ESGRO Complete™ Products for Mouse Embryonic Stem Cell Culture in Serum-Free and Feeder-Free Conditions

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A major challenge in harnessing the potential of embryonic stem (ES) cells for both *in vitro* and *in vivo* use is the controlled and reproducible self-renewal of ES cells in the laboratory. Currently, the routine culture of mouse ES cells largely relies on the addition of serum and in some cases the presence of a feeder layer. Serum is an inherently heterogeneous, undefined product that has batch to batch variability, while the use of feeder cells is cumbersome and adds extra variability. A fully defined and easy to use culture system would lead to more systematic and uniform approaches to large scale screening projects. We have developed a fully defined, serum and feeder free system based on the work of Ying *et al.*[1] that allows for the efficient self-renewal of mouse ES cells. This system enables the researcher to successfully passage, freeze and thaw ES cells in a serum free environment while maintaining their pluripotency. Unlike other systems, ES cells grow efficiently at clonal density making this system suitable for transfection and targeting experiments. Here we describe observations on ES cell growth characteristic of this system and the differences with those cells grown in serum.

Background

Stem Cell Sciences plc (SCS) is a leading biotechnology company in the embryonic stem cell field, working in close collaboration with the Institute for Stem Cell Research (ISCR) in Edinburgh Scotland. The publication of the work of Ying *et al.*[1] demonstrates the role of STAT3 and SMAD signaling as revealed in serum free culture conditions. SCS has developed and optimized this serum-free and feeder-free system for the general maintenance of self-renewal of mouse ES cells in their pluripotent state that has universal utility in the stem cell field. This technology has been exclusively licensed by Millipore. It is available in a convenient ready to use format and marketed under name ESGRO Complete.

THE SERUM-FREE AND FEEDER-FREE SYSTEM CLONAL ASSAY TEST

The efficiency of ESGRO Complete Clonal Grade Medium to support the self-renewal of mouse ES cells without the presence of feeders is routinely tested in a clonal assay performed over 5 days. This type of assay shows the efficiency of this culture system in the maintenance of the clonal growth of the cells compared to other systems, creating an ideal environment to conduct reproducible and comparable genetic manipulations between different laboratories and researchers. For example, it is very easy to pick individual colonies after transfection of the cells in targeting experiments and lends itself to the development of high throughput screening procedures.

In this test, we use early passage mouse E14Tg2a ES cells that were previously grown in serum. These cells are directly plated into serum free medium at a clonal density (1 x 10^3 cells) in a 10 cm tissue culture dish pre-coated with ESGRO Complete Gelatin. Each single cell gives rise to a "pickable" colony within 5 days of plating. Colony formation is observed as undifferentiated cells often as tight round colonies (Figure 1A) or somewhat flatter colonies (Figure 1B) but all with a distinct cytoplasmic and nuclear morphology. More rarely, some colonies may have differentiation at their edges in the form of large flat cells which we call a "skirt". If the colonies are left for more than 5 days, many will overgrow and start to differentiate. However, if the colonies are picked prior to overgrowth, then self-renewal of the cells from the colonies is easy to maintain on subsequent passage. In those colonies with a differentiated "skirt," any differentiated cells gradually become less and less noticeable during passage as the self renewing cells predominate.

The standard assay to distinguish self renewing cells from any differentiated colonies is to fix and stain the colonies on day 5 for alkaline phosphatase. This marker is expressed in ES cells and, although it is not a specific marker for self renewal, it is an easy and economical test to perform on a routine basis. The ES cell colonies stain intensely red with this assay (Figure 2A), while any differentiated cells do not stain (Figure 2B). When alkaline phosphatase positive colonies are counted during this clonal assay, the cloning efficiency is around 20%, so typically we count around 200 alkaline phosphatase positive colonies per plate.

The efficiency of this system is demonstrated by comparative clonal assays: in standard ES cell medium containing 10% Fetal Calf Serum (FCS), and in ESGRO Complete. Cells plated in 10% FCS containing medium tend to form colonies with a more irregular morphology and have more colonies that are clearly differentiated. When the plates are stained with alkaline phosphatase, the colonies grown in 10% FCS, in general, show less intense staining with many colonies displaying different levels of alkaline phosphatase staining (data not shown).

PASSAGING THE CELLS

While colony formation has been used as a routine test for clonal growth, we also routinely test the media during serial passage of ES cells. Mouse ES cells have a high metabolic rate and, therefore, we find the best results when the media on the cells is changed every 2 days. We also find that the cells prefer to be passaged prior to reaching 70 - 80% confluence, as overgrowth of the cells leads to differentiation. Standard trypsin used in passaging ES cells grown in standard serum containing media is not advised. Over trypsination causes

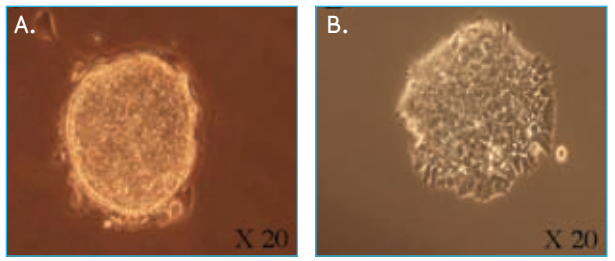

Figure 1. Undifferentiated ES cells colonies with a distinct cytoplasmic and nuclear morphology (day 4 of a clonal assay). (A) Tight round colony, (B) flatter colony.

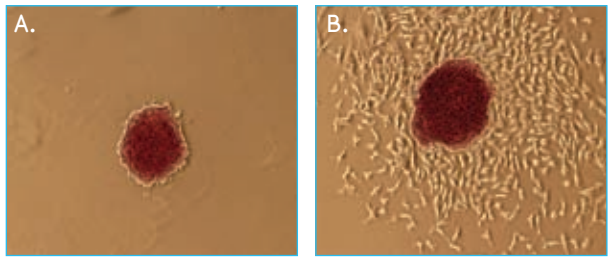

Figure 2. Alkaline phosphatase staining allows for an easy distinction between undifferentiated ES cells (red) and differentiated cells (unstained) on day 5 of the clonal assay. (A) ES cells colony with no differentiation, (B) differentiated cells at the edge of an ES cells colony.

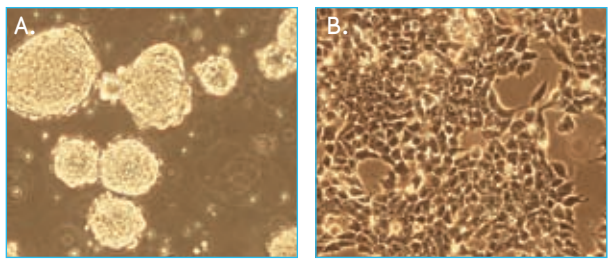

Figure 3. Standard Trypsin use causes the ES cells to lift off the plates in serum free conditions (A) whereas the use of Accutase allows for an efficient and gentle way to routinely passage the cells (B).

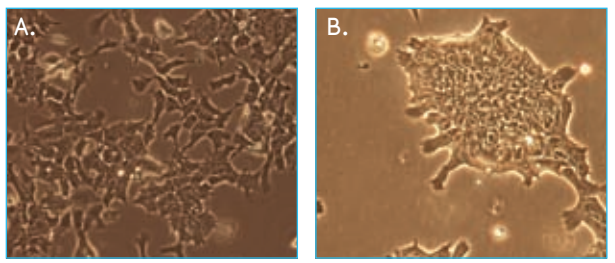

Figure 4. n serum containing medium, ES cells spread out with spaces between cells (A); whereas in serum free conditions ES cells grow as tight colonies with cells closely packed together (B). Less differentiation is seen in serum free conditions when compared to serum containing medium.

the cells to lift off the plates to form "ball" like structures reminiscent of embryonic bodies (Figure 3A). This is probably due to over trypsination affecting cell adhesion. To avoid this, we assayed for the best method of passaging the cells. ESGRO Complete Accutase was found to be ideal in giving a rapid dissociation of cells from the plates without any detrimental effects on cell morphology (Figure 3B). In addition, we developed ESGRO Complete Trypsin by the titration of porcine trypsin activity in a proprietary formulation and obtained results similar to the use of Accutase. We also developed ESGRO Complete Enzyme Free Dissociation Solution to avoid exposure to any enzymatic based products. This system takes longer for the cells to dissociate from the plastic, but the plating efficiency is also very good (not shown).

When cells passaged in ESGRO Complete Clonal Grade Medium are compared to cells passaged in standard ES cell media containing 10% FCS, we find that cell morphology was again more irregular with irregular cell spacing and high levels of spontaneous differentiation (Figure 4A). When the same cells are grown in ESGRO Complete Clonal Grade Medium, they remain as tight colonies with the ES cells closely packed together. Very little if any differentiation was noticed within colonies with any spontaneous differentiation being restricted to the outer edges of the colonies (Figure 4B).

To confirm pluripotency of ES cells after many passages in ESGRO Complete Clonal Grade Medium, cells were stained for alkaline phosphatase positivity and antibody stained for Oct-4 (Figure 5A) and Nanog (Figure 5B). Karyotype and germ line competency were confirmed (not shown).

FREEZING AND THAWING THE CELLS

We have optimized standard freezing solutions for the cryopreservation of the mouse ES cells to optimize recovery of cells grown in ESGRO Complete Clonal Grade Medium. ESGRO Complete Freezing Medium was devised by comparison of a number of different freezing protocols and was selected for its ease of use and high recovery rate. It is based on standard DMSO freezing techniques but is serum-free. Sub-confluent mouse ES cells were dissociated using ESGRO Complete Cell Dissociation Solution, resuspended in ESGRO Complete Clonal Grade Medium and counted. Cells were then spun down and resuspended in ESGRO Complete Freezing Medium at approximately 1 x 10^6 cells/ mL per cryotube and frozen in liquid nitrogen. Cells were thawed gently and washed in ESGRO Complete Clonal Grade Medium and plated into a small tissue culture flask precoated with ESGRO Complete Gelatin. We observed again that the cells prefer to grow as tight colonies, with the ES cells closely packed together (Figure 6).

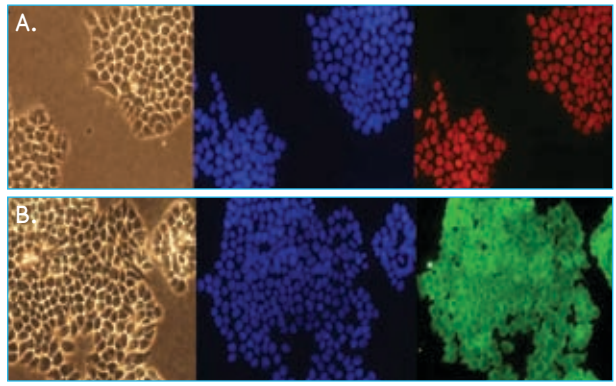

Figure 5. Immunostaining of mouse ES cells grown in ESGRO Complete Clonal Grade Medium. From left to right for the top panel: phase contrast, Hoechst staining and Oct-4 staining. From left to right for the bottom panel: phase contrast, Hoechst staining and Nanog staining.

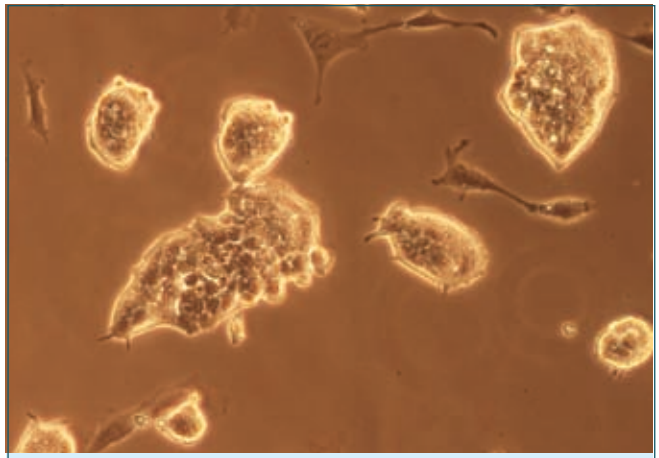

Figure 6. Mouse ES cells previously frozen in ESGRO Complete Freezing Medium and thawed in ESGRO Complete Clonal Grade Medium (4 days post- thawing).

Conclusion

ESGRO Complete Clonal Grade Medium is an innovative serum-free cell culture medium that provides a convenient method to grow mouse ES cells. The ability to form clones makes it suitable as a universal serum-free system for genetic manipulations and growth of these cells. Cells have different growth characteristics than in serum being tighter and more closely packed together with less differentiation. Cells can be successfully passaged, frozen and thawed while maintaining their pluripotency (Oct-4 and Nanog expression) with a normal karyotype and germ line competency.

Reference

1. Ying QL, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell.* 2003 Oct 31; **115(3)**:281-92.

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