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Use of a 96-well plate reader to evaluate proliferation of equine satellite cell clones in vitro

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Abstract. We have adapted a methylene blue staining assay to measure proliferation of equine satellite cell clones in a 96-well format. This technique allows rapid and accurate measurement of proliferating satellite cells which is a considerable enhancement over manual counting methods. Methylene blue is incorporated into the nuclei and intracellular matrix of satellite cells and then released into the aqueous phase. Absorbance of stained cells is read at 620 nm and correlates with increasing numbers of cells (range tested from 1×10^3 to 4×10^4). This method was used to determine the response of equine satellite cells to FGF, both human recombinant and bovine, and to IGF-1. This format is very efficient in measuring and comparing the proliferation of equine satellite cells. However, fusion of cells to form multinucleated myotubes cannot be assayed using this method because it lacks the sensitivity and specificity to differentiate multinucleated from mononucleated cells, and to detect expression of myogenic proteins. The assay could be accurately applied from 0 to 144 hours, before significant fusion and differentiation takes place. Using this assay will reduce analysis time to quantitate the proliferation response of equine satellite cells to different growth factors.

Key words: Equine, FGF, IGF, Methylene blue, Satellite cell

Abbreviations: rhFGF = recombinant human fibroblast growth factor basic; bFGF = bovine fibroblast growth factor basic; IGF-1 = insulin-like growth factor-1

1. Introduction

Cell proliferation can be measured by a variety of techniques using proliferation markers. These markers can be either radioactive labels incorporated into dividing cells or non-radioactive reagents which are only metabolized by proliferating cells. Radioactive labels, such as tritiated thymidine, require special handling and expensive disposal. Radioactive labels also require that the cells be harvested from the cultureware which eliminates the possibility of viewing the cells directly and visualizing their morphology. The need to harvest the labeled cells onto filter mats also limits its use to non-adherent cells or an additional trypsinization step. Newer non-radioactive assays are available commercially, but are often cost prohibitive, lack the necessary level of sensitivity, and may not be applicable to all cell types.

Oliver et al. [1] characterized a simple, inexpensive methylene blue staining assay which was applied to fibroblast culture. Although this procedure has proven effective for fibroblasts, it has not been evaluated for other cell systems including clonal cultures of equine satellite cells. In previous studies, we have used laborious manual counting methods to measure the proliferation of primary cultures of equine muscle [2, 3]. Satellite cells have a level of cellular complexity which would require more of the assay system [4]. Specifically, satellite cells can reach a stage in vitro when they stop proliferating, start differentiating, and fuse to become multinucleated myotubes. Because of this, any assay attempting to measure the level of proliferation in cultures of equine satellite cells would need to able to detect the time at which multinucleated myotube formation began, since this time would define the terminal point of the usefulness of the proliferation assay. Traditionally, satellite cells have been grown in 24-well plates and nuclei counted visually after Giemsa staining to measure proliferation. This paper defines the applicability of the methylene blue proliferation assay to satellite cell clones grown in a 96-well format and its limitations on detecting multinucleated cells.

2. Materials

A. Equipment
1. Incubator, CO_2, model No. 3326.[1]
2. Laminar flow hood, model No. SG-600.[2]
3. Microscope, inverted with phase contrast, model No. Nikon TMS.[3]
4. Cell counter, model No. 07-905.[4]
5. Hemocytometer, model No. 02-671-5.[4]
6. 1–20 μl Pipetman, model No. P-20.[5]
7. 1–200 μl Pipetman, model No. P-200.[5]
8. 100–1000 μl Pipetman, model No. P-1000.[5]
9. pH meter, model No. 8200.[6]
10. ELISA plate reader, model No. DU 640B.[7]
11. Plate rotator, model No. R4140.[8]
12. Chemical balance, model No. BB3000.[9]
13. Stirrer/hot plate, model No. PC320.[10]
14. Multichannel digital pipettor, model No. 4172-317.[11]

B. Cell culture medium
1. Dulbecco's modified Eagle's medium (DMEM), Cat. No. D-7777.[12]

C. Cell culture reagents
1. Penicillin/streptomycin, Cat. No. 15145-014.[13]
2. Pig skin gelatin, Cat. No. G1890.[12]
3. Fetal calf serum, Cat. No. A-115-L.[14]
4. Trypsin, Cat. No. T-2021.[12]
5. AraC, Cat. No. C-6645.[12]
6. Basic fibroblast growth factor, recombinant human, Cat. No. 4125-70.[15]
7. Basic FGF, bovine, Cat. No. 133-FB-025.[16]
8. Insulin-like growth factor-1, recombinant human, Cat. No. H-5555.[17]
9. IL-6, recombinant human, Cat. No. 206-IL.[16]

D. Staining supplies
1. Methylene blue, Cat. No. JTQ473-5.[18]
2. Ethyl alcohol, Cat. No. JT9401-3.[18]
3. Borate, Cat. No. JT0091-1.[18]
4. Formaldehyde, Cat. No. JT2106-1.[18]
5. Giemsa stain, Cat. No. GS-IL.[12]
6. Methanol, Cat. No. JT9070-3.[18]
7. Sodium hydroxide, Cat. No. JT3722-11.[18]
8. Hydrochloric acid, Cat. No. JT9535-3.[18]
9. Sodium chloride, Cat. No. JT3624-5.[18]
10. Potassium chloride, Cat. No. JT3040-1.[18]
11. Sodium phosphate, dibasic, Cat. No. S0876.[12]
12. Potassium phosphate, monobasic, Cat. No. JT3246-1.[18]
13. EDTA, Cat. No. JT8993-1.[18]

E. Plastic and glassware
1. Pipet, 10 ml glass, Cat. No. 13-674-M.[4]
2. Pipet, 5 ml glass, Cat. No. 13-675-K.[4]
3. Plastic pipet tips, 200 μl, Cat. No. 21-197-86.[4]
4. Tissue culture plate, 96-well flat bottom, Cat. No. 3072.[19]
5. Culture flask, 25 cm^2, Cat. No. 83.1810.[20]
6. Culture flask, 75 cm^2, Cat. No. 25110-75.[10]
7. Pipet tips, 1000 μl, Cat. No. P-3250-1.[21]
8. Filter, sterile, 0.22 μm, Cat. No. 8-0301-73.[22]
9. Media bottles, Cat. No. 1395-500.[10]
10. Plastic tanks, Cat. No. H16191.[4]
11. Centrifuge tubes, 15 ml, Cat. No. 05-539-5.[4]
12. Centrifuge tubes, 50 ml, Cat. No. 334959.[23]
13. Reagent reservoir, autoclavable, Cat. No. 22-26-580-6.[24]
14. Plastic funnel, 121 ml capacity, Cat. No. 4283-0080.[22]
15. Filter paper, diameter 15.0 cm, grade 1, Cat. No. 1001-150.[25]

3. Procedures

A. Preparation of materials and solutions
1. DMEM media
   a) Dissolve a package of DMEM and 3.7 g $NaHCO_3$ in 900 ml of ddH_2O.
   b) Adjust pH to 7.08 with HCl/NaOH.
   c) Bring volume to 1000 ml with ddH_2O.
   d) In a laminar flow hood, filter sterilize the DMEM by passing it through a 0.22 μm filter into a 1 liter sterile bottle.
   e) Incubate the DMEM at 37 °C for 48 hours to check sterility.
2. Stock solutions of growth factors
   a) Stock solutions of growth factors
      (1) bFGF
      Lyophilized powders of bovine FGF were resuspended in 1 ml of sterile PBS containing 0.1% bovine serum albumin to give a final concentration of 25 μg/ml.
      (2) rh FGF
      Lyophilized powders of rhFGF were resuspended in 3.125 ml of sterile DMEM to give a final concentration of 8 μg/ml.
      (3) IGF-1
      Lyophilized powders of IGF-1 were resuspended in 1 ml of 0.1 M Acetate buffer to give a final concentration of 25 μg/ml.
   b) DMEM-10% fetal calf serum (FCS)
   Prepare 100 ml of serum-containing media by adding 10 ml FCS, 1 ml of 100X Pen/Strep to 89 ml of DMEM.
   c) Daily preparation
   Dilute growth factors to final concentrations with DMEM-10% FCS in a 15 ml sterile centrifuge tube and add to culture wells with multichannel pipettor and reagent reservoir.
3. Trypsin-PBS/EDTA
   a) Prepare PBS/1 mM EDTA by adding 0.37 g of $Na_2EDTA \cdot 2H_2O$ to 1 liter of PBS. Sterilize by autoclaving for 60 min at 121 °C on liquid cycle.
   b) Add 10 ml of sterile trypsin (25 g porcine

trypsin in 1 liter of 0.9% NaCl) to 90 ml of sterile PBS/EDTA.

4. Borate buffer
   a) 10× borate buffer (0.1 M)
      (1) Dissolve 6.18 g of Boric acid in 900 ml of ddH_2O.
      (2) Adjust pH to 8.3 with HCl/NaOH.
      (3) Bring volume to 1000 ml with ddH_2O.
   b) 1× borate buffer (0.01 M)
      Dilute 100 ml of 10× borate buffer to 1000 ml volume with ddH_2O.
5. 0.15 M saline solution
   a) 5 M NaCl stock solution
      Dissolve 292.2 g of NaCl in 900 ml of ddH_2O. Bring volume to 1000 ml.
   b) 0.15 M saline solution
      Dilute 30 ml of 5 M NaCl stock solution to 1000 ml volume with ddH_2O.
6. Formaldehyde solution
   Add 100 ml of 37% formaldehyde and 100 ml of 10× PBS in 700 ml of ddH_2O. Bring volume to 1000 ml.
7. 1% (w/v) methylene blue solution
   a) Dissolve 1 g of methylene blue in 90 ml of 1× borate buffer.
   b) Bring volume to 100 ml with 1× borate buffer.
   c) Filter through plastic funnel lined with filter paper.
8. Gelatin solution
   a) Add 0.1 g of pig skin gelatin to 100 ml of ddH_2O.
   b) Autoclave for 20 min at 121 °C on liquid cycle.
9. Giemsa stain
   Add 1 ml of stock Giemsa solution to 14 ml of ddH_2O for a working solution of 1:15.
10. PBS
    a) 10× PBS
       (1) Dissolve 100 g of NaCl, 2.5 g of KCl, 14.4 g of Na_2HPO_4, and 2.5 g of KH_2PO_4 in 900 ml of ddH_2O.
       (2) Adjust pH to 7.08 with HCl/NaOH.
       (3) Bring volume to 1000 ml.
    b) 1× PBS
       (1) Dilute 100 ml of 10× PBS to 1000 ml volume with ddH_2O.
       (2) Place the PBS into a 1 liter bottle and autoclave for 60 min at 121 °C.
11. Elution solution
    Mix 95% ethanol and 0.1 M HCl at 1:1 ratio.

B. Cell culture methods

Glassware was sterilized before use for 30 min at 121 °C, except where indicated. Any media or solutions were either filter sterilized using 0.22 µm filter or autoclaved for 30 min at 121 °C.

1. Trypsinization
   a) Grow equine satellite cells in DMEM-10% FCS at 37 °C and 5% CO_2.
   b) When the cells are 80–90% confluent, remove the growth media and wash cell monolayer 1× with 10 ml of prewarmed PBS. Remove PBS by pipetting. Add 5 ml of Trypsin-PBS/EDTA and incubate the flask for 5 min at 37 °C until all cells detach.
   c) Add 5 ml of prewarmed DMEM-10% FCS to the flask to neutralize trypsin. Triturate cells gently with pipet for 10 times and transfer to a 50 ml sterile centrifuge tube.
   d) Use 5 ml of the same media to wash the flask and combine (c) and (d).
2. Counting
   Count cells using the eight outside squares of standard hemocytometer chamber and an inverted light microscope. Average the counted squares and correct for dilution and volume by using the formula: (Total cells counted in 8 squares/8) × 104 = cells/ml.
3. Gelatinized plates
   Coat 96-well flat bottom plates with 100 ml/well of 0.1% pig skin gelatin for at least 2 hours at room temperature. The excess was removed by quick and forceful inversion.
4. Plate cells in DMEM-10% FCS for 24 hours
   Dilute cells to 2 × 104 cells/ml in DMEM-10% FCS and add 100 µl/well with a multichannel pipettor and reagent reservoir.
5. Media changes
   Refeed cells every 24 hours with DMEM-1% FCS plus desired growth factors.

C. Methylene blue staining

1. Culture media was removed from the plates by quickly and forcefully inverting over the sink basin. Plates were then submerged briefly in plastic tanks containing 500 ml of 0.15 M saline.
2. 100 µl of a 10% formaldehyde saline solution was added with a multichannel pipettor and the plates were incubated at room temperature for 30 min.
3. The formaldehyde solution was removed into a separate hazardous waste container by inversion.
4. 100 µl of methylene blue stain was added and the plates were incubated for 30 min at room temperature. The dye was removed by inversion and then washed 4 times by immersion in plastic tanks containing 500 ml of borate buffer.
5. 100 µl of elution solvent solution was added to release the cell bound dye.
6. Absorbance was then read at 620 wavelength after correcting for background absorbance with wells containing only 100 µl of the elution solvent.

D. Giemsa staining

1. Remove media by quickly and forcefully inverting over the sink basin.
2. Wash cells with PBS.
3. Add methanol and incubate for 45 min at room temperature.
4. Remove methanol by inversion.
5. Add Giemsa stain and let incubate for 30–60 min at room temperature.
6. Remove Giemsa by inversion.
7. Wash 3 times with ddH_2O.

4. Results and discussion

Isolation of equine satellite cell clones and culture
Satellite cells were isolated as primary cultures from biopsies of equine muscle as described by Greene et al. [3]. Equine satellite cell strains were established by the same methods previously for cloning and propagating specific ovine satellite cell populations [5–7]. Clones were isolated as described by Erickson et al. [8] and the procedure outlined in Figure 1. Equine muscle tissue was digest for 1 hour in pronase and subjected to three differential centrifugations as described [3]. Between 300 to 750 primary cells were plated on 15 cm cell culture plates. Individual cells were ringed by 1 cm sterile cloning rings [5, 6]. Following clonal expansion to approximately 100 cells, cells contained within the cloning rings were lifted off the plate with trypsin and transferred to new plates. Colonies were allowed to proliferate to 50% density. At this point, nearly half of the cells of the colonies were lifted from the expansion plate and cryopreserved in DMEM with 20% horse serum and 10% DMSO and stored in liquid nitrogen [3]. The remainder of the cells were subsequently used for the experiments described herein. Myogenic clones were identified as satellite cells by their ability to fuse and form multinucleated myotubes. Two equine satellite cells have been cloned by this method and were designated SE-2 and SE-4 [8]. SE-2 cells were used for all the studies described in this paper. Values represent the average of triplicate samples. Standard errors did not graph differently than the datapoint so are not shown.

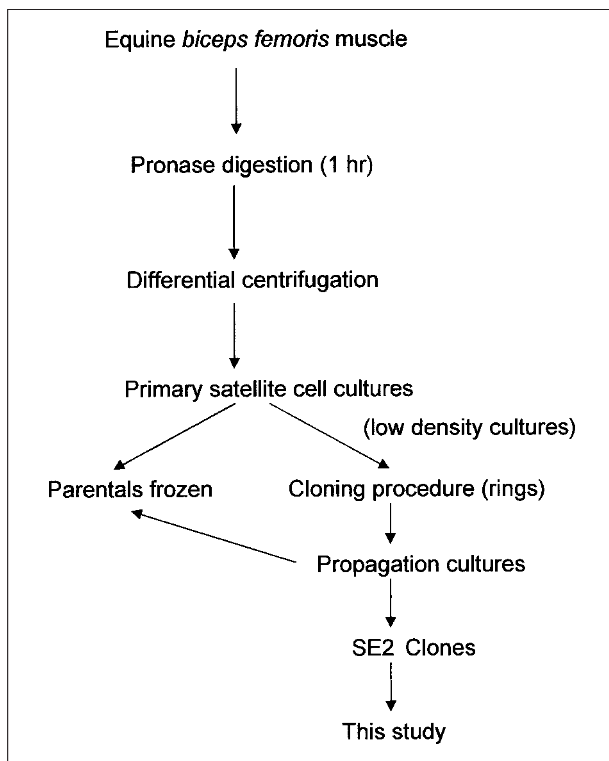

Figure 1. Flow diagram of the major steps involved in the isolation and propagation of clonal equine satellite cell cultures.

Clones were grown in tissue culture flasks coated overnight with gelatin. For proliferation assays, cells were cultured in gelatin-coated, 96-well microtiter plates containing DMEM with 10% fetal calf serum and no growth factors for the first 24 hours. Subsequently, the plating media was removed and the cultures washed three times before media containing the specific growth factors was added. Growth medium was changed every 24 hours.

Methylene blue staining
Oliver et al. [1] describe the methylene blue staining paper for use on rat and human fibroblast and lung cells. The assay is inexpensive, particularly applicable to adhered cells, and there is a high degree of correlation between cell number and methylene blue staining. For this project, the staining assay described had to be adapted from that reported for primary cultures [4] to satellite cell growth dynamics. Satellite cells also differ from the cell lines previously used to define this assay because they have the potential to differentiate under the right culture conditions to form multinucleated myotubes.

The proliferation assay is based on the cellular staining properties of methylene blue which can then be released from the cell into solution by lowering the pH, and then quantitated by measuring absorbance. This method is very effective for measuring cell numbers because it releases stain from the cells and measures stain incorporation by all the cells in the well, thereby eliminating cell to cell field variation that would occur in a solid phase immunohistochemical methodology and standard counting. The speed of the assay allows quick comparisons between clones and their different response to growth conditions, and is particularly applicable to mechanical analysis situations used in high volume cell culture laboratories.

To accurately apply this assay to equine satellite cell clones, we needed to establish the growth kinetics of the clone and evaluate the sensitivity and specificity to cell numbers. To demonstrate that the amount of staining correlated with the number of equine satellite cells in the well, 1×10^3 to 4×10^4

cells were added to gelatin-coated wells with DMEM and 10% FCS and incubated for 24 hours to allow them to adhere with minimal proliferation. A parallel plate was also incubated overnight to be stained with Giemsa and the cells counted manually and evaluated for fusion. Cell numbers per well were calculated by averaging the counted number of nuclei per 3 fields under the 40× objective and multiplying by the number of field areas per well [9]. Results are shown in Figure 2 and indicate that the absorbance of methylene blue stained equine satellite cells at 620 nm wavelength corresponds to increasing plated cell numbers.

After showing this relationship, the correlation of absorbance with cell proliferation in extended cultures was then demonstrated. Figure 3 presents the results from equine satellite cells grown in varying concentrations of fetal calf serum. SE-2 cells were plated on gelatin-coated 96-well plates at a cell density of 2×10^3 cells per well. This cell concentration was determined optimal for equine satellite cells based on a pilot study with representative plating densities (data not shown). The cells spent the first 24 hours of culture in DMEM with 10% FCS to allow adherence. The next day, adherence medium was removed, the cultures washed, and the medium changed the next day to either 1%, 2.5%, 5%, or 10% sera. As shown in Figure 3, the amount of absorbance after methylene blue staining correlates with cell concentration ($r = 3$, $p < 0.05$), and the assay is sensitive enough to identify proliferation differences between the lower concentrations of sera.

In a second experiment, IGF-1 (200 ng/ml), recombinant human FGF (50 ng/ml) and bovine FGF (50 ng/ml) were applied to cells over a 96 hour period with one plate being stained with methylene blue every 24 hours. Fusion of cells accounted for 0% to 0.07%. Concentrations of factors were chosen based on a maximum proliferation effect seen in titration experiments (data not shown). Applying this technique, we can demonstrate the growth dynamics of equine satellite cell response to high levels of IGF-1 and are able to compare the relative effectiveness of bovine and recombinant human FGF at equally high concentrations (Figure 4). Based on the absorbance at the high concentrations used, IGF-1 appeared to be less effective than either recombinant human or bovine FGF at inducing proliferation up to 96 hours. This effect will be examined more closely in future studies since these results seem contrary to that obtained in other species. There was also an observed delay in the proliferation of equine satellite cells to bovine FGF versus human recombinant FGF. This delay was substantiated with lower cell number counts as well as less methylene blue staining. Based on these results, however, methylene blue staining is an efficient method which can be used to characterize the effectiveness and activity of new growth factors.

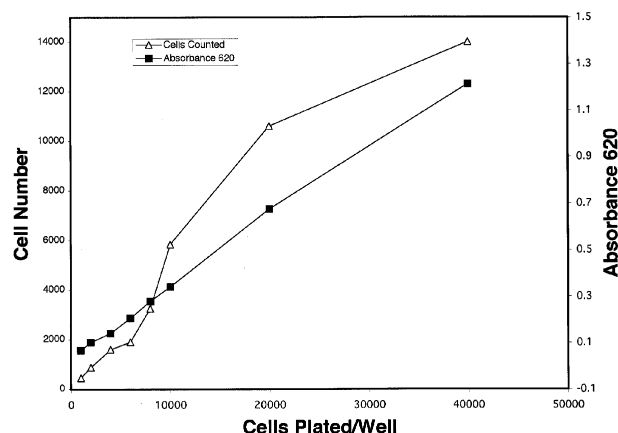

Figure 2. Methylene blue uptake is proportionate to increasing cell numbers.
Satellite cells were added to parallel sets of gelatin-coated plates at concentrations between 1×10^3 to 4×10^4 cells/well. The following day, one plate was stained with methylene blue and the absorbance measured while the second plate was stained with Giemsa and the number of cells per well were determined. The darkened triangles represent the average absorbance of triplicate samples. The light squares represent the total number of cells per well, averaged for three replicates.

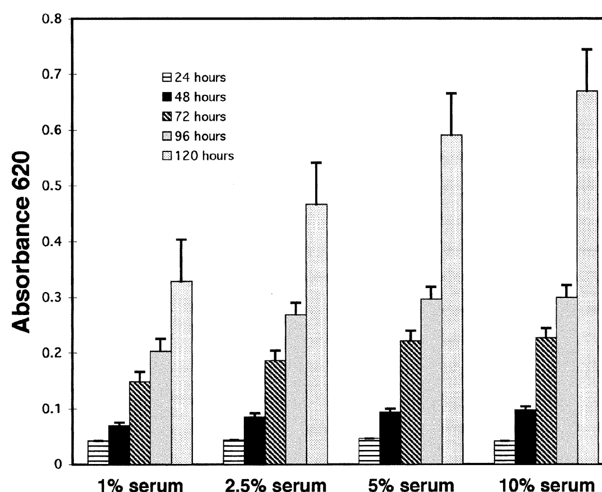

Figure 3. Methylene blue staining to measure proliferation in response to different sera concentrations.
Equine satellite cells at 2×10^3 cells per well were plated on gelatin coated plates with 10% sera for the first 24 hours. Subsequently, the media was changed to contain 1, 2.5, 5, or 10% serum. Absorbance after methylene blue staining was measured at 24, 48, 72, 96, 120 hours.

The 96-well plate assay has proven to be an efficient and time-saving format for many assays. Applying this method to measure equine satellite cell growth and proliferation enables the evaluation of multiple growth factors at a range of concentrations on many different clones. Based on our results, this assay is specific and sensitive enough to warrant its use in satellite cell cultures. However, there is one unique aspect of satellite cells which limits its appli-

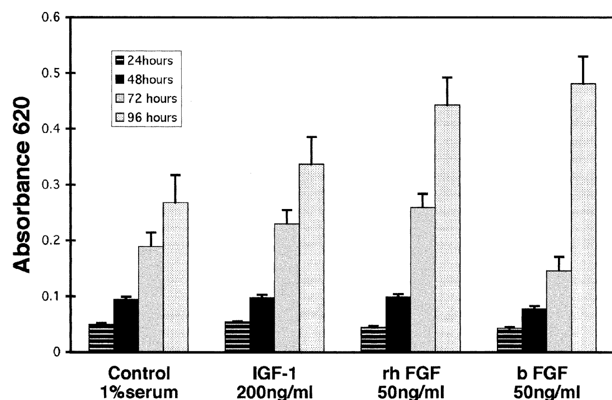

Figure 4. Growth kinetics with specific growth factors. Equine satellite cells at 2×10^3 cells per well were plated on gelatin coated plates with 10% sera for the first 24 hours. Subsequently, the media was changed to contain 1% sera with either 200 ng/ml IGF-1, 50 ng/ml bFGF, or 50 ng/ml rhFGF. Absorbance after methylene blue staining was measured at 24, 48, 72, 96, 120 hours.

cation. Satellite cells are defined by their ability to fuse and form multinucleated myotubes. The appearance of fused, multinucleated cells in a culture must be determined to define the application of the methylene blue assay to accurately measure proliferation. For equine satellite cells under the present culture condition, this time limit was optimally 96 hours, but could be extended to 120 or 144 depending on growth conditions.

In the studies presented, the absorbance as it related to cell number and level of fusion was also confirmed by manual counting of cells. The staining differences in the small number of fused cells was ultimately diluted by the methylene blue release from the large number of unfused, undifferentiating cells. This is an advantage of the assay in that it measures the absorbance after the dye is released from all the stained cells, making the effect of a few differentiated on the overall measurement unnoticeable. It also limits its sensitivity to detect early increases in protein concentration or in a small percentage of cells. However, our future experiments will examine the applicability of methylene blue staining on measuring the number of well-differentiated myotubes in satellite cell cultures, as well as other assays to quantitate early differentiation in equine satellite cell clones.

Notes on suppliers

1. Forma Scientific, Marietta, OH, USA
2. Baker, Sangord, ME, USA
3. Nikon, Melville, NY, USA
4. Fisher, Pittsburgh, PA, USA
5. Rainin, Woodburn, MA, USA
6. Sargent-Welch, Skokie, IL, USA
7. Beckman, Palo Alto, CA, USA
8. Baxter, Deerfield, IL, USA
9. Mettler, Hightstown, NJ, USA
10. Corning, Corning, NY, USA
11. LabSystems/Finnipipette, Helsinki, Finland
12. Sigma, St Louis, MO, USA
13. Gibco-BRL, Gaithersburg, MD, USA
14. HyClone, Logan, UT, USA
15. Integen, Purchase, NY, USA
16. R&D, Minneapolis, MN, USA
17. Bachem, Philadelphia, PA, USA
18. JT Baker, Phillipsburg, NJ, USA
19. Falcon, Franklin Lakes, NJ, USA
20. Sarstedt, Newton, NC, USA
21. Intermountain Scientific, Kaysville, UT, USA
22. Nalgene, Rochester, NY, USA
23. Nunc, Naperville, IL, USA
24. Brinkman, Westbury, NY, USA
25. Whatman, Fairfield, NJ, USA

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