BlueKit™
Powered by Hillgene

Ver202409

Instructions for Use of Cell Cytotoxicity Assay Kit (Adherent Target Cells)

Cat.No. HG-CKK002

CONTENTS

1. Product Description

Cell Cytotoxicity is a critical quality attribute (CQA) for the biological activity of immune cell therapy products (e.g., NK cells, CAR-NK cells, Tm cells, CAR-T or TCR-T cells, etc.), and it is an important element of product quality research and quality control. Compared with cell cytotoxicity methods such as lactate dehydrogenase (LDH) assay, 51Cr release assay, luciferase reporter gene assay, RTCA assay, the detection of CFSE- and 7-AAD-labelled cell killing by flow cytometry is safer, more convenient, faster and more stable.

The Cell Killing Assay Kit (Suspension Target Cells) is used to determine the cytotoxicity of effector cells to relevant target cells and is suitable for the detection of target cell killing in suspension culture. It consists of CFSE (Carboxyfluorescein Succinimidyl Ester, Carboxyfluorescein Diacetate Succinimidyl Ester, a green fluorescent dye that can penetrate cell membranes and is used to identify target cells in mixed cell populations), 7-AAD (7-Amino Actinomycin D, which binds to the DNA of membrane-damaged cells and is used to label dead cells) , medium A and Assay Buffer composition.

Immune effector cells are co-cultured with CFSE-labelled target cells for a certain period of time and then labelled with 7-AAD. Since CFSE and 7-AAD have different spectra, it is possible to separate target cells from effector cells by flow cytometry and to identify live and dead cells. The cell populations were divided into four categories: CFSE-7-AAD- for live effector cells, CFSE-7-AAD+ for dead effector cells, CFSE+7-AAD- for live target cells, and CFSE+7-AAD+ for dead target cells, thus allowing the assessment of the killing activity of the immune cells at the single-cell level.

2. Application

It is suitable for the evaluation of the killing function of immune cell products such as NK cells, CAR-NK cells, CAR-T cells, TCR-T cells, Tm cells, etc. on different tumour cell lines (adherent target cells).

3. Specifications

1 Kit

4. Components

Cat.	Components	Specification	Temperature	Position
HG-MD002	Medium B	100 mL	2~8°C	Kit I
HG-FW001	Assay Buffer	100 mL	2~8°C	
HG-CFSE	CFSE	1 vial	-20°C	Kit II
HG-7-AAD	7-AAD	1 vial	-20°C	

△ *Note：Before the experiment, please read the instructions carefully and check the reagents in the kit.*

5. Storage Conditions and Expiry Date

Each component of the kit is stored according to the corresponding storage temperature, and the expiry date is detailed on the outer packaging of the kit.。

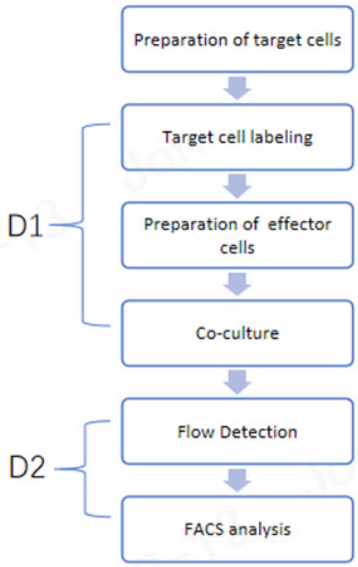
△ Note：CFSE and 7-AAD dyes are recommended to be used once after rethawing, to avoid repeated freezing and thawing.

6. Materials Required but Not Supplied

- Flow cytometer (equipped with 488nm excitation light)
- Centrifuge (96-well plate)
- Pipettes and tips
- FACS tubes
- Aseptic spiking tank
- Effector and target cells
- 0.25% Trypsin
- Carbon dioxide incubator
- Water bath
- U-bottom and Flat 96 well plate
- Tin foil
- ddH2O
- Complete medium for target cells
- 1×PBS

7. Flowchart and reagent preparation

7.1. Flowchart



7.2. Preparation of CFSE working solution: Use Assay Buffer to dilute the CFSE stock solution (green cap, Cat.: HG-CFSE) into 1×CFSE at 1:500, use it once and avoid repeated freezing and thawing.

△ Note: If the CFSE fluorescence signal is high or low, the dilution ratio of CFSE staining working solution can be increased or decreased to find out the more appropriate dilution ratio according to different cell types.

7.3. Preparation of 7-AAD working solution: Use Assay Buffer to dilute the 7-AAD stock solution (red cap, Cat.: HG-7-AAD) into 1×7-AAD at 1:1000, use it once and avoid repeated freezing and thawing.

8. Preparation of target cells

8.1. According to different target cell growth rates, target cells should be resuscitated at least one week in advance, and after resuscitation, target cells should be cultured for 2-3 times.

8.2. The target cells should be passaged once 1-2 days before plate spreading, and make sure that the target cell viability is ⩾ 90% and the quantity is sufficient when spreading the plate。

9. Day1 Co-culture of effector and target cells

9.1. Target cell labeling

9.1.1. Open the biosafety cabinet and UV irradiate for 30 minutes in advance, meanwhile turn on the water bath to

stabilize the water temperature at 37°C .

9.1.2. After preparation, remove the target cells from the CO2 incubator (T75 culture flask for example) and transfer to the biosafety cabinet. Discard the medium in the culture flask, add 10 ~15 mL 1×PBS to wash once and discard the supernatant.

9.1.3. 2 mL of 0.25% trypsin was added to the culture flask, and the flask was placed in a CO2 incubator at 37.0 ± 1°C , 5.0 ± 0.5 % for cell digestion for 3 ~ 5 min. Observe the adherent cells in the microscope, most of them are moving in a sandy shape, that is to judge that their digestion is complete.

9.1.4. After the cells have been digested completely, add 10 mL of target cell complete medium to terminate the digestion, use an electric pipette to blow and mix the cells, and transfer to a 15 mL centrifuge tube.

△ Note: In the process of target cell blowing and mixing, the operation should be kept gentle to prevent the death ratio of target cells in the control group from being too high due to excessive blowing force.

9.1.5. Place a 15 mL centrifuge tube in a centrifuge and centrifuge at 400 g for 5 minutes.

9.1.6. Discard the supernatant, add 10 mL of target cell complete medium, blow and mix and take a small amount of cell suspension for counting.

9.1.7. According to the result of cell counting, take 1E6~3E6 cells (quantification is required, the amount of cells is determined according to the amount of target cells used for plate spreading) to 15 mL centrifuge tube, centrifuge at 400 g for 5 minutes.

9.1.8. Discard the supernatant, add 10 mL of Assay Buffer to the cell precipitate and resuspend, centrifuge at 400 g for 5 minutes.

9.1.9. Discard supernatant, add 10 mL of Assay Buffer to the cell sediment, and centrifuge at 400 g for 5 minutes.

9.1.10. Discard the supernatant, add 1~3 mL (the specific volume according to the cell volume in 9.7, if the cell volume is 1E6, then add 1 mL of staining workup) of prepared 1×CFSE staining workup to the cell precipitate, so that the cell density during staining is controlled to be around 1E6 cells/mL, and gently blow with a pipette to mix well.

9.1.11. Wrap the centrifuge tube with tinfoil and incubate in a bath at 37 °C for 15 minutes, shaking the bottom of the centrifuge tube every 5 minutes or so to make the cell staining uniform. uneven CFSE staining will affect the results of killing.

9.1.12. After incubation, wipe and sterilize the centrifuge tube with 75% alcohol and transfer it to a biological safety cabinet. Open the cap of the centrifuge tube and add 10 mL of medium B. Blow gently to mix.

9.1.13. Place the tube in a centrifuge and centrifuge at 400 g for 5 minutes.

9.1.14. Discard the supernatant, add the appropriate amount of medium B to resuspend the cell precipitate, and adjust the cell density to 2E5 cells/mL.

9.1.15. Prepare a 96-well flat-bottomed plate, transfer the cell suspension to a sterile spiking tank, and add 100 µL of target cells to the experimental group and the control group, respectively, with a target cell spread of 2E4 cells/well. At least 2 parallel wells were set up for each group. If there were more than one target ratio in the experimental group, one group could be set up for the control group.

9.1.16. At the end of target cell spreading, place the 96-well flat-bottomed plate temporarily in a 37.0 ± 1° C, 5.0 ± 0.5 % CO_2 incubator.

9.2. Effector cell preparation and co-culture

9.2.1. Resuscitate effector cells or use fresh effector cell suspension directly.

△ Note: Effector and target cell resuscitation should be thawed quickly in a 37°C water bath (thawing time is

controlled within 2 minutes), cells in a 37°C water bath for too long will lead to a decrease in cell viability.

9.2.2. Take an appropriate amount of effector cell suspension into a centrifuge tube, add 2~10 times the suspension volume of medium B and mix well. Place in a centrifuge and centrifuge at 400 g for 5 minutes.

9.2.3. Discard the supernatant, add 10 mL of medium B to resuspend the cell precipitate, gently blow and mix well, and then put it back into the centrifuge, centrifuge at 400 g for 5 minutes.

9.2.4. Discard the supernatant, resuspend the cell precipitate with a small amount of medium B (in order to facilitate the adjustment of the effector cell density, the amount of effector cells can be predicted in this step first, to ensure that the density in this step is greater than that of the effector cells of the optimal targeting ratio), and take an appropriate amount of cell suspension for cell counting.

△ Note: The viability of effector cells has a certain impact on the killing results, ensure that the viability of effector cells is ⩾ 70% when spreading the plate.

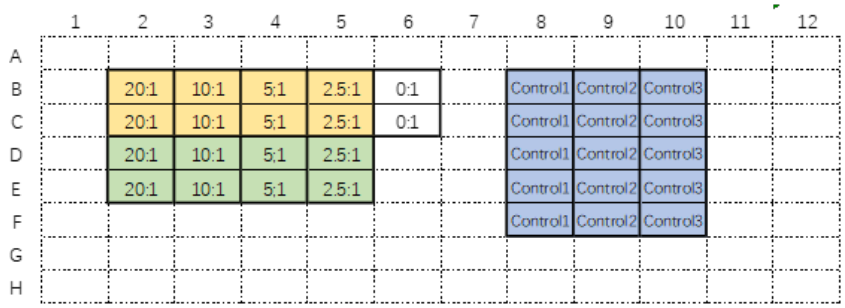
9.2.5. Adjust the effective live cell density (NK, CAR+ or TCR+ cell density) to 4E6 cells/mL according to the counting results (according to the effector-target ratio of 20:1, which can be adjusted according to the actual demand).

9.2.6. Gradient dilution of the above effector cells using medium B. Set up multiple sets of effector-target ratios according to actual needs.

9.2.7. Perform effector cell addition according to the following table, add 100 µL of effector cells per well for the experimental group, and the effective effector cell spreading amount is 4E5 cells/well (take the effect-target ratio of 20:1 as an example). In the experimental group, 100 µL of target cells and 100 µL of effector cells were added to each well; in the control group, 100 µL of medium B and 100 µL of target cells were added to each well.

Group	Experiment (based on an effect-target ratio of 20:1)	Control (0:1)
Target cell	100 µL	100 µL
Effector cell	100 µL	/
Medium B	/	100 µL

9.2.8. Reference plate layout (The plate layout is based on 20:1, 10:1, 5:1 and 2.5:1 efficiency target ratios as an example, which can be adjusted according to the experimental situation).



9.2.9. After cell addition is completed, gently shake or tap the 96-well flat-bottom plate (do not shake excessively to prevent splashing of the cell suspension in the wells) in order to achieve a uniform distribution of cells in each well.

9.2.10. Place the 96-well flat-bottomed plate in a CO_2 incubator at 37.0 ± 1 °C 5.0 ± 0.5 %, and the co-culture time is recommended to be 20 hours (the actual co-culture time can be mapped out according to different target cells and effector cells).

10. Day2 Flow Detection and Analysis

10.1. Flow Detection

10.1.1. At the end of the co-culture, remove the 96-well plate from the CO_2 incubator and place in a centrifuge. centrifuge at 400 g for 5 minutes.

10.1.2. Quickly invert the 96-well plate and discard the supernatants or use a lance to aspirate the supernatants (be careful not to touch the bottom cell sediment).

10.1.3. Add 200 μL of 1×PBS to each well and place back in the centrifuge at 400 g for 5 minutes.

10.1.4. Discard the supernatant, add 50 μL of 0.25% trypsin per well, and place in a carbon dioxide chamber to digest for 3-5 minutes.

10.1.5. After the target cells are completely digested under the microscope, add 200 μL of medium B to each well, gently blow and mix, then transfer to a 96-well U-bottom plate or flow-through tube and centrifuge at 400 g for 5 minutes.

△ Note: After adding trypsin, you need to observe the digestion of target cells in time, and terminate the digestion operation only after the target cells are completely digested; do not use pipette to forcefully blow down the adhered target cells.

10.1.6. Discard the supernatant, add 200 μL of 1×7-AAD staining solution to each well/tube, blow and mix well, and then incubate in the refrigerator at 2-8°C for 15 minutes under light.

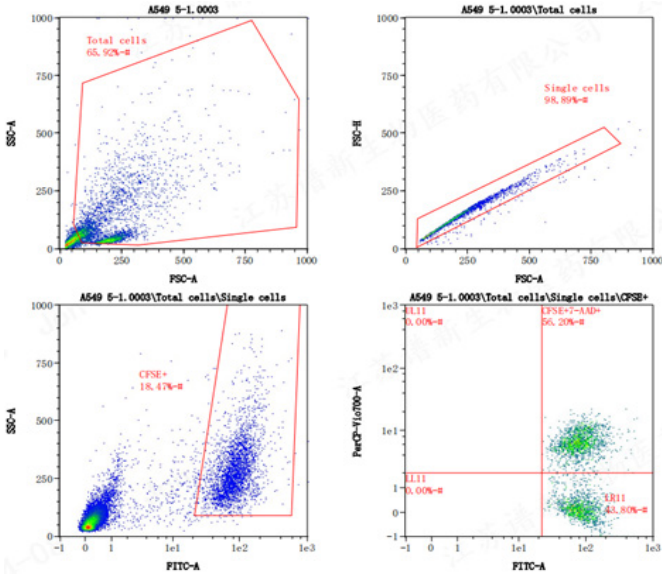
10.1.7. Immediately after incubation, perform flow-through assay and collect at least 5000 Events under CFSE+ gate.

11. FACS analysis

11.1. Total cells are circled using FSC-A and SSC-A. Single cells were circled from the total cells using FSC-A and FSC-H to exclude the effect of adherent cell populations on the results.

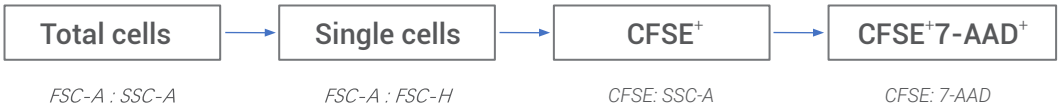
11..2. Circle CFSE-labeled target cells from Single cell population using CFSE (FL-1) and SSC-A, and then circle 7-AAD-labeled dead cells from target cell population by CFSE (FL-1) and 7-AAD (FL-3), and the proportion of the damned cell population is the CFSE+7-AAD+ proportion.

11.3. Diagram of the gate strategy:



△ The flow meter model used in the schematic diagram is Milrenyi Biotec MQ10, other machines refer to each operating instructions for the gate strategy.

11.4. Gate Strategy



11.5. Calculation formulae

Cell killing rate (%) = Experiment: $CFSE^+7\text{-}AAD^+$ (%)-Control: $CFSE^+7\text{-}AAD^+$ (%)

12. Single Positive Tube Preparation and Flow Cytometer Voltage and Compensation Adjustment

12.1. Control 1 (CFSE single-positive tubes): CFSE-labeled target cells were plated according to the treatment of the D1 control. Cells were resuspended using Assay Buffer at the time of sample collection and detection. This group was used for voltage adjustment of CFSE (FL-1) channel and compensation adjustment of FL-3 channel.

△ Note: Control 1 group target cells are only labeled with CFSE and no 7-AAD staining.

12.2. Control 2 (7-AAD single-positive tubes): the unlabeled CFSE target cells were plated according to the treatment of the D1 control group. To collect samples, cells were digested and transferred to 1.5 mL centrifuge tubes and incubated in a 70° C water bath (or metal bath) for 5 minutes. At the end of the incubation centrifuge at 400 g for 5 min. Cell precipitates were resuspended using 200 µL of 1×7-AAD staining workup. This set was used for voltage regulation of 7-AAD (FL-3) channels and compensatory regulation of FL-1 channels.

△ Note: Control 2 group target cells are not labeled with CFSE, only 7-AAD staining.

12.3. Control 3 (blank tube): the unlabeled CFSE target cells were plated according to the treatment of D1 control group. For the D1 control, cells were resuspended using 200 µL Assay Buffer at the time of receipt of the sample for detection.

△ Note: Control 3 group target cells are not labeled with CFSE and are not stained with 7-AAD.

12.4. Adjustment of flow cytometer voltage and compensation was performed using Control 1, Control 2 and Control 3 group cells.

△ Note: To ensure enough samples for flow cytometer parameter adjustment, it is recommended to prepare 3~5 replicate wells for each Control group.

13. Example diagrams

A

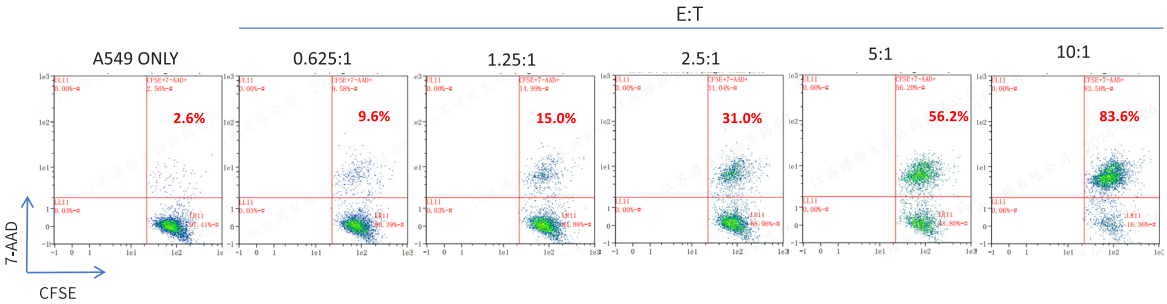


Figure A. FACS analysis of the killing rate of NK cells against CFSE-labeled A549 target cells

B

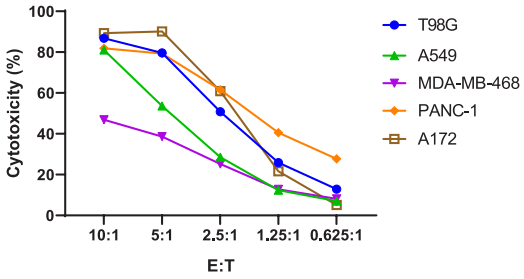


Figure B. FACS analysis of the killing rate of NK cells against CFSE-labeled target cells of different solid tumors

Figure A shows a flow chart of cell killing of CFSE-labeled A549 cells co-cultured with NK cells at different potency-target ratios for 20 h and stained with 7-AAD for 15 min. CFSE+7-AAD+ represents dead target cells. The killing effect of NK cells on A549 cells was subsequently enhanced as the potency-target ratio increased.

Figure B Flow chart of cell killing of CFSE-labeled target cells of different solid tumors co-cultured with NK cells at different potency-target ratios for 20 hours and stained with 7-AAD for 15 minutes. As the potency-target ratio increased, the killing effect of NK cells on different tumor cells was subsequently enhanced.

Note: E:T (Effector:Target) is effective effector:target cell.

14. Troubleshooting

question	Possible causes	method settle an issue
CFSE+ group is at edge of scatterplot or outside maximum ranges of FL-1 channels	High CFSE concentration	Increase the dilution of CFSE working solution
	Inappropriate parameter setting for FL-1 axis	Adjust the FL-1 axis parameters so that the cell population is displayed in its entirety.
No difference in effector cell killing results for different potency-to-target ratios	Poor target and/or effector cell status leads to	Re-resuscitate target or effector cells, paying attention to experimental manipulation to ensure cell viability;
	Effector cells are not cytotoxic to target cells	Replacement of effector cells with specific killing toxicity for experiments.
Higher proportion of $CFSE^{+}7\text{-}AAD^{+}$ in the control group	Improper handling of target cell labeling or too long plate spreading time	Read the instructions carefully, pay attention to all experimental operations, and complete the spreading of the plate quickly.
	Insufficient tryptic digestion time or excessive blowing during sample collection	Prolong the tryptic digestive time and microscopically observe complete digestion before proceeding to the next step, all blowing operations should be gentle.
	Medium B is not suitable for this target cell culture	Seeking technical support

15. References

Russell, J. H. , and T. J. Ley. "Lymphocyte-mediated cytotoxicity." Annual Review of Immunology 20.6(2002):323-370.

16. Contacts

Address：1463 Wuzhong Avenue, Yuexi Street, Wuzhong Economic Development Zone, Suzhou, Jiangsu Province
Postal code：215104
Tel：400-900-1882
E-Mail：info@hillgene.com
Website：https://www.hillgene.com

17. Notice to buyers

Our products are for research use only. They may not be used for any other purpose, including, but not limited to, use in humans, for therapeutic or diagnostic purposes, or for any commercial purposes. Our products may not be transferred to third parties, resold, modified for resale or used to manufacture commercial products or provide services to third parties without our consent.

Our products may not be transferred to a third party, resold, modified for resale or used to manufacture commercial products or provide services to third parties without our prior written approval.

Your use of this product must also comply with any applicable license requirements described in the product pages on the Official Site(https://www.hillgene.com). It is your responsibility to review, understand, and comply with any restrictions set forth in such notices.

For more product, intellectual property and restricted use information, visit https://www.hillgene.com.

Annex 1: Safety Precautions

● General Instructions

Failure of the user to use this product in the manner described in this manual may result in personal injury or damage to the instrument or equipment. Ensure that the person using this product has received the general laboratory safety instructions and safety information provided in this document.

（1）Before using the instrument or equipment, read and understand the safety information in the user documentation provided by the instrument or equipment manufacturer.

（2）Read and understand all applicable Safety Data Sheets (SDS) and use appropriate personal protective equipment (gloves, protective clothing, goggles, etc.) before handling chemicals.

● potential biohazards

（1）7-AAD is a potential carcinogen. For your safety and health, wear lab coat, gloves, and eye and face protection when handling to avoid 7-AAD contact with skin and eyes. If this product is inhaled, swallowed, or comes into contact with skin, eyes, or clothing, dispose of it immediately and wash it thoroughly.

（2）Samples used in accordance with this method that were contacted during the experiment may be considered biohazardous. When handling biohazards, use appropriate hazardous waste disposal methods.

● Biohazard

（1）Biological samples, such as tissues, body fluids, infectious agents, and blood from humans and other animals, have the potential to transmit infectious diseases. Perform all work in a facility equipped with appropriate safety equipment (e.g., biosafety cabinets). Safety equipment also includes personal protective equipment such as gloves, jackets, coveralls, shoe covers, boots, respirators, face masks, and safety glasses or goggles.

（2）Prior to working with potentially biohazardous materials, individuals should be trained in accordance with local regulations and company/agency requirements.

● Hazardous Waste (from Instruments)

Waste from instruments is potentially hazardous. Follow the guidelines in the preceding “Biohazard” warning.

Annex 2: Related products (more products are available at Hillgene Bio https://www.hillgene.com)

categories	Pseudolaricin	Product Code
analysis and detection	Cell Killing Assay Kit (Suspension of Target Cells)	HG-CKK001
	Human Interferon gamma (IFN-γ) ELISA Assay Kit	HG-IF001
	CRS Cytokine ELISA Assay Kit	HG-HC001
	HIV-1 p24 ELISA Assay Kit	HG-P001
	CAR/TCR Gene Copies Assay Kit (qPCR-Fluorescent Probe Method)	HG-CA001
	RCL (VSVG) Gene Copy Number Assay Kit (qPCR-Fluorescent Probe Method)	HG-RC001
	Mycoplasma DNA Assay Kit (qPCR-Fluorescent Probe Method)	HG-ZY002
cell expansion	CD19 CAR-T Cell Preparation Kit	HG-POC001
	NK Cell Expansion Kit	HG-POC004
cotransfection agent	Viral E-hancer A	HG-PTD001-A
	Viral E-hancer B	HG-PTD001-B
	Viral E-hancer C（ROU）	HG-PTD001-C-R
	Viral E-hancer C（GMP）	HG-PTD001-C-G
	Viral E-hancer D	HG-PTD001-D

CELL THERAPY
INNOVATION INSPIRED

BlueKit™
Powered by Hillgene

Welcome to order

Jiangsu Hillgene Biopharma Co.,Ltd

A : Building 4, Yue Wang Park, No. 1463, Wuzhong Avenue, Wuzhong District, Suzhou , Jiangsu, China.

T : 400-900-1882

E : info@hillgene.com

W : www.hillgene.com/www.bluekitbio.com

2409