I. PURPOSE

The purpose of this procedure is to outline the T-cell cloning performed in Ragon laboratories.

II. SAFETY

This procedure needs to be carried out in the BSL2+ laboratory following all BL2+ regulations.

III. SCOPE

This procedure applies to all employees, students, contractors and visitors that perform T-cell cloning.

IV. REQUIREMENTS

Training to be obtained by qualified trainer or Subject Matter Expertise (SME).

V. RESPONSIBILITIES

A. The Ragon Institute **qualified trainers** are responsible for the overall implementation of this procedure and ensuring compliance and for periodic review of this procedure. Updates if any may be initiated by the qualified trainers or Subject Matter Expertise (SME).
B. **All employees, students, contractors and visitors** are required to follow this procedure.

VI. REAGENTS

Reagent	**Vendor**	**Catalogue #**
RPMI-1640 media	Sigma	R0883
L-Glutamine	Mediatech	25-002-C1
Pen/Strep (5000IU Pen/5000ug/ml strep)	Mediatech	30-001-C1
FBS	Sigma	Actual tested in PAR lot
IL-2	NIH AIDS Research &Reference Reagent Program	Cat#136
96- well plates (round bottoms)	Westnet	3799

VII. PROCEDURE

CLONING OF PEPTIDE-STIMULATED PBMCS

1. Make fresh R10/50 for cloning before starting procedure.
2. Dilute effector cells to 100,000 cells/ml (0.1M/ml) to be cloned.
3. Take out the correct number of sterile 96 well culture plates. Open them inside the hood only to preserve their sterility.
4. One plate will contain the following:
   a. 10 million irradiated allogeneic feeders (you can out 15M if you have)
   b. Anti-CD3 mAb (12F6). You can use either 0.1 µg/ml of 12F6 or 0.25 µg/ml of PHA. Do not add stimulator more than 10 minutes before seeding cells on the plate.
   c. Effectors—30 µl, 10 µl, 3 µl or 1µl depending on the chosen dilution.
   d. 20 ml of fresh R10/50.

   For 30 cells/well:
   Total volume on the plate is 200 µl/well x 96 wells = .2 ml.
   To convert the solution to cells/ml, 30 cells/well x 5 = 150 cells/ml
   1 plate = 100 wells x 200 µl = 20 ml
   X (100,000 cells/ml) = 20 ml (150 cells/ml)
   x = 30 µl

   For 10 cells/well:
   10 µl of 100,000 effector cells/ml to be cloned in 20 ml of feeder's solution.

   For 3 cells/well:
   Use 3 µl of 100,000 effector cells/ml to be cloned in 20 ml of feeder's solution.

5. Place 200 µl in each well. Do not feed for 6-7 days. After that, exchange R10/50 IL-2 medium (100 µl out, 100 µl in). Make sure not to disturb cells at the bottom when feeding.
6. Feed twice a week. Discard pipette tips between rows (avoid cross contamination)
7. After 14 days in culture, single wells will start to grow (15-35% at 30 cells/well, 12-18% at 10 cells/well). Observe daily and mark growing wells.
8. Keep feeding for 21 days. After that, transfer growing wells into a 24 well plate with 1 million irradiated allogeneic feeders per well in R10/50 and anti-CD3.
9. Make sure that on well will be your feeder control.
10. Culture it for 14 days and test for cytolytic activity in Cr51 assay. See Protocol 26-00.
11. **Note**: if you only need to test one peptide, then the Cr assay can be done directly from the 96 well plates. Only 2 targets need to be checked—blank (no peptide) and the chosen peptide. In this case, take 25 µl of the effector suspension from the 96 well plate for each target. Add medium back onto plate afterwards.
12. If you need to check more than 2 peptide targets, then the cells need to be expanded first.

13. Keep expanding growing and active clones to freeze at least 10x5million vials. The first badge of freshly isolated clones is valuable.