I. PURPOSE

The purpose of this procedure is to outline the Chromium 51 release assay performed in Ragon laboratories.

II. SAFETY

This procedure needs to be carried out in the BSL2+ laboratory following all BL2+ regulations. The individual performing this procedure must have radiation training at MGH and be certified for Isotope Usage. Assay cannot be done without a Radiation Badge. Only few Biological Hoods in each tissue culture room at Ragon Institute are certified for usage of radioactive isotopes.

III. SCOPE

This procedure applies to all employees, students, contractors and visitors that work on the chromium 51 release assay.

IV. REQUIREMENTS

Radiation Badge obtained from MGH Radiation Office upon completion of required training. Retraining is required every year.

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. BACKGROUND INFORMATION

B958 is a T cell line which was initiated by exposing marmoset lymphocytes to EBV extracted from human leucocytes line from elderly person with transfusion induced infectious mononucleosis. It releases high titer of EBV transforming virus.

VII. PROCEDURE

If BCL lines are used as antigen presenting cells: Feed the EBV cell line 1:1 the day *before* the assay -- this will lower spontaneous release of targets.

Separate radioactive waste very carefully from regular waste. Monitor your work area after each use of this or any other radioactive isotope. Check lead holder for radioactivity each time you use

that by removing plex vial at least 1 M away and checking empty lead with Geiger counter. Record usage of ^{51}Cr every time (in TC room and when signing out the Cr). When the vial reaches half its total volume, it is a good idea to verify the actual amount of ^{51}Cr in the tube with pipette -- sometimes there is less ^{51}Cr in the vial because somebody forgot to record when they removed some.

1. Before starting, please be sure to sterilize hood, reagents, and pipettes with 70% ethanol (spray first, then wipe down with gauze). **Safety glasses, full gown, and double gloves are required at ALL times in the hood**. Media, cultures, and relative chemicals should only be opened INSIDE of the hood. Before starting experiment make sure that D-125 buckets and spray bottles have been changed **within the past 7 days**.
2. **BEFORE USING CHROMIUM:** Survey hood, the top and bottom of the Cr bullet, and surrounding areas with the Geiger counter to ensure that there have not been any previous radioactivity spills. Please clean any areas above background and survey again. **HOOD PREPARATION**: Line hood with blue chuck. Depending on the amount of waste you anticipate, use 3-6 50 mL conical tubes labeled as "Cr51 Waste" filled ¼ full with D125 as waste basins for pipettes, tips, Eppendorf tubes, etc. Anything that touches or is exposed to chromium, media containing chromium, and cells containing chromium, must be deposited into these tubes as radioactive waste.
3. Select the appropriate targets (for example EBV line) expressing the restricting HLA class I allele and transfer the required number of cells into a 15 ml conical tube. Transfer 1-1.5 million cells per target into a 15 ml conical tube, depending on how many targets you will need for the assay; plan the assay in advance so that you will know how many cells you need. If you are planning big screening and might need a lot of cells, count before .
4. Spin cells down for 10 min at 1500 rpm.
5. Aspirate supernatant to about 200 µl and add peptide at 10 µg/ml FC.
6. Add 50 □Ci of ^{51}Cr per target (usually 10 µL. Be aware of the date (radioactive decay) and the initial volume/concentration of the Chromium), then resuspend cells with the same pipette tip you used to add the chromium.
7. Incubate cells for 1 hour at 37 ^{0}C.
8. In the meantime, count the effectors and resuspend them at the proper concentration, which is most likely 1M/ml or 0.5M/ml for an Effector:Target ratio of 10:1 or 5:1. Place these tubes in the incubator until you are ready to make the effector plate.
9. Take out a 96-well rounded bottom plate and write all the necessary information on it. This will help you put the correct cells into the correct wells. Make sure to put markings on the plate itself as well as the lid to avoid confusion with the lids from multiple plates.
10. After the hour is up, wash the targets three times with 12 ml **cold** R10 at 1000 rpm for 7 min in a **cold** centrifuge.
11. Count targets and resuspend at 1 x 10^5 cells/ml (.1M/ml). Place on ice and prepare the effector plate.

12. When the effector plate is ready, add 100 □l targets to each well using a multichannel pipettor or a repeater pipet. Remember to make a Max wells in triplicate. Do it on a separate plate to avoid potential splash hazard. (Max wells are made with 100 µL targets and 100 µL of 5% Triton)
13. Incubate plates for 4 hours at 37 degrees C.
14. After 4 hours, harvest 30 µl of supernatant onto a Luma plate using a multichannel pipettor.
15. Add 30 µl of 5% Triton per well and let the Luma plate dry overnight at room temperature in a hood with the blower left on.
16. Add 30 µl of D125 to the chromium plate. Aspirate all liquid waste into the chromium vacuum flask and then dispose of the plate as chromium dry waste. Write on flask how much Chromium you are aspirating and do not allow the flask to become too full with liquid, or above 1000 uCi. Full flask (with less than 1000uCi) can be autoclaved and after dispose in designated sink. See if there is a sign above the sink for radioactive disposal.
17. The next day, expose of 96 well plate, all chromium waste from previous day, and the ols/used luma plate inside of a plastic bag labeled "Cr 51 Waste" with your initials and date. Put bag into radioactive waste buckets and record the amount of waste on the log.
18. Measure the Luma plate the next morning on the Wallac counter. (the plate is OK for few weeks so it is no problem if it is not read immediately.

VIII. PREPARATION OF TARGETS FOR CHROMIUM ASSAY

Note: media and centrifuges should be kept at 4 ^{0} C.

1. Count viable and non-viable cells from vaccinia infected target cells following protocol. Greater than 70% viability is needed for a successful assay.
2. Remove 1.5 million cells and place in a 15 ml conical centrifuge tube, one for each target.
3. Spin at 1500 rpm for 10 min.
4. Aspirate all but 200 µl of the supernatant.
5. Add 50 µCi of ^{51}Cr. (As of 2002, 10 µl of ^{51}Cr = 10 µCi.)
6. Incubate for 45 min at 37 ^{0} C.
7. Wash 3 times with 12 ml of cold R10, centrifuging at 4 ^{0} C for 7 minutes at 1000 rpm.
8. After the final wash, add 3 ml of R10. Resuspend gently and remove a small aliquot (less than 200 µl) for counting.
9. Count viable and nonviable targets.
10. Dilute targets to the correct final concentration of 1 x 10^5 cells/ml (.1M/ml) and keep them on ice until you are ready to add them to the plate.
11. Prepare the effectors plate with the correct peptides and effectors as the chosen ratio. Take care to avoid contaminating adjacent wells when adding peptides to the plate.
12. Add targets last to the plate and incubate them in the incubator for 4 hours, 6 hours if you are doing the assay on fresh PBMCs.
13. Harvest 30µl of supernatant for Luma plates. Remember to add 30µl of 5% triton to each well of the Luma plate. Dry Luma plates overnight in the hood before counting the next day.
14. When you put your plates on Wallac to be counted make sure that you have written the file number that the assay will be saved under.

15. Dispose your radioactive plates appropriately. It is your responsibility to transfer them to the lower shelf and bag them all and tag them for proper disposal when full. If you need instruction on this, just ask!
16. Clean and dispose radioactive waste after **each** assay! There is no radioactive storage in the tissue culture room at all!

IX. RADIOACTIVE WASTE DISPOSAL AFTER ^{51}Cr ASSAY

1. All Cr51-contaminated waste must be decontaminated with D 125 first. Aspirate the liquid into the chromium flask, then put the solid waste into a plastic bag (we use empty bags from centrifuge tubes). These are the strongest bags we have available. They are to be sealed and marked "Cr51".
2. DRY WASTE: The plastic bags should be placed in the radioactive disposal barrels. Disposal barrel is on the eighth floor, the radioactive waste barrel is located in the freezer alcove across from the waste disposal room. Be sure to record the number of bags you add and the radioactivity level of the barrel. Do not overfill them--3/4 is enough!)
3. Extra large bags to line the barrels are kept next to the barrel, which is labeled as the chromium storage area. As of JULY 2017, only 8th floor at 400 technology is using Chromium and has Waste Area.
4. When the barrel is full, the large bag should be sealed and 'tagged' using yellow radiation waste disposal tags. Activity is usually less than 200 mCi for solids must be written on the barrel, so that the person sealing them knows exactly what amount to write on the disposal tag.
5. LIQUID WASTE: All liquid waste must be aspirated into a special chromium flask. This flask is found on the Chromium cart, accompanied with lead, which you should put around the flask when in use.
6. The flask is then autoclaved, and the waste is dumped down the disposal sink. This should be documented and you cannot dispose of more than 1000 uCi down the sink at one time. This must be done after each assay!
7. This flask should then should be left on the cart with radioactive supplies for other to use.
8. Solid radioactive waste is picked up when full. Please notify Alicja or your floor manager that the barrel is full, so they can arrange a pick-up.
9. All supplies for radiation disposal such as jugs and bags can be obtained free from the Radiation Safety Office by calling.
10. Chromium waste MUST be removed from TC room after each assay.
11. When general barrel is full, close that, tag that and call Radiation Safety Office to arrange pick up .