I. PURPOSE

The purpose of this procedure is to outline the T-cell re-stimulation procedure 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 work on the CTL Feeding protocol.

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

Item	**Manufacturer**	**Order Number**
RPMI-1640	Sigma	R0883
Pen/Strep (5000 IU Pen/ 5000ug/mL Strep)	Mediatech	30-001-Cl
L-glutamine (200mM; 29.2 mg/mL)	Mediatech	25-002-Cl
HEPES (1M; 238.3mg/mL)	Mediatech	25-060-Cl
FBS, Heat-inactivated	Sigma	F4135 (a lot tested for here)
IL2	Hoffmann-La Roche	Ro 23-6019
PBS	Sigma	D8537
PHA	Fisher	R30852801
Anti-CD3 Antibody	NIH AIDS Reagent Rental	
15 ml propylene tubes	Falcon	352063
T25 flasks with 0.2 ul filter	Corning	3056

VII. PROCEDURE

If you want to maintain the culture of T cells continuously for longer period, it is a good idea to restimulate every week by maintaining more than one batch of cells for each cell line. You will always keep older badges up so you can take 1-2M from those flasks for restimulation. Since clones grow differently restimulation to restimulation (due to different badges of fresh feeders), this will increase your chances of having enough cells for an experiment or for freezing.

Use Buffy coat cells (from Blood Bank at Main Campus) to restimulate or PBMC from HIV negative donor if smaller restimulation is planned (that can be requested through RAGON CLINICAL Platform. Follow protocol 00-03) for cloning use same day fresh blood/buffy coat.

Note: Always make fresh R10/50 for restimulation! Use fresh 12F6 (anti CD3 ab) or PHA. (These should not be stored in the refrigerator for more than 2 weeks. Stock is kept in -80 ^{0} freezer.)

1. Ficoll buffy coat or peripheral blood form HIV negative donor. See protocol .
2. After final wash, count cells and resuspend them at 20 million cells/ml of R10.
3. Irradiate at 3000 cGY in the XRAD irradiator on 9th floor room 917
4. Place 1-2 million CTLs in each T25 flask. Do not exceed 2 million CTLs per flask.
5. Add 20 million irradiated feeders in 20 ml of volume of R10/50 medium.
6. Add either 12F6 at 0.1 □g/ml or PHA at 1:1000 dilutions.
7. Warning: do not restimulate more than 3-5 flasks at once! You can make 3-5 place them in the in incubator and start new badge. This will ensure that the clones spend a minimal amount of time outside their desired incubator conditions. If you are a beginner, it's a good idea to do even fewer simultaneously because you are slower.
8. In keeping with that warning, make sure that your CTLs are not in the incubator which is opened frequently. This will help keep the incubator temperature and CO2 levels as optimal as possible.
9. After CTLs have been established, they are fed twice a week (Monday /Friday) by medium exchange. Transfer flasks gently into the hood, being careful not to disturb the cells on the bottom. Aspirate about 7 ml of media off the top, and then add about 7 ml of fresh R10/50 to each flask. You can feed with media which is not older than 2 weeks.
10. Flasks are fed in this manner for several weeks or as long as the clone is growing.
11. Small numbers of cells can be removed and restimulated to make a fresh batch. Old cells will remain active as long as they are fed.
12. The recommended wait time for doing a Cr51 assay on restimulated CTLs is 7 days after restimulation. After that, the clone can be used as long as it is in good condition, which varies from clone to clone, batch to batch.
13. If testing specificity by Flow /Tetramer 12 days or more waiting period is recommended, unless you will use dead cell dye to sort out dead feeders.