sigma Plot). If a test sample's absorbance value falls outside the standard curve ranges, that test sample needs to be reanalyzed at a higher or lower dilution as appropriate.

Example:

This standard curve was generated at NKMAX for demonstration purposes only. A standard curve must be run with each assay.

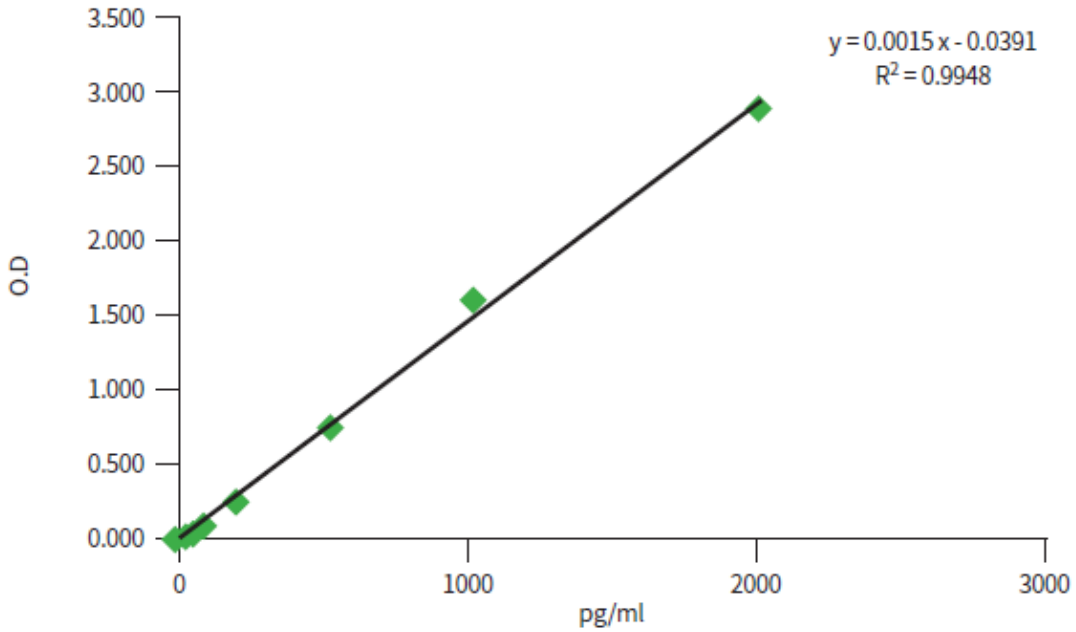

Figure 4. Example of standard curve

Tip: if a sample shows an IFN-γ concentration >2,000 pg/mL, and there is a need to determine its precise concentration, dilute the plasma sample 1:10 with Diluent and re-assay by ELISA.

Quality Control:

The accuracy of test results depends on the generation of an accurate standard curve. Therefore, results derived from the standards must be consistent before test sample results can be interpreted.

For the ELISA to be valid:

(1) Raw absorbance value (not blanked) of 2,000pg/mL must be ≥1.2

(2) Raw absorbance value (not blanked) of 0pg/mL must be ≤0.2

(3) The correlation coefficient (R2) of the linear regression of the standard curve must be ≥0.98

If these conditions are not satisfied, the test results are not valid and a new test must be conducted.

Calculation of Sample Concentration:

Calculate the concentration of the samples using the average of the absorbance values and the equation generated by linear regression analysis (y=ax+b). For instance, if the latter is y=0.0015X-0.0391 (as shown in the example), and the sample average absorbance is 1.516, then the sample concentration is calculated as follows;

Sample concentration (pg/mL) = (average absorbance of the sample-b) / a

Sample concentration (pg/mL) = (1.516+0.0391) / 0.0015 = 1036.73 (pg/ml)

7. TROUBLESHOOTING

Problem	Possible cause
High Background	• Background wells were contaminated. • Matrix used had endogenous analyte. • Adhesive film improperly used or sealed. • Plate was insufficiently washed. • TMB Substrate Solution was contaminated.
No signal	• 'Activator' was left for too long at room temperature before blood collection causing 'Activator' inactivation • Blood was contaminated. • Blood was contained Immunosuppressant reagent. • Incorrect or no antibodies were added. • Streotavidin HRP was not added. • Substrate solution was not added. • Wash buffer contained sodium azide.
Low or poor signal for the standard curve	• Standard was stored improperly. • Standard dilution error may have occurred. • Reagents were added to wells with incorrect concentrations. • Plate was incubated with inappropriate temperature, timing or agitation.
Signal too high, standard curves saturated	• Standard dilution error may have occurred. • One or more reagent incubation steps were too long. • Plate was incubated with inappropriate temperature, timing, or agitation
Sample readings out of range	• Samples contain no or below detectable levels of analyte. • Samples contain analyte concentrations greater than highest standard point.
High variations in samples and/or standards	• Pipetting errors may have occurred. • Plate washing was inadequate or non-uniform. • Samples were not homogenous. • Samples or standard wells were contaminated.

8. TECHNICAL SERVICE

For customer technical service please contact:

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