

3T3-L1A Cell Line Protocol

1) Introduction:

- ⇒ The 3T3-L1As may be kept in a pre-adipocyte state by following a standard splitting and feeding protocol.
- ⇒ If an adipocyte phenotype is desired the pre-adipocytes must be differentiated into adipocytes, a process that takes two weeks following the seeding of the cells into the experimental plates.
- ⇒ The morphology of the pre-adipocytes is fibroblast like. Once the differentiation process has started the cells look post-confluent, balled up and “icky”. Two-three days post-differentiation the cells begin looking like adipocytes - rounding up, making and storing fat.
- ⇒ The most important factor in the differentiation process is the cell density prior to differentiation. The seeding densities for a variety of experimental plates are listed below.
- ⇒ Once the differentiation process has begun the cells may not be split/passaged as they are no longer dividing.

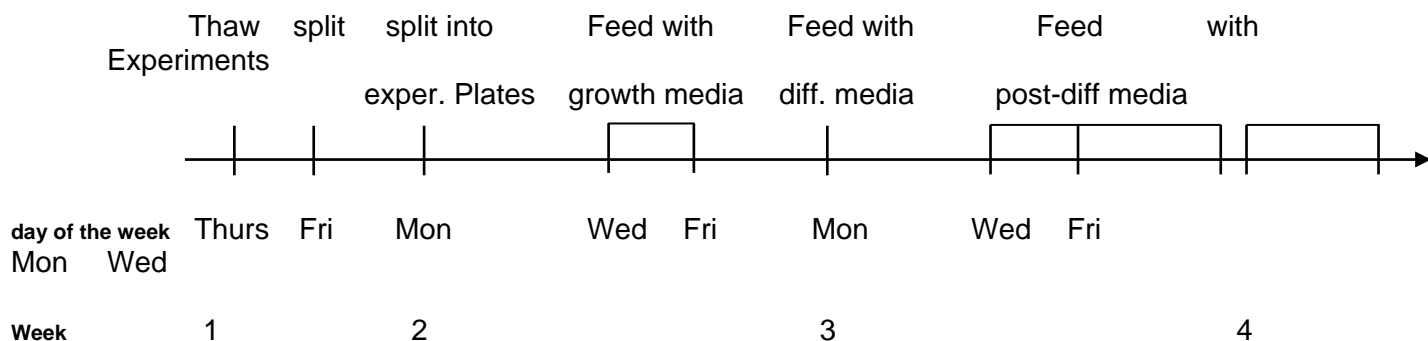
2) Time Line:

To carry:

- ⇒ If the procedure detailed below is followed the cells can be thawed on a Thursday, split the next day (Fri) to get them through the weekend and build up the number of plates/cells required for experimentation. The cells can then be split and plated into the experimental plates on Mon (see densities under “splitting” below)
- ⇒ Carry dishes can be maintained in 10 cm dishes. Those dishes seeded on Mon for carrying - $1.2-1.5 \times 10^5$ cells/10 cm dish - should be passaged again on Fri. With 2×10^5 cells added per dish on Fri, the dish will need to be split again on Mon.....
- ⇒ See “feeding” below for the feeding schedule for the carry dishes.

To differentiate:

- ⇒ Experimental plates that have been seeded (see densities under “splitting” below) on a Monday will be fed with complete growth media on Wed and Fri.
- ⇒ These cells will be confluent by Friday and on the Monday following will be post-confluent and will look balled up and ‘ugly’ – they are now ready to differentiate with IBMX-DEX-INS.
- ⇒ These cells are differentiated on Mon, fed with post-differentiation media on the next Wed, then maintained (Fri and Mon feedings) in the post-differentiation media until they are used for an experiment 7-10 days following the start of the differentiation process.



3) To thaw cells:

- Thaw one tube of cells quickly at 37°C (2 min).
- Rinse with 70% ethanol.
- Add cells to **10-20 mls** of “pre-conditioned” complete growth media (media that has been placed in a 10 cm **Costar** dish and equilibrated in a 37°C incubator for approximately 15 min to reach proper pH and temperature).
- Once cells sit down (usually takes 2-4 hrs) do a 100% media change into complete growth media to remove the DMSO and any dead cells.
- Once cells have reached a pre-confluent state, usually 1-2 days after thawing, cells can be split to carry or for differentiation.

4) Splitting:

Prior to confluence (80-90% confluent):

- Aspirate media and rinse cells once quickly with 1X PBS @ RT (on window sill in TC room).
- Aspirate and add 2 ml of trypsin/EDTA and incubate for 2-5 min @ 37°C.
- Add complete growth media up to 10 ml and triturate approximately 10 times to break up cells.
 - Add 10-11 μ l of cell dilution to each side of a hemacytometer
 - Count cells in the 1 mm center square (=25 squares, each containing 16 smaller squares) and 4x1 mm corner squares on each side of the hemocytometer.
 - The average cell count = # of cells x 10^4 /ml
 - The total # of cells/ml = (average count per square)x(the dilution factor)x (10^4).
Ex = 20 cells per square x 10 x 10^4 = 200 x 10^4 cells/ml = 2.0×10^6 cells/ml.
Therefore the total number of cells obtained = 2.0×10^6 cells x 10 ml = 20×10^6 .
- One 10 cm dish can be used to seed 8-10, 10 cm dishes for carrying.
- I use Costar plates for carrying and differentiation.

To carry:

- Plate cells at $1.2-1.5 \times 10^5$ cells per each 10 cm dish. At this density cells will need to be split every 5 days.

For Differentiation:

- When using T25 flasks:
 - Plate 2×10^5 cells/T25 flask.
 - 30 ml per flask.
 - ★ There are approximately 5×10^6 cells/flask by 7-10 days post differentiation.
- When using 6 well plates:
 - Plate 0.8×10^5 cells/well.
 - 4 ml per well.
- When using 24 well plates:
 - Plate 0.2×10^5 cells/well.
 - 1 ml per well.

★ There are approximately $4\text{-}5 \times 10^5$ cells/well by 7-10 days post differentiation.

- When using 96 well plates:
 - Plate 2×10^3 cells/well.
 - 100 μl per well.

5) Feeding:

To carry:

- Feed cells every 2-3 days by doing a **complete** (100%) media change with complete growth media. This works out to be Mon, Wed, and Fri feedings with the time line above.

For differentiation:

- For the first week after seeding, experimental cells are feed with the same media and on the same schedule as the cells to be carried (see above).

6) Differentiating:

- Three days after the cells are confluent (Mon) cells will be balled up and looking “icky”. Differentiate by changing the media (100% change) to the differentiation media containing IBMX-DEX-INS.
- Leave the differentiation media on the cells for **48 hrs** (Mon-Wed).
- On Wed change the media to the post-differentiation media containing insulin **with out IBMX and DEX.**
- The subsequent feedings on Fri and Mon are done with a complete media change into post-differentiation media containing insulin.
- *****Be careful when feeding after the differentiation has started as the media gets very fatty and thick. For the 24 well plates use a blue tip and pipet aid to remove the media, do not use the vacuum to aspirate the media as the fat sticks to the cells and if it is removed too quickly the cells will come off with the media. For the flasks, turn them upside down so the media runs off of the cells and then carefully vacuum the media off.
- The cells can then be used for experiments. Fatty acid oxidation assays are performed **7-10 days** after differentiation with IBMX-DEX-INS media (on Mon). Assays exploring triglyceride synthesis/TG inhibitors or stimulators are performed 3 days following the media change to the post-differentiation media (on Wed).

7) Freezing down:

Prior to confluence (80-90% confluent) trypsinize as described above.

- Combine cells from each 10 cm dish and pellet (spin for 2-3 min at 1000 rpm).
- Re-suspend the pellet in 1 ml of complete media + 10 % DMSO – 1 ml per each 10 cm dish.
- Aliquot 1 ml of re-suspended cells/vial and freeze slowly in isopropanol @ -80°C then store in the liquid N_2 freezer.

8) Medias:

a. Growth Medium

Remove 22 mls from 1 L of D-MEM and add the following components:

Reagent	[stock]	volume added	[final]
FBS		110 ml	10%
Pen/Strep		11 ml	1%
D-Biotin	**	8.0 $\mu\text{g/ml}$	0.008 $\mu\text{g/ml}$

Filter sterilize

**To make 8.0 µg/ml Biotin: add 0.01g to 1.25 ml of ddH₂O. Add one drop of 10 N NaOH from a yellow tip to solubilize.

b. **Differentiation Medium**

Solutions required:

day of use

3-ISOBUTYL-1-METHYLXANTHINE (IBMX) ---- Make fresh on

For stock solution add 11.5 mg/ml = 51.8 mM to ddH₂O, then add 1 N KOH or NaOH until it dissolves.

(Powder is kept in -20°C on tissue culture shelf in a desiccator)

DEXAMETHASONE (DEX)

For stock solution dissolve 0.39 mg/ml = 0.994 mM (FW=392.47) in ddH₂O.

(Powder is kept @ RT on my bench and solution aliquots are kept in door of tissue culture frig. @ 4°C

INSULIN

Stock solution = 10 mg/ml

Kept @ 4°C (Frig C)

OR to make from powder:

For stock solution dissolve in water. May need to add acid to get it in solution – 1 drop conc. HCl from yellow tip/1 ml solution.

(Powder is kept at -20°C on tissue culture shelf – store aliquots t in -20°C)

filter sterilize

Add the following reagents to 100 ml of growth medium on the differentiation day:

	Reagent	[stock]	volume added	[final]
	IBMX	51.8 mM	1 ml	0.5 mM final
	DEX	0.994 mM	100 µl	1 µM final
final	Insulin	10 mg/ml	100-200 µl	10-20 µg/ml

filter sterilize

c. **Post-differentiation Medium**

Add 100 - 200 µl of insulin stock (10 mg/ml) to 100 ml of complete growth medium (10-20 µg/ml final insulin concentration). Add insulin **fresh** to media - each day of feeding.

9) **Product Ordering Information:**

- D-MEM (with L-Glutamine, high glucose, w/ pyridoxine hydrochloride, w/o sodium bicarbonate);

Gibco #11965084.

- Penicillin/Streptomycin; 10,000 U/ml Penicillin, 10,000 µg/ml Streptomycin; Gibco 15140-148.
- Biotin, approx. 99%, cell culture tested; Sigma B4639-1G.
- Fetal Bovine Serum (FBS), characterized; Hyclone; SH30071.03HI. Hyclone will heat inactivate if you include the HI when ordering.
- Insulin; 10 mg/ml; Sigma I-0516.
- 3-Isobutyl-1-methylxanthine (IBMX); Sigma 15879.
- Dexamethasone – water soluble (DEX); Sigma D-2915.
- Corning 10 cm dishes (100x20mm); Fisher 430167.
- Costar 24 well plates; Fisher 3526.