Sep 13, 2019 Version 2

UC Davis - Basic Adipocyte Culture Protocol V.2

DOI
dx.doi.org/10.17504/protocols.io.7bvhin6

K.C. Kent Lloyd[1]

[1]University of California, Davis

Mouse Metabolic Phenotyping Centers
Tech. support email: **info@mmpc.org**


Lili Liang

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.7bvhin6

External link: https://mmpc.org/shared/document.aspx?id=82&docType=Protocol

Protocol Citation: K.C. Kent Lloyd 2019. UC Davis - Basic Adipocyte Culture Protocol. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.7bvhin6**

Protocol status: Working
We use this protocol and it's working

Created: September 13, 2019

Last Modified: September 13, 2019

Protocol Integer ID: 27733

Keywords: Basic Adipocyte Culture Protocol , basic adipocyte culture protocol summary, adipocyte, protocol, culture

Abstract

Summary:

Basic Adipocyte Culture Protocol

Materials

MATERIALS

- Phosphate-hepes buffer
- Nalgene incubation jars (30 ml for mice) **Fisher Scientific Catalog #**02-925-1a
- Nytex filters 250 um **Catalog #** PGC 34-1800-03
- 450 um for subcutaneous depot **Catalog #**PGC 34-1800-09
- Long needles (6) **Fisher Scientific Catalog #** 14-825-16H
- 1 ml pipet tips (6 boxes)
- 0.2 ml pipet tips (1 box)
- surgical equipment (3-5 small scissors 3 large scissors 3 forceps)
- 500 ml reagent jars 250 and 100 ml reagent jars

Note:

Fisher Scientific, RRID:SCR_008452

Reagent Preparation:

Reagent 1: **Hepes Phosphate Buffer**
1000 mL or 500 mL Recipe

For 1000 mL recipe add ~200 mL ddH_2O to a 400 mL beaker.
For 500 mL recipe add ~100 mL ddH_2O to a 250 mL beaker.
Add BSA located in FC refrigerator:

1000 mL Recipe			500 mL Recipe
20 g	Bovine Serum Albumin	Omega BA-90	10 g

Let BSA stir in a small beaker while mixing the rest of the chemicals.

For 1000 mL recipe add ~600 mL ddH_2O to a 1000 mL beaker.
For 500 mL recipe add ~300 mL ddH_2O to a 500 mL beaker.
Then add these chemicals:

1000 mL Recipe			500 mL Recipe
7.900 g	NaCl	Fisher S271-3	3.950 g
0.323 g	$CaCl_2*2H_2O$	Fisher C79-500	0.1615 g
0.308 g	$MgSO_4*7H_2O$	Fisher M63-500	0.154 g
0.061 g	KH_2PO_4	Mallinckrodt 7100	0.0305 g
0.3086 g	Na_2HPO_4 (see note)	Fisher BP-332	0.1543 g
0.900 g	D-glucose	Sigma G7021	0.450 g
10 mL	1M HEPES (FC refrig.)	Sigma H0887	5 mL

Note: can use 0.291 g $Na_2HPO_4*7H_2O$ instead of 0.1543 g Na_2HPO_4

Add the BSA solution after the chemicals have been dissolved. Adjust the pH to 7.2-7.4 with NaOH, then adjust the volume to 1000 mL or 500 mL with ddH_2O using a graduated cylinder. The buffer is then filtered into an autoclaved bottle using a 0.2 µm Nalgene bottle top filter under sterile conditions. Label, date, and initialize the buffer bottle.

Troubleshooting

1 **Day Before Preparation:**
Make phosphate-hepes buffer (instructions posted on chem shelf or *last page of this document*).

Prepare and autoclave supplies: Nalgene incubation jars (30 ml for mice--Fisher 02-925-1a), nytex filters (250 um PGC 34-1800-03, 450 um for subcutaneous depot PGC 34-1800-09), long needles (+6—Fisher 14-825-16H), 1 ml pipet tips (+6 boxes), 0.2 ml pipet tips (1 box), surgical equipment (3-5 small scissors, 3 large scissors, 3 forceps), 500 ml reagent jars, 250 and 100 ml reagent jars.

Cut 6 inch long PE tubing for aspirating needles (Clay Adams PolyEthylene tubing - PE-205- fit to needles) and sterilize under uv.

Clear and clean hood, turn on uv light.

2 **Day 1 Media Preparation:**
Place buffer in incubator to warm.
Turn on shaking water bath to 37 degrees.
Place 6 ml (pre-aliquoted and frozen) tubes of FBS (Omega FB-02), nystatin (N1638 Sigma), 3 ml penicillin/streptomycin (15140-122 GIBCO) (all in FC freezer) in incubator to thaw.
Get 500 ml bottle of DMEM (11885-084 GIBCO) from walk-in cold room (**check glucose content—need low glucose (5mM)!!**!).

Place microfuge tube of insulin stock in hood to thaw (-80 freezer#1, top shelf, FC insulin box).
Place microfuge tube of 100uCi/ml C14 glucose (New England Nuclear NEC-042) stock in hood to thaw (-20 freezer, FC C14 glucose stock).

Turn on hood light in order to turn off uv.
Make basic media by adding 6 ml each of FBS, nystatin, 3 ml penicillin/strep and 6 ml nonessential amino acids (11140-050 GIBCO in FC refrigerator) to 500 ml DMEM.
Make medias (depending on the experimental reagents to be added, this can be the most difficult, intensive, and time-consuming part). Assume that 3ml media are needed per well (2ml added on Day 0, and .3 ml replaced at Day 1,2,3.). In order to make up .02 uCi/ml media for lipogenesis work, add .2 ul/ml media.

Prepare insulin.
- Dilute the 1mg/ml stock insulin (SIGMA I5523—original dilution 10mg in .01M HCl—150ul aliquots) 10Xs (.1 ml to 1.0 ml)
- Sterilize with .2 um syringe filter.

- Label it 160 nM insulin stock.
- Dilute 160 nM insulin stock 100Xs (.1 ml to 10 mls).
- Label it 1.6 nM insulin stock.
- Mix well.
- Dilute 1.6 nM insulin stock to 0.48 nM stock and label (1.5 ml to 5 mls).
- Dilute 1.6 nM insulin stock to 0.16 nM stock and label (1ml to 10 mls).

Add the appropriate amount of insulin to medias. 10 microliters of the insulin stock added to 1 ml of media = conc of stock label insulin media (i.e. 100 microliters of 1.6 nM insulin stock to 10 ml of media = 1.6 nM insulin media).
Mix medias well, loosen lids, and store in incubator (6% CO2) until needed.
Place extra DMEM in incubator until needed.

3 **Prepare for animal kill.**

- Cut lab covering for each carcass and label with animal #, absorbent side up.
- Label two sterile 60 mm culture dishes with animal # for each animal.
- Place 1 dry set by microbalance.
- Add buffer to other set.
- Place surgical equipment in beaker with 70% EtOH.
- Fill a 15 ml labeled conical tube with buffer.
- Label one edta purple top vacutainer with ID
- Set aside lid and place small plastic funnel in tubes.
- Get ice for bloods.
- Set up and place FC notebook by microbalance.

Prepare collagenase (Worthington 4197 -- 5 gram dry-bottle in FC refrigerator).

- Mice collagenase concentration =0.625 mg/ml
- Need 2 ml/gram of fat
- Assume 1 gram of fat/mouse.
- Transfer to 50 ml conical tube.
- Add 8 ml buffer/5 mg dry collagenase (Standard 6-10 mouse recipe = 12.5 mg collagenase/20 ml of buffer)
- Mix collagenase well and sterilize with steriflip.
- Store in incubator until needed.

Ready to kill:
Add isoflurane to kill jar.
Place animal in kill jar.
When unconscious, weigh and record.
Deccapitate, and collect truncal blood in funnel and edta tube.
Place lid on blood tube, invert, store on ice until centrifuging and separating is possible.
Place animal on kill cloth and take to hood.

4 **Fat Digestion:**

Remove epididymal and/or mesenteric fat pad using buffer-rinsed surgical equipment and place in labeled culture dish with buffer.
Tare dry labeled culture dish with microbalance.
Under hood, transfer fat pad to culture dish using buffer-rinsed forceps.
Weigh and record.
If fat pad weighs more than 4-4.5 grams, remove extra fat using buffer rinsed scissors.
Record suspension fat pad weight on culture dish.
Add 2 ml of collagenase/gram of fat to labeled suspension jars.
Transfer fat pad to lid of culture dish.
Set timer.
Mince fat for 1-2 minutes (one minute when experienced, two when novice).
Using cell scraper, transfer minced fat to incubation jar with collagenase.
Set timer and record incubation start time on lid of jar.
Parafilm lid of jar.
Place in 37 degree shaking (motor on 6) water bath for 30 minutes.
Replace buffer container in incubator between uses.

5 **Fat Cleaning:**

During 30 minute incubation, prepare for filtration.

- For each mouse (or suspension with less than 1 gram fat) label a 15 ml conical.
- Remove lid and place a 250 um filter on top of tube.
- Use a 10 ml pipette to force filter into tube.

At 30 minute incubation (+/- only 1 minute) remove suspension jar from bath.
Add 10 ml buffer and pipette up and down 4 times to mix.
Transfer suspension to filter, making sure pipette is in filter, not in a fold.
Allow suspension to drain.
Making sure gloved hands are sterile, gently scrape filter into conical tube (skip this step if volume of cell suspension already collected is adequate for culture needs).
Add buffer up to 14 mls Centrifuge at 900 rpms--check setting—for 6 minutes.

During centrifuging prepare syringes for cleaning steps.
For each suspension label a 20 ml syringe.
Place a long autoclaved needle (6 inch 18 gauge Fisher 14-825-16H) on syringe.
Place a PE tube plastic cover on needle.
Place syringes with needles upright—3 to a 600 ml beaker—to keep sterile.
Label a large beaker for waste.
At end of 1st spin remove the buffer and stromal vascular layer from underneath cell layer with needle and syringe. Place this buffer in waste beaker.
Add fresh buffer to 10-14 ml depending on quantity available.

Centrifuge at 900 rpms for 6 minutes.
At end of spin remove buffer and replace with 8-10 ml warm basic media.
Centrifuge at 900 rpm for 6 minutes.
At end of centrifuge remove media and add fresh up to no more than 14 ml.
Place in incubator and start a timer.
Incubate for at least 30 minutes before plating.

6 **Plating in 6 well plates:**
During incubation prepare collagen (Cohesion Technologies 00701)
Calculate the amount needed figuring .3 ml/well and plus an extra 3-5 ml.
Transfer that amount to an appropriate-sized sterile container (50 ml conical, 100 ml reagent bottle, or collagen bottle). To minimize collagen waste, pouring is better than pipeting.
Add 1 ml 10X DMEM stock (GIBCO 41500-034 -- 50 ml conical tubes in door of FC refrigerator—1 packet reconstituted to 100 ml w/.22 grams bicarbonate, sterilized) per 10 ml collagen.
Add 10 and 1M NaOH to collagen to get pH = 7, using a red color to judge (not orange, not pink).
Sometimes it is safe to start with 1ul of 10M NaOH/ml of collagen and then fine tune with 1M NaOH.
However, collagen pH can vary by lot and this “safe” quantity can change. Add 1M NaOH only 1-5 ul at a time.
Try not to overshoot/undershoot too many times since this affects ability of collagen to set.
Try not to take too long, as the collagen can start to set if it gets too warm.
Set up for collagen pipeting by having a 1 ml pipet set for .3ml for wells and a 200ul pipet set for .15ml for vials. Also set up a 200ul pipet for .05ml media.
Have a 50 ml conical tube set up to hold each pipet and keep sterile (minimizes the need for fresh tips with each pipeting and wasting expensive collagen).
Prepare plating plan based on amount of fat in suspensions, and culture objectives and priorities.

At end of incubation (the length can be up to 3 hours without affecting cell activity) and when collagen, plates are ready, prepare suspension 1 for plating. Remove media with long needle from under cell pack. Centrifuge 2 minutes at 500 rpm to bring extracellular lipid to top of cell layer. Remove extracellular lipid from top and media from bottom of cell pack.

The amount of isolated adipocytes added to each well is dependent on age and leanness of animals and the depot used. 50 ul of epididymal cells is sufficient for young lean mice (less than 24 grams). 75-100 ul epididymal cells is better for older and fatter animals.

Pipet 0.3 ml collagen in middle of all wells of one plate. Add .05 ml basic media to top of collagen mound in each well. In order to measure and account for media evaporation, a “no cell” well is pipeted with collagen/media only, as just described. It will be treated/sampled identically to all other wells.

Use an accurate 200 ul pipette (check for accuracy) with a sterile wide-bevel tip for fat pipeting (ART 200G pipet tips, Fisher 21-236-1A). Draw up 50ul (or 25-200 ul) of fat suspension from center of cell pack and pipet back into cell well. Draw up 50ul (or 25-200 ul) of fat suspension from center of cell pack with same pipet tip and carefully check the pipet tip of adipocytes for homogeneity. If tip is full, without pockets of extracellular lipid, pipet directly on collagen/media droplet. Using same tip, add adipocytes to all wells in the plate (except the “no cell” well), or vials in the set. Replace lid on plate, label with plate #, FC# and Suspension #. Spread collagen throughout bottom of wells with back and forth motion, but do not get too vigorous. Place plate in incubator immediately to set.

Finish the plating for all suspensions. Carefully, without disturbing cell layer, add 2 ml of appropriate media to each well (including “no cell” wells) and label the wells. Double check to make sure each plate is appropriately labeled and return to incubator. Return the leftover media to incubator. END OF DAY 0

Day 1:
Set up a 6 microfuge tubes for each plate and number as per culture run sheet. One plate at a time, sample from each well and place sample in appropriately labeled microfuge tube. Change pipet tip between each sample. Replace sampled media with .3 ml of the appropriate media. Freeze samples until glucose/lactate or leptin or adiponectin analysis.

Day 2:
Set up a 6 microfuge tubes for each plate and number as per culture run sheet. One plate at a time, sample .3 ml from each well and place sample in appropriately labeled microfuge tube. Change pipet tip between each sample. Replace sampled media with .3 ml of the appropriate media. Freeze samples until glucose/lactate or leptin or adiponectin analysis.

Day 3:
Set up a 6 microfuge tubes for each plate and number as per culture run sheet. One plate at a time, sample .3 ml from each well and place sample in appropriately labeled microfuge tube. Change pipet tip between each sample. Replace sampled media with .3 ml of the appropriate media. Freeze samples until glucose/lactate or leptin or adiponectin analysis.

Day 4—Final Samples:

Set up a 6 microfuge tubes for each plate and number as per culture run sheet. One plate at a time, sample 1 ml from each well and place sample in appropriately labeled microfuge tube. Change pipet tip between each sample. Freeze samples until glucose/lactate or leptin or adiponectin analysis.

We determine the intracellar lipid in the 96 hour cultured cells. Media is removed and as much extracellular lipid as possible. Depending on how firmly the cells are attached to the collagen, this can be an easy task or a challenging task. When the cell/collagen pack is firm it is easy (and this is why it is best to wait at least 40 minutes before adding the 2ml of media to the just plated cell on Day 0). Aspirate remaining media with extracellular lipid with a vacuum flask/Pasteur pipet setup. Add 1ml of warm basic media for a rinse, and aspirate again. Wipe lipid from edge of well with a cotton swab on stick. Add 4 ml of methanol to each well. Parafilm the plate and cover with lid. Refrigerate until transfer to folch tubes using the adipocyte transfer protocol.

If the cell/collagen layers are not firm it is necessary to protect them during aspiration with a 250um mesh circles cut to the size of the wells. Carefully remove as much media and extracellular lipid as possible with pipet (aspirating is too risky) and without disturbing or losing cells. Place a 250um mesh circle in each well. Add 1-2 ml of warm basic media for a rinse. Swirl the well contents and then let the plate sit uncovered, allowing time for the extracellular lipid to rise to top of media. Aspirate media as above. Wipe lipid from edge of well with a cotton swab on stick. Add 4 ml of methanol to each well. Parafilm the plate and cover with lid. Refrigerate until transfer to folch tubes using the adipocyte transfer protocol.

7

Determination of Total Intracellular Lipid and (Glucose to Lipid)*

Folch Method

***(Glucose to Lipid) steps are *highlighted (BOLD)* and are skipped if only intracellular lipid is to be determined.**

1. All work with chloroform containing Folch tubes must be done under the fume hood.

2. Add dH20 to within 0.5 inches of Folch tube top 24 hours after addition of chloroform.

3. Reseal tube and invert 2-3 times. Be aware that sometimes tubes leak when inverted and labeling disappears. Check and relabel when necessary. Keep tubes in sequential order so relabeling is possible.

4. When chloroform layer is clear, tubes are ready for the rest of the procedure.

5. Aspirate the top water/methanol layer off the chloroform layer. ***Make sure to use aspirating flasks that are labeled with radioactive tape when aspirating from tubes containing C14.*** Retain as much as of the chloroform layer as possible (as least 8 mls), while trying to remove all traces of the top layer. Use an un-aspirated tube to judge the amount of chloroform lost.

6. When all tubes are aspirated, let settle while preparing Folch pans, pipets and ***scintillation vials***. Some Folch tubes are for lipid determination only, therefore do not contain C14, and scintillation vials are not needed. If you do not know if this is the case, ask Kimber or James.

7. Pre-labeled pans are in the Plexiglas desiccators.

8. Pre-made data sheets are taped to side of desiccators. Label data sheet with FC#, date, your name.

9. Trays are on top of desiccators. Make sure the tray is lined with clean aluminum foil. Use gloves when handling trays and pans.

10. Weigh pans one at a time, shutting door of desiccators after the removal of each pan. Record the weight to the 4th place and # of each pan on the data sheet and load the weighed pan on tray, left to right, front to back. Need 1 pans per tube for duplicates.

11. Keep a foil cover over weighed pans.

12. Load clean scintillation vials in racks. Label 1 vial for each tube using black Sharpie. Each tube must be labeled with FC# as well as tube # FC 230, p1-1, p1-2,p1-3....

13. ***Collect one 1 ml pipette for each tube in a pipette basket,*** and one 5 ml pipette for each tube in a different basket. Check the pipette labels carefully!! Talk to Kimber or James about allowable substitutions if not enough pipets. Record tube # under Sample ID on data sheet, and make sure the size of the pipette used for each sample is recorded.

14. Move dirty pipette soaker, which is stored by sink in FC lab, near working station under the hood.

15. ***Pipette 1 ml of chloroform into corresponding scintillation vials.*** The folch tubes should be left open only long enough to transfer aliquot.

16. Place used pipettes in the pipette soaker, tip up.

17. Set the vials aside under hood to allow chloroform to evaporate overnight.

18. Place tray of pans under hood behind folch tube rack.

19. Pipette 5 ml of chloroform from each tube into the corresponding pan. Record the size of the pipette. Use strips of foil to keep all rows of pans covered except the row into which you are pipeting.

20. Leave the filled pans under the hood to evaporate overnight with the foil cover set lightly on top.

21. When chloroform has evaporated from vials, add 15 ml Ecolume scintillation fluid to each scintillation vial, put on lid and mix well.

22. Prepare a scintillation vial of 100 ul of each media that corresponds to the Folch tubes. Add 15 ml of the Ecolume scintillation fluid in the blue jug to each media vial.

23. Load vials onto scintillation counter. Make sure the first vial is a blank, marked PJH C14 Rm3426. Sign the logbook. To start counter, hit "ready" 2xs, then "2" enter, "2" enter.

24. Remove the vials the next day, and carefully label DPM data sheet with FC # and each count with vial label.

25. When chloroform has evaporated from pans, place folch pans into desiccators.

26. They can be weighed 2+ hours later. Use gloves when weighing pans and touch outside rims only. Record weight on data sheet. Substract Pan from Pan+TG and multiply by 2 (if used a 5 ml pipette) to determine total intracellular lipid.

27. Place data sheet and vial DPM counts into the FC CPM data binder #2.

8 **Glucose Oxidation Protocol**

All work areas and equipment used will be defined/labelled with tape "Caution-Radioactive Materials." Personnel will wear lab coats, closed-toed shoes and disposable gloves.
The 1000 uCi of C14 glucose is reconstituted in 1 ml sterile dd water and transferred to sterile 125 ml reagent jar. Use 4 more exact 1 ml pipeting of sterile dd water to clean C14 vial, transferring each to the reagent bottle. Add 95 ml sterile dd water to reagent bottle to dilute C14 to 10 uCi/ml. Aliquot into 200 sterile microfuge tubes, 500 ul each, and stored in labeled freezer box in the -20 freezer.

The work bench of the sterile hood in room 3430 is lined with absorbent counter paper and the dry C14 waste container is moved to the vicinity of the hood. The C14 glucose is added to culture media at a concentration of 0.03 uCi/ml (3ul per ml) using disposable pipet tips in a disposable 50 ml falcon tube. Any unused portion of the C14 glucose glucose stock is marked T (for thawed) on top of tube and returned to the freezer box and the –20ºC freezer. All large volume pipeting of radioactive media is done using disposable pipets. All pipets, tips and empty tubes are disposed in the dry C14 radioactive waste immediately after use. All prepared media are labeled with radioactive label tape and stored in an incubator which is also labeled with radioactive tape.

Cells isolated from rat adipose tissue are plated in 25 ml glass scintillation vials and 2 ml of the radioactive media (0.03 uCi/ml) is applied. The vials are capped using a rubber stopper which has been fitted with a polystyrene hanging well containing a 2 cm x 8 cm strip of Whatman #1 filter paper. Vials are gassed with 5% CO2 for 15 seconds using vent/delivery needles. Cells are maintained for 48 hr in a 37ºC incubator which is labeled as containing radioactive material.

After 48 hr, 1000 ul of media is removed from each vial using a needle and 1 cc syringe. To minimize waste, the same needle and syringe is rinsed with plain/old DMEN and used for all samples. Used rinse media is placed in a labeled C14 waste bottle. Needles are discarded into a C14 radioactive-labeled sharps box and syringes are discarded into a dry C14-labeled radioactive waste bin. The 1000 ul media samples are placed in microfuge tubes and stored in a radioactive-labeled freezer box (also labeled as oxidation samples--FC XX). After media samples are removed, 200 ul of benzethonium hydroxide, a chemical which binds carbon dioxide, is applied by needle and syringe onto the filter paper in the hanging well through the rubber stopper (same needle is used for all samples). The needle is discarded into a C14 radioactive-labeled sharps box and the syringe is discarded into a dry C14 labeled radioactive waste bin. The C14 glucose oxidized by the cells to CO2 is absorbed onto the benzethonium hydroxide-soaked filter paper. Cells are lysed by the addition of 3 drops sulfuric acid delivered with needle/syringe through rubber stopper (same needle is used for all samples). All needles are placed in a radioactive-labeled sharp box and syringes are placed in the dry radioactive waste.

After an overnight wait, the rubber stopper is removed from the vial and the hanging well is clipped with small wire cutters into a clean 25 ml scintillation vial containing scintillation fluid and the radioactivity is counted by a scintillation counter. One hundred ul aliquots of the unconditioned treatment media is also counted in 25 ml aqueous based scintillation fluid to determine total dpms added to vials.

The rubber stopper is disposed in the dry radioactive waste. The wire cutters are washed three times with RBS, and the RBS is discarded into liquid C14 radioactive waste. Both the scintillation vials used for culture and used for counting are labeled as

radioactive and stored in vial flats until they are collected by EH&S. All procedures (adding media, removing media samples, adding sulfuric acid, adding benzethinium hydroxide, removing stopper and well) are performed on absorbent counter paper, which is surveyed for contamination and placed in dry radioactive waste if contaminated. Paper that becomes contaminated with spilled radioactive media is cut out and disposed of into dry C14 radioactive waste. A count rate survey meter will be used to check work areas for contamination during and after each experiment. Paper that becomes contaminated with spilled radioactive media is cut out and disposed of into dry C14 radioactive waste.

24 hours later vials can be loaded onto scintillation counter. Make sure the first vial is a blank marked PJH C14 Rm3426. Sign the logbook. To start counter, hit ready 2xs, then 2 enter, 2 enter.

Remove the vials the next day, and carefully label DPM data sheet with FC # and each count with vial label