***In vitro* and *in vivo* Limiting Dilution Assay for Colorectal Cancer**

L Agro^{1,2} and C O'Brien^{1,2,3,4}

¹Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada

²Princess Margaret Cancer Centre, The University Health Network, Toronto, Canada

³Department of Surgery, University of Toronto, Toronto, Canada

⁴Department of Physiology, University of Toronto, Toronto, Canada

Abstract

The *in vitro* limiting dilution assay is used to determine the colorectal cancer initiating cell (CC-IC) frequency of a CC-IC enriched suspension culture, grown in growth factor enriched serum free media. The *in vivo* limiting dilution assay is used to determine the colorectal cancer initiating cell frequency of a primary colorectal cancer sample or an established suspension cell line using immunocompromised murine xenograft models. *In vitro* and *vivo* limiting dilution assays (LDAs) can be used to determine the effect of a specific treatment or genetic knockdown strategy on the initiating cell frequency of a population of CC-ICs or colorectal cancer sample, respectively.

Part I. *In vitro* CRC LDA

Materials and Reagents

1. Centrifuge tubes (BD Falcon, 352096, 352070)
2. Serum free cell culture media (see Recipes)
3. Wash media (DMEM/F-12) (ThermoFisher, 11320-033)
4. 0.4% Trypan blue solution (Life Technologies, 15250-061)
5. Permanent marker (VWR, 52877-310)
6. 7× sterile, non-tissue culture 96 well U-bottom plates (Falcon, 351177)
7. 0.25% Trypsin (Life Technologies, 25200-056)

Equipment

1. 37 °C Incubator
2. Light microscope (Leica, DM IL LED)
3. Hemocytometer (Hausser Scientific, 1483)
4. Pipette aid (Integra, 155 000)

5. Pipettes (BD Falcon, 357530)
6. Flow cytometry assisted cell sorter (FACS) (see Recipes)

Procedure

Note: The range of cell concentrations used will depend on the frequency of initiating cells in your fraction. An initial LDA using a wide range of concentrations can be used to find the best range of cell doses, ideally it should include doses with 100% sphere formation down to cell doses with no sphere formation, as well as, a range of doses in between. For an LDA to be statistically valid one must first test whether their sample complies with the Poisson single-hit model, which assumes that the number of biologically active units in a culture varies according to Poisson distribution. For addition information and calculations please refer to Extreme Limiting Dilution Analysis (ELDA) site (<http://bioinf.wehi.edu.au/software/elda/>), and the accompanying paper¹. Ex: if the initiating frequency is approximately (1:50–1:200), then the range indicated in table 1 will be sufficient to obtain statistically significant results.

A. Seeding for an in vitro LDA

1. Grow cells to confluence in a T175 flask at 37 °C.
2. Centrifuge cells (300–450×g) for 5 min at room temperature, then remove supernatant.
3. Resuspend cell pellet in ~3 ml of trypsin and incubate at 37 °C for the necessary time to obtain single cells.
4. Remove cells from incubator and resuspend with ~10 ml of wash media to stop trypsin reaction.
5. Centrifuge cells (300–450×g) for 5 min at room temperature, then remove supernatant.
6. Completely resuspend pellet in enough cell media so that you will have a final cell concentration of about 1×10^6 cells/mL in order to make cell counting on the hemocytometer easier for you.
7. Mix 10 μ l of cell mixture with 10 μ l of trypan blue solution and load mixture onto a hemocytometer to count the number of live cells using a light microscope. Be sure to count cells in all four coordinates and divide by 2 to obtain the concentration of cells in your solution ($__ \times 10^4$ /ml). Please refer to <http://www.abcam.com/protocols/counting-cells-using-a-hemocytometer> for a further explanation on how to use a hemocytometer.
8. Seed cells either manually or by using a FACS machine.

Manual cell seeding

- a. Serially dilute cells to obtain the following final cell concentrations
 - i. Tube 1 (1,000 cells/well): 27,000 cells in 5.4 ml media

- ii. Tube 2 (100 cells/well): 10,000 cells in 20 ml media
- iii. Tube 3 (10 cells/well): 2,000 cells in 40 ml media
- iv. Tube 4 (1 cell/well): 300 cells in 60 ml media
- b. Thoroughly mix cell solutions each time, before dispensing 200 µl of each into sterile non-tissue culture 96 well U-bottom plates according to table 1.
- c. Centrifuge plates containing single cells (300–450xg) for 5 min at room temperature, and using a microscope, determine which wells are confirmed to have only one cell. Using a marker, circle those wells containing single cells.
- d. Return plates to 37 °C incubator as soon as possible.

Flow cytometry assisted cell sorter (FACS) cell seeding

- a. Remove $\sim 2\text{--}3 \times 10^6$ cells from your cell solution and put it into a centrifuge.
- b. Centrifuge cells (300–450xg) for 5 min at room temperature.
- c. While cells are spinning, mix sytox blue with PBS + 2% FBS to obtain a final working concentration of 10 nM–1 µM depending on your cell line (please refer to product manual for further information). Avoid exposure of sytox blue solution to light.
- d. Remove supernatant and completely resuspend pellet in 2–3 ml sytox blue working solution.
- e. Filter cell/sytox blue solution through a 0.35 µm filter to ensure a single cell suspension before putting cells into a FACS tube.
- f. Keep the cell solution on ice and in the dark.
- g. Add 200 µl of cell media to 7 sterile non-tissue culture 96 well U-bottom plates.
- h. Use a FACS machine to sort cells into 96 well plates containing cell media, gating out the dead cells that are positive for sytox blue (see Table 1). Sytox blue has an excitation of 444nm and an emission of 480nm (please refer to product manual for further information on how to read sytox blue on a FACS machine).
- i. Centrifuge plates containing single cells (300–450xg) for 5 min at room temperature, and using a microscope, determine which wells are confirmed to have only one cell. Using a marker, circle those wells containing single cells.
- j. Return plates to 37 °C incubator as soon as possible.

B. Scoring an *in vitro* LDA

- 1. After ~2–4 weeks remove the plates from the incubator and using a microscope, determine which wells contain cell spheres. For the 1 cell/well plates, be sure to only score those wells that were originally confirmed to contain a single cell.

2. Use ELDA: Extreme Limiting Dilution Analysis online software to calculate the cancer cell initiating frequency and significance (<http://bioinf.wehi.edu.au/software/elda/>).

Part II. *In vivo* LDA

Materials and Reagents

1. Centrifuge tubes (BD Falcon, 352096, 352070)
2. 1 ml syringe without needle (Restek, 22766)
3. 100 × 1 ml syringes with (28^{1/2}G) needles (BD, 329420)
4. 50× NOD-SCID or NSG mice
5. ~5 ml matrigel (Corning, 354248)
6. Serum-free cell culture media (see Recipes)
7. Wash media (DMEM/F-12) (ThermoFisher, 11320-033)
8. 0.4% Trypan blue solution (Life Technologies, 15250-061)
9. 0.25% Trypsin (Life Technologies, 25200-056)

Equipment

1. 37 °C Incubator
2. Light microscope (Leica, DM IL LED)
3. Hemocytometer (Hausser Scientific, 1483)
4. Pipette aid (Integra, 155 000)
5. Pipettes (BD Falcon, 357530)
6. Mouse ear punch (Kent Scientific, !NS750075-5)

Procedure

Note: The range of cell concentrations used will depend on the frequency of initiating cells in your fraction. An initial LDA using a wide range of concentrations can be used to find the best range of cell doses, ideally it should include doses with 100% tumor formation down to cell doses with no tumor formation, as well as, a range of doses in between. For an LDA to be statistically valid one must first test whether their sample complies with the Poisson single-hit model, which assumes that the number of biologically active units in a culture varies according to Poisson distribution. For addition information and calculations please refer to ELDA site (<http://bioinf.wehi.edu.au/software/elda/>), and the accompanying paper¹. Ex: if the initiating frequency is approximately (1:1,000 – 1:5,000), then the range indicated in table 2 will be sufficient to obtain statistically significant results.

A. Preparing cell syringes

1. Grow cells to confluence in a T175 flask at 37 °C.

2. Centrifuge cells (300–450xg) for 5 min at room temperature, then remove supernatant.
3. While cells are spinning, thaw matrigel on ice.
4. Resuspend pellet in ~3 ml of trypsin and incubate at 37 °C for the necessary time to obtain single cells.
5. Remove cells from incubator and resuspend with ~10 ml of wash media to stop trypsin reaction.
6. Centrifuge cells (300–450xg) for 5 min at room temperature, then remove supernatant
7. Completely resuspend pellet in enough cell media so that you will have a final cell concentration of about 3×10^6 cells/mL in order to make cell counting on the hemocytometer easier for you. Ensure that your cell concentration is no less than 2×10^6 cells/mL to allow for the cell concentrations listed in step 9.
8. Mix 10 μ l of cell mixture with 10 μ l of trypan blue and load mixture onto a hemocytometer to count the number of live cells using a light microscope. Be sure to count cells in all four coordinates and divide by 2 to obtain the concentration of cells in your solution ($__ \times 10^4$ /ml). Please refer to <http://www.abcam.com/protocols/counting-cells-using-a-hemocytometer> for a further explanation on how to use a hemocytometer.
9. Serially dilute cells so that you have the following final cell concentrations
 - a. Tube 1 (50,000 cells/injection): 600,000 cells in 360 μ l media
 - b. Tube 2 (10,000 cells/injection): 120,000 cells in 360 μ l media
 - c. Tube 3 (1,000 cells/injection): 12,000 cells in 360 μ l media
 - d. Tube 4 (100 cells/injection): 1200 cells in 360 μ l media
 - e. Tube 5 (10 cells/injection): 120 cells in 360 μ l media
10. Prepare syringes (with needles) by removing the plunger and adding necessary labels (*i.e.* cell concentration, treatment *etc.*).
11. Using a syringe (without a needle), aspirate the matrigel and deposit a small drop of gel (~200 μ l) into the base of syringe.
12. Thoroughly mix the desired cell solution before adding 30 μ l to the droplet of matrigel.
13. Carefully reinsert the plunger, making sure not to expel the matrigel solution out of the syringe.
14. Keep the loaded syringes on ice until ready to use.
15. Repeat this until you have 100 syringes (20 syringes per cell concentration), as shown in Table 2.

B. Injecting mice

1. Lightly sedate mice with isoflurane and ear notch them to identify each mouse (See Laboratory Animal Medicine, 2015, for more information on animal identification).
2. Inject mice subcutaneously with cells on both the right and left flank, using a new syringe for each injection (see figure 2). Each mouse should have two injections, one on each flank (see Table 2).
3. Return mice to their cage to recover.

C. Scoring an *in vivo* LDA

1. After ~4–8 weeks, determine which mice have developed tumors.
2. Continue to monitor mice with 0–1 tumors for up to 6 months, or as long as ethically permitted, to ensure there is no late tumor formation.
3. Use ELDA: Extreme Limiting Dilution Analysis online software to calculate the cancer cell initiating frequency and significance (<http://bioinf.wehi.edu.au/software/elda/>).

Notes

- a. Results will vary slightly when repeating a LDA, however when comparing treated groups, the LDA results should convey the same overall result each time. For example; treatment 'A' always produces a lower, statistically significant, initiating frequency compared to a control treatment 'B'.
- b. Increasing the number of replicates in both *in vitro* and *in vivo* LDAs will increase the preciseness of the assay.

Recipes

1. If using FACS for cell sorting you will need:
 - a. Sytox blue (Life Technologies, S11348)
 - b. Phosphate-buffered saline (PBS) + 2% fetal bovine serum (FBS) (Life Technologies, 10010023, 26140-111)
 - c. FACS tubes with 0.35 μ M filter (BD Falcon, 352235)
2. Serum free cell culture medium:
DMEM/F-12 (1:1 ratio) supplemented with:
 - a. Penicillin-streptomycin-amphotericin B (1%) (Life technologies, 15240-062) 5ml/500
 - b. L-glutamine (2mM) (Invitrogen, 25030081) 5ml/500
 - c. Non-essential amino acids (1X) (Hyclone, SH3023801) 5ml/500ml
 - d. Sodium Pyruvate (1mM) (Hyclone, SH3023901) 5ml/500ml

- e. HEPES (1X) (Invitrogen,15630080) 5ml/500ml
- f. Heparin (4ug/ml) (Sigma, H3149-100KU)
- g. Lipids 1ml/500ml, (Sigma, L0288)
- h. EGF (20ng/ml) (Biomart co., RKP01133)
- i. bFGF (10ng/ml), (Biomart co., RKP09038)
- j. N2 supplement-A 5ml/500ml, (Stem cell, 07152)
- k. NeuroCult SM1 Neuronal 2ml/500ml, (Stem cell, 05711)

References

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Figure 1.
In vitro LDA schematic.

Figure 2.
Subcutaneous injection sites on the right and left flank of a mouse

Figure 3.
In vivo LDA schematic

Table 1Cell seeding for an *in vitro* limiting dilution assay

Cell concentration (#cells/well)	Number of 96 well plates	Number of wells
1,000	0.25	24
100	1	96
10	2	192

Table 2Cell concentrations and injections for an in *vivo* limiting dilution assay

Cell concentration (#cells/injection)	Number of injections (2 injections/mouse)	Number of mice
50,000	20	10
10,000	20	10
1,000	20	10
100	20	10
10	20	10
TOTAL:	100	50