Cellular Technology Limited

PROTOCOL

Human Natural Killer Target cell Visualization Assay (TVA™)

CONTENTS

- *CTL TVA™ Dye*
- *CTL-Wash™ Supplement 10x Medium*
- *CTL-Test™ Medium*
- *CTL-LDC™ Cell Counting Reagent*
- *Plates: 96-well, clear, flat-bottom polystyrene; and 96-well, clear, round-bottom polystyrene plates*
- *Adhesive plate sealing sheets*
- *Hemocytometers; 1x2 chambers, disposable*
  1 plate kit: 5 slides
  5 plate kit: 25 slides
  10 plate kit: 50 slides
- *Protocol*

PROCEDURE

1. PREPARATION OF TARGET CELLS — STERILE CONDITIONS

a) Resuspend 2.5x10^5 – 1x10^6 K562 cells in 1ml of *CTL-Test™ Medium* and add 1µl *TVA™ Dye* directly to the tube. Protect from light. ***Note:*** *You will need approximately 2.5x10^5 K562 cells for four Effector samples.*

b) Mix gently and incubate at 37°C for 10-15 minutes.

c) Centrifuge at 330g for 10 minutes and decant supernatant.

d) Resuspend cells in 5ml *CTL-Test™ Medium.*

e) Repeat Steps **c)** and **d)** for a total of two washes to remove all unbound dye.

f) Resuspend Target cells in 2ml (per 1ml of K562 used initially) of *CTL-Test™ Medium.*

g) Count Cells using the CTL Cell Counting Software. *(Refer to Section 4)*

h) Centrifuge and resuspend stained K562 cells at a final concentration of 5x10^4 cells /mL in *CTL-Test™ Medium.*
- **Demo Kit (1 plate):** Final volume of Target cells required: 5ml (4 Effector samples)
- **5 plate kit:** Final volume of Target cells required: 20ml (20 Effector samples)
- **10 plate Kit:** Final volume of Target cells required: 40ml (40 Effector samples)

i) PREPARATION OF EFFECTOR CELLS — STERILE CONDITIONS

a) If using cryopreserved PBMC, thaw PBMC according to CTL Thawing Protocol (included). If using fresh blood, isolate PBMC using standard protocol. ***Note:*** *If using cryopreserved PBMC it is highly recommended to use CTL Anti-Aggregate Wash™ 20x instead of CTL-Wash™ Supplement 10x during the thawing procedure to prevent cell clumping and aggregation (20ml for 5 plate kit, 40ml for 10 plate kit) (Catalog # CTL-AA-001/5).*

b) Centrifuge the samples at 330g for 10 minutes and reconstitute in *CTL-Wash™ Medium* or *CTL Anti-Aggregate Wash™ 20x* at approximately 0.5-3x10^6 cells/ml. ***Note:*** *Approximately 1x10^6 PBMC is needed per donor, therefore it is advisable to start with a slight excess to account for any loss of cells due to thawing and/or washing steps; if samples will be run in replicates, cell numbers will need to be calculated accordingly.*

c) Count cells with the *CTL-LDC™ Reagent* and the CTL Cell Counting Software. *(Refer to Section 5)*

d) Centrifuge and resuspend PBMC (Final concentration: 5x10^6 cells/ml) in *CTL-Test™ Medium.*

j) TARGET CELL VISUALIZATION ASSAY — STERILE CONDITIONS

a) Add 100µl of Effector Cells **(2-d)** to Row A in a 96-well, round-bottom culture plate *(see **Figure 1** on reverse for sample Plate Layout).*

b) Prepare serial dilutions of Effector PBMC (100µl per well) down 6 more wells in the culture plate ***(Figure 1).*** ***Note:*** *To save unused portions of the culture plate for future assays, use the adhesive plate sealing sheets (included) and store in sterile conditions.*

c) Add only *CTL-Test™ Medium* to the 8th well.

d) Add 100µl of stained K562 **(step 1-h)** cells to each well.

e) Using a plate adapter, centrifuge the plate at 330g for 5 minutes.

f) Incubate for 3-4 hours at 37°C

g) Resuspend cells in the plate and transfer 50µl of cell solution from each well of the culture plate to a corresponding well in a 96-well, flat-bottom imaging plate. Repeat for 2 additional wells per sample *(triplicates for each sample; **Figure 2**).*

h) Tap the sides of the plate gently to ensure homogenous distribution of cells in the well.

i) Image the remaining fluorescent Target K562 cells with the NK-TVA™ Application of the CTL Cell Counting Software using an ImmunoSpot® S6 Fluorescent Analyzer.

See other side for Counting Protocols, Plate Layouts, and Technical Tips.

4. COUNTING TARGET CELLS (K562) — REFER TO #15 UNDER TECHNICAL TIPS

a) Use cells from step **1-f.**
b) Resuspend cells by inverting the tube with the Target cell solution two times just before taking the sample for counting.
c) Aspirate 10µl of cell solution and fill a hemocytometer chamber with a steady stream of cell solution to ensure even flooding of the hemocytometer chamber.
d) Repeat for a total of two chambers.
e) Cells can be counted using either a fluorescence-capable microscope or a suitable ImmunoSpot® Analyzer. *(Live cells will fluoresce green at 500nm excitation and 525nm emmission wavelengths.)*
f) Use the wizard-guided CTL Cell Counting Software and the "Tumor Cells" plate type for counting the samples and for quality control and calculations.

5. COUNTING EFFECTOR CELLS (PBMC) — REFER TO #15 UNDER TECHNICAL TIPS

a) Use cells from step **2-b.**
b) For each Effector sample to be counted add 50µl *CTL-LDC™ Reagent* per well to a separate round-bottom, 96-well plate. *(Do not use the Culture plate included with the kit.)*
c) Resuspend cells by inverting the tube with the Effector cell solution two times just before taking the sample for counting.
c) Take 50µl of the cell suspension and add to the 50µl of the *CTL-LDC™ Reagent* and resuspend by pipetting up and down three times.
c) Using the same pipette tip, aspirate 15µl of cell and dye mixture and fill the hemocytometer chamber with a steady stream of cell solution to ensure even flooding of the hemocytometer chamber.
d) Repeat for two more chambers.
e) Cells can be counted using either a fluorescence-capable microscope or a suitable ImmunoSpot® Analyzer. *(Live cells will fluoresce green at 500nm excitation and 525nm emmission wavelengths; dead cells will fluoresce red at 530nm excitation and 620nm emmission wavelengths.)*
f) Use the wizard-guided CTL Cell Counting Software and the "PBMC" plate type for counting the samples and for quality control and calculations.

TECHNICAL TIPS

1. After serial dilution of the PBMC, tumor cells can be added with multi-channel pipettes to an entire column.
2. In order to maintain sterility when centrifuging the plate, use a plate adapter and tape the sides of the plate to avoid accidentally dislodging the lid.
3. When transferring cells from the culture plate to the imaging plate, cells need to be resuspended by pipetting up and down 5-10 times to ensure homogenous distribution of cells in solution and to avoid clumping of tumor cells.
4. When tapping the sides of the plate, tap gently to avoid splashing into neighboring wells.
5. For standardized results, use the CTL Cell Counting Software to determine the viability of PBMC and the tumor cells. The Software will also calculate the volume for resuspending the cells.
6. To reduce clumping of the Effector cells, PBMC should be washed with CTL-Wash™ Medium.
7. We highly recommend the use of serum-free CTL-Test™ Medium for the TVA™ assay to prevent manipulation of the Natural Killer cells in the PBMC population.
8. Deviations from the specified wash steps, concentrations, timing requirements, and reagent requirements may alter the performance of the assay.
9. Upon successful completion of the assay viable tumor cells will be stained green.
10. If cells appear to be concentrated along the walls of the well, remove plate from the Analyzer and gently tap the sides.
11. The ImmunoSpot® Analyzers and Software have advanced features that permit objective recognition, counting, and analysis.

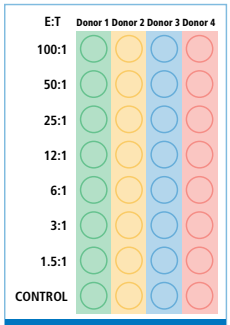

Figure 1: Plate Layout—Culture plate 5x10³ K562(T) per well + PBMC(E).

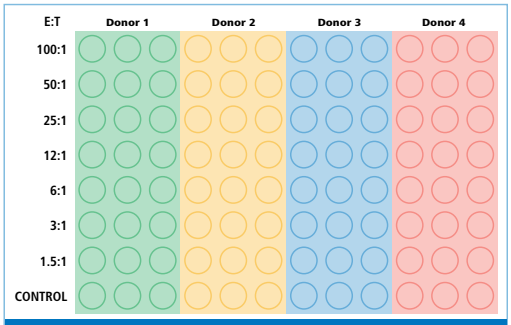

Figure 2: Plate Layout—Imaging Plate 1.25x10³ K562(T) / well + PBMC(E).

12. When using ImmunoSpot® Analyzers, use the Cell Counting Software and the NK-TVA™ Application. The wizard will guide you through the counting process and analyze the data, which can then be exported to an Excel sheet.
13. Once the cell-dye mix has been added to the hemocytometer chamber, samples should be counted within 15 minutes, afterwhich time, drying may occur.
14. Continuous exposure of the stained cells will cause photobleaching. The samples can be counted up to five times without bleaching. To afford count stability, some ImmunoSpot® Analyzer models automatically eject tray when counting is not ongoing.
15. When counting cells using the Cell Counting Software, please note that the dilution factor for Target cells and Effector cells will be different.

See other side for Contents and TVA™ Procedure.
For laboratory research use only. Not for use in therapeutic or diagnostic procedures.

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