Basic Tick Cell Culture Methods

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General Considerations

Medium Preparation:

The tick cell culture medium L-15B was formulated to grow tick cells with reduced concentrations of serum and other supplements (Munderloh and Kurtti, 1989). The addition of amino acids, a tricarboxylic acid, glucose, minerals and vitamins to Leibovitz's L-15 (Leibovitz 1963) resulted in growth rates that were comparable to those in L-15 with 20% FBS when tested on several established cell lines from ixodid ticks. The medium also facilitated isolation of new cell lines from *Ixodes scapularis* ticks (Munderloh et al. 1994), grasshoppers (Munderloh et al. 1994), and *Aedes vexans* mosquitoes (Mazzacano et al. 1991). The original formulation which specified 12 mM glucose, was subsequently modified to increase this amount to 80 mM, resulting in further improvement of tick cell growth. During the initial attempts to culture *Anaplasma phagocytophilum* (formerly the human granulocytic ehrlichiosis agent, HGE) from patient blood samples, we discovered that these bacteria were sensitive to osmotic pressure, and would not grow in L-15B unless diluted by adding one third volume of sterile water, to achieve approximately 300 mOsm/L (Munderloh et al. 1999). This medium is referred to as L-15B300. Some of the tick cell lines (e.g., ISE6, IRE11, DAE100) are not or only minimally affected by diluting the medium, and others prefer the full-strength formulation (e.g., IDE12, CCE1, 2 and 3). We therefore routinely use the diluted formulation for the former, and the undiluted medium for the latter, supplemented with 5% FBS, 5% TPB and 0.1% lipoprotein (1% of 10 fold diluted stock). For cultivation of *Anaplasma, Ehrlichia* and *Rickettsia* the diluted medium, L15B300, is always used, and is buffered by addition of HEPES and $NaHCO_3$. It is not necessary to adapt the cell lines to the medium used for bacterial culture. In fact, tick cell lines do poorly when grown continuously in the presence of bicarbonate, yielding low rickettsial numbers in turn. The ability of tick cells to tolerate wide ranges in pH, osmolarity and periodic presence of bicarbonate is likely related to their life cycle alternating between inactive stages that are characterized by hemolymph osmotic pressures of up to 500 mOsm/L in unfed females, and actively feeding

stages when blood (containing bicarbonate) is imbibed and hemolymph osmotic pressure drops precipitously.

Table 1 lists the ingredients for making the undiluted L-15B containing 80 mM glucose, and how to store them. All ingredients should preferably be cell culture tested, or at least of the highest grade available.

Upon receipt, all ingredients should be dated, and stored as indicated in Table 1. When preparing L-15B, lot or batch numbers for all ingredients should be recorded in a media log book, and subsequent medium batches be made using the exact same (hopefully known good) ingredients. It is important to write down actual, weighed out amounts of ingredients added, rather than the amount called for in the recipe, to help track any errors and problems.

The quality of the water used to make L-15B is extremely important. Researchers first attempting tick cell cultivation may be best advised to purchase cell culture grade water (type 1) from a supplier such as Invitrogen or Protide. Water "polishing" systems such as the Milli-Q system from Millipore fitted with an ultrafiltration cartridge to remove endotoxins are capable of supplying water of satisfactory purity if serviced regularly as directed by the manufacturer. To prepare L-15B, it is best to use a volumetric flask that has been high heat (180 ºC or higher, for at least 2 hours) sterilized to remove endotoxins. Five hundred to 700 ml of sterile, cell culture grade water is preloaded into the volumetric flask, and ingredients are weighed out and added in the order listed. The flask should be agitated intermittently to swirl the liquid and aid in the dispersal of the powders. Once all components have been incorporated, the volume is brought up to 1 Liter, and a clean stir bar is added. While the medium is being stirred slowly for the next 1.5 hrs (not longer), it is advisable to wrap the volumetric flask in aluminum foil and protect the medium from light. Upon completion, the L-15B is filtered through a 0.22 μm filter into storage bottles. If bottled cell culture water was purchased from a commercial supplier, it is convenient to use these bottles to store the medium. Filter units that incorporate storage bottles are an excellent choice as well. Glass bottles should be prepared as described below. Using separate pipets for each storage bottle, 4-5 ml of medium from each bottle are removed into a sterile tube, and incubated at 37 ºC to check sterility. An aliquot of medium that will be discarded is used to measure the *p*H which should be between 5.5-6.5. If an osmometer is available, ensure that the

mOsm/L is 415 mOsm/L plus or minus 10. The medium should be stored protected from light at 4 ºC, and will keep for months.

L-15B300 is prepared by simply adding cell culture grade, sterile water to L-15B in a volume that amounts to 33% of additional water in the diluted medium. To achieve this, the volume of L-15B to be diluted is divided by the number 3, and the result is the quantity of water that should be added. For example, when starting with 300 ml of L-15B, 100 ml of water is the correct amount to add in order to make 400 ml L-15B300. The osmotic pressure of the medium will be reduced to approximately 300 mOsm/L.

Mineral and Vitamin Stocks

The preparation of these has been described in detail (Munderloh and Kurtti 1989). There have been no changes in the formulations and they will not be repeated here. One of the goals of improving the culture conditions for tick cells was reduction of undefined supplements such as serum and TPB by as much as 75%. These additives likely were the major source of trace elements in Leibovitz's medium which does not contain any, and we anticipated that it would be necessary to replace those we considered to be required by arthropods whose sole source of nutrition is blood. Presumably, iron is key among minerals ticks would pick up with a blood meal, and was therefore incorporated as ferrous sulfate at a concentration of $1.8X10^{-3}$ m*M* along with cobalt, copper, manganese, zinc, molybdate, and selenate in the 10^{-5} to 10^{-6} m*M* range. In the presence of 5% FBS, 5% TPB and 0.1% lipoprotein concentrate, these supplements added to L-15B have supported continuous cultivation of cell lines from every tick species tested so far (Table 2).

Other additives

Tryptose phosphate broth (TPB) is an enzymatic digest of casein commonly used for culture of fastidious bacteria, but has been widely employed in invertebrate cell culture as well. Besides peptides and amino acids, it also appears to provide purines and pyrimidins that can be utilized by cells to make DNA via salvage pathways (Kurtti and Munderloh, 1989). While some well-established tick cell lines can grow in the absence of TPB, primary cultures and young lines usually require this supplement. Because TPB is both inexpensive, easy to prepare, and beneficial, it is routinely included. We have found that the powdered Difco brand TPB from

Beckton Dickinson is the best quality for tick cell culture. It should be prepared as stated on the label, and aliquoted into 100 ml-bottles. Care must be taken to autoclave TPB for only 15-20 min at 121 ºC, and not longer. The autoclaved liquid must be a pale straw color. If it has been autoclaved for too long and has turned dark, it will be toxic to the cells. The liquid TPB should be added to L-15B300 at 5% (v/v).

Fetal bovine serum (FBS) is the only type of serum used for tick cell culture, and is routinely heat-inactivated, although this step is not an absolute requirement. As with all other ingredients, sera with low levels of endotoxins are preferred, and all batches should be tested for their ability to support growth of tick cell lines in comparison with a known good serum. Most suppliers will make test samples available free of charge if the buyer guarantees purchase of a minimum quantity, commonly 6 L. Fetal bovine serum can be stored for extended times (years) at -20 ºC or -70 ºC. Depending on quantities used over a given period, and to avoid repeated freezing-thawing cycles, it is recommended to subdivide volumes of 500 ml at a time into 50-100 ml aliquots before heat-inactivating (30 min at 56 ºC in a water bath) and freezing. It is added to L-15B/L-15B300 at a concentration of 5 - 10% (v/v), concentrations which support both primary cultures as well as established lines. Higher concentrations do not necessarily improve growth, and should be carefully evaluated. The amount needed depends on the quality of the FBS.

Bovine Lipoprotein Cholesterol Concentrate (BLC) is used as a partial serum-replacement for reduced serum or serum-free mammalian and tick cell cultures. It allows significant cost savings while reducing the percentage of undefined supplements in the culture system, which reduces variation in experimental results when a new lot of serum is introduced. Lot-to-lot variation in sera can completely change the outcome of assays aimed at determining, e.g., nutritional requirements of either the tick cells themselves, or microbes growing in them. The lipoprotein that we prefer to use was temporarily unsatisfactory, due to problems with contaminating bacteria. The manufacturer seems to have addressed this successfully (the product now comes sterile-filtered), and we again recommend the bovine lipoprotein cholesterol concentrate from MP Biomedicals (formerly ICN; catalog number 191476). Upon arrival, it should be aliquoted and stored frozen. Before use, it is diluted 10-fold in L15B/L-15B300 (or L15C/

L15C300, and stored in 10-50 ml aliquots at 4 ºC, protected from light. This diluted working stock should be incorporated into the final medium at 1-2% (v/v).

Antibiotics and Antimycotics

We do not routinely include antimicrobials in tick cell culture media. When needed as a precaution such as for shipping cells to another laboratory, or when cells are regenerated from liquid nitrogen storage, antibiotics commonly available for mammalian cell culture may be used at the recommended concentration. Examples include gentamycin (50 μg/ml final concentration, from Sigma), or a mixture of penicillin and streptomycin (50-100 IU/ml and 50-100 μg/ml, respectively), and fungizone (0.125-0.25 μg/ml), available from BioWhittaker. These antibiotics and antifungals may also be useful for the first few weeks after initiating primary cultures from tick eggs or other tissues. It is however advisable to exercise caution, as some, e.g., gentamycin, may interfere with cell growth over the long term.

Glassware preparation.

It is especially important that all glassware, pipets, culture vessels, media and components are free of or low in endotoxins. All glassware used in tick cell culture must be prepared following strict standards, although the necessary equipment need not be expensive or elaborate. A portable household dishwasher that can be hooked up to regular tap water and to deionized water is useful. The following is the procedure used in our lab. Glassware is washed once with a detergent designed for laboratory glassware washers, and then rinsed once with tap water and twice with deionized water using the washer and full wash cycles. The glassware is then unloaded, and soaked over night twice in deionized water (by filling all bottles, beakers, Erlenmeyer flask, etc. to the top), and once in Milli-Q cell culture grade water. All glassware that can be baked at 200 ºC is dried, covered with two layers of aluminum foil, and sterilized at 180-200 ºC for 2 h, counting from the time the oven has actually reached 180 ºC. Screw cap bottles

are filled with Milli-Q water and autoclaved 30 min at 121 ºC. Before use, the water is discarded from the bottles, or can be used in applications that require sterile cell culture water. If this is not practical, sterile cell culture plastic ware may be substituted.

Sensitive Components

` Table 1 shows the storage conditions for the ingredients to make L-15B, which may differ from the instructions on the label. From experience, we have found that glutamic acid cannot be used if it is older than 1 year after purchase, and must be discarded after this period, as it will inhibit cell growth. Note: we have switched to using a different grade of glutamic acid (the highest purity available), namely NOT the cell culture tested grade. We had problems with cytotoxicity using the cell culture tested grade. It is important to remain aware of possible problems with glutamic acid. Tryptose phosphate broth powder should be stored desiccated in the cold to avoid microbial growth, especially where laboratories may become warm and humid. Reduced glutathione will keep for over a year when stored desiccated at 4 ºC. Ferrous sulfate should be stored the same way, and is sensitive to oxidization, turning toxic for cells. The color of the granules should be evenly turquoise, and not have turned a paler shade. If this has happened, it must be replaced. The same applies to the other metal salts. Likewise, it is important to prepare mineral stock D, which contains the ferrous sulfate, by dissolving the ingredients in the order listed, i.e., first ascorbic acid, followed by reduced glutathione, and lastly the iron salt. Mineral stock D should be aliquoted into 1-2 ml tubes, depending on the volume of L-15B that is made at a time, and frozen in a non-defrosting freezer to avoid thawing cycles. The frozen stock should be nearly completely white, with no more than a slight haze of brown. If it turns dark, it can no longer be used, and must be discarded.

Tick Cell Culture Procedure

Tick cells are maintained at temperatures close to mammalian skin surface conditions, i.e., 30-34 ºC. At lower temperatures, growth and metabolism is considerably reduced (Kurtti and Munderloh 1982; Bell-Sakyi 1991). For routine maintenance of stock cultures, sealed 25 cm^2 flasks with 5 ml of complete growth medium are the most commonly used culture vessel, and incubation in a CO_2 atmosphere such as used for mammalian cells is not necessary. The population doubling time of tick cells varies among lines, but is at least 3 days, and usually more.

However, well established lines like ISE6 or IDE2 can be diluted up to 50-fold, although a 1:5 or 10 dilution will work well for most tick cell lines. Seeding tick cells at higher densities depresses growth, but may be done for specific experimental reasons. Before subculture, the medium in the culture flask should first be replaced with fresh growth medium, i.e., L-15B/L15C or L-15B300/L15C300 containing 5% - 10% each FBS and TPB, and 0.1-0.2% of BLC (1-2% of 10-fold diluted stock). The cells are then flushed off the growth surface using a 5-ml pipet or Pasteur pipet to direct a stream of medium at the cell layer until all have been detached. The cell suspension is diluted in complete medium, and 5 ml added to new flasks. Flasks are incubated growth surface down in a 30-34 ºC incubator until the cultures have reached the desired degree of confluency. Depending on the cell line, a flask seeded with a 5-fold diluted cell suspension will be ready for use or another subculture in 1-3 weeks. It should be noted that many tick cell lines will not produce as a monolayer, and start to pile on top of each other in clumps even before the flask bottom is completely covered. This is normal. Also, lines that grow in this manner (e.g., IDE8), cannot be resuspended into a single cell suspension. Enzymatic treatment to separate the cells (e.g., using trypsin) is not recommended as it will damage the cells. When subculturing cells, some should be keep in the parent flask. If something goes wrong, the cells will survive better in the original (parent) flask then in the daughter flasks.

It is commonly held that mammalian cells should preferably be subcultured during the logarithmic growth phase to achieve optimal growth and survival rates. In contrast, tick cells seem to benefit from a period of rest between subcultures, and if pushed through passages too fast, a cell population may collapse and die out. Indeed, cultures can be maintained without passage for weeks as long as the medium is replaced weekly. However, for routine maintenance, tick cell cultures should be split (diluted) at a ratio of at least 1:5, and do well when diluted up to 1:20. Subculturing tick cell lines at split ratios of 1:3 or 1:2 for long periods results in eventual deterioration of the cultures. Tick cells tolerate a wide rage of *p*H conditions, but below a *p*H of 7 and above *p*H 8 there is little growth. Ideally, the starting *p*H value should be set near 7.5 using sterile 1 N NaOH. The different cell lines maintained in our laboratory vary in their capacity to produce lactic acid, and minor adjustments of the starting *p*H may be needed depending on the particular cell line used. However, it is not important that the *p*H be maintained at a constant value between medium changes. When the cells are used to propagate tick-borne pathogens, special requirements governing the growth of these organisms should be considered.

Cold Storage

We routinely use two methods to store tick cell lines. One is to place freshly-fed cultures that should be of high cell density into a refrigerated incubator set to 12 ºC. This provides an excellent solution for intermediate term storage of weeks to months without the need to feed the cultures, and the time cultures survive under these conditions depends on the cell line in question. The other method is cryopreservation in liquid nitrogen using methods similar to those developed for mammalian cells. Medium for freezing the cells is composed of complete growth medium but with the concentration of FBS raised to 20%, and including 10% dimethylsulphoxide (DMSO; from Sigma). The growth medium is removed from a culture and cells resuspended in freezing medium at room temperature. A densely grown 25-cm^2 flask will contain enough cells for freezing five ampoules with 1-1.5 ml of cells in freezing medium (Nunc CryoTube vials). Any device that achieves a controlled rate of freezing with the temperature decreasing by 1 ºC/min starting from room temperature will provide satisfactory results. However, it is not sufficient to place ampoules in a box wrapped in layers of newspaper at the bottom of a -70 ºC freezer. After a minimum of 2 h, the frozen vials can be transferred to the liquid or vapor phase of a liquid nitrogen tank as available.

To regenerate cells, an ampoule of frozen cells is rapidly thawed in a 37 ºC water bath, the cell suspension in freezing medium is added to 5 ml complete medium, and the flask is incubated over night at 34 ºC. The culture should be fed the next day with a complete change of growth medium. During the first week after thawing cells, it may be desirable to include an antibiotic solution such as gentamycin (50 μg/ml final concentration), or a mixture of penicillin (50-100 IU/ml) and streptomycin (50-100 μg/m/). Continuous use of antibiotics is not recommended, and prolonged use of gentamycin can interfere with cell growth.

Table 1. Components of basal L-15B medium. (Numbers for L15C are listed in parentheses)

Ingredient	Amount	Storage	Comments
Cell culture water	to 1 Liter (1 L)	room temperature	endotoxin-free, cell culture grade
L-15 powder	1 package (1 pkg)	4 ºC	
L-aspartic acid	0.299 g (0.449 g)	4 ºC	
L-glutamine	0.292 g (0.500 g)	-20 ºC	
L-proline	0.300 g (0.450 g)	4 ºC	
L-glutamic acid	0.490 g (0.490 g)	4 ºC, desiccated	< 1 year old!
α-ketoglutaric acid	0.299 g (0.449 g)	4 ºC, desiccated	
D-glucose	14.4105 g (18.018 g)	room temperature	
mineral stock D	1 ml (1 ml)	-20 ºC	Munderloh & Kurtti 1989
vitamin stock	1 ml (1 ml)	-20 ºC	Munderloh & Kurtti 1989
10 N NaOH	0.5 ml (0.75 ml)	-20 ºC	

Table 2. Mineral and Vitamin Stocks

Ingredient	Weight (mg/100ml)	Final molarity in L-15B
Stock solution A ($x10^5$)		
$CoCl_2.6H_2O$	20	$8.4x10^{-9}$
$CuSO_4.5H_2O$	20	$8.0x10^{-9}$
$MnSO_4.H_2O$	160	$9.5x10^{-8}$
$ZnSO_4.7H_2O$	200	$7.0x10^{-8}$
Stock solution B ($x10^5$)		
$Na_2MoO_4.2\ H_2O$	20	$8.3x10^{-9}$
Stock solution C ($x10^5$)		
Na_2SeO_3	20	$1.2x10^{-8}$
Stock solution D ($x10^3$)		
Ascorbic Acid	1000	$5.68x10^{-5}$
Glutathione (reduced)	1000	$3.3x10^{-5}$
$FeSO_4.7H2O$	50	$1.8x10^{-6}$
Stock Solution A	1 ml	----
Stock Solution B	1 ml	----
Stock Solution C	1 ml	----
Vitamin Stock ($x10^3$)		
p-aminobenzoic acid	100	$7.3x10^{-6}$
Cyanocobalamine (B12)	50	$3.7x10^{-7}$
d-Biotin	10	$4.1x10^{-7}$

Table 3. Tick Cell Lines Available from the University of Minnesota

Species of Origin	Designation	Approx. Passage #	Reference
Amblyomma americanum	AAE2, AAE12	30	Munderloh & Davidson
Carios capensis	CCE1, 2, 3	30	Kurtti unpublished
Dermacentor albipictus	DALBE3	30	Policastro et al. 1997
Dermacentor andersoni	DAE15, DAE100	50-80	Simser et al. 2001
Dermacentor nitens	ANE58	50	Munderloh & Kurtti 1989
Dermacentor variabilis	DVE1	50	Kurtti & Munderloh
Ixodes scapularis	*IDE2, *IDE8, *IDE12	50-100	Munderloh et al. 1994
	ISE18, ISE6	40, 100	Munderloh et al. 1994, 1999
Ixodes ricinus	IRE11	40	Simser et al. 2002
Rhipicephalus appendiculatus	RA243	150	Varma et al. 1975
	RAE25	100	Munderloh & Kurtti 1989
Rhipicephalus (Boophilus) microplus	BME26	60	Munderloh & Kurtti 1989
Rhipicephalus sanguineus	RSE8	40	Kurtti & Munderloh 1982

*Note: *I. scapularis* cell lines designated "IDE" are chronically infected with an orbivirus of unknown pathogenicity. Refer to: Attoui et al. 2001. Complete sequence characterization of the genome of the St. Croix River virus, a new orbivirus isolated from cells of *Ixodes scapularis*. J. Gen. Virol. 82: 795-804.

Suppliers

Water:
Protide Pharmaceuticals
1311 Helmo Ave.
St. Paul, MN 55128

Tel.: 651-730-1500; 800-552-3569
Fax: 651-730-8900
email: Protide@protidepharma.com

or: Invitrogen (see below)
catalog No.: 15230-162

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MP Biomedicals Life Science Division
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1 (800) 334-6999 (fax)
custserv@mpbio.com
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Note that the new lipoprotein concentrate is supplied sterile, and does not require filtering.

Fetal Bovine Serum:
Invitrogen/Gibco, Lonza, or Sigma. Other sources may be suitable.
Each lot must be tested for its ability to support tick cells prior to purchase.

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E-mail: OC_DOM_HC@sial.com
800-325-3010

Note: we have switched to using a different grade of glutamic acid (the highest purity available), namely NOT the cell culture tested grade. We had problems with cytotoxicity using the cell culture tested grade.

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