Product Manual

StemTAG™ 96-Well Stem Cell Colony Formation Assay (Cell Recovery Compatible)

Catalog Number

CBA-325	96 assays
CBA-325-5	5 x 96 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

CELL BIOLABS, INC.
Creating Solutions for Life Science Research

Introduction

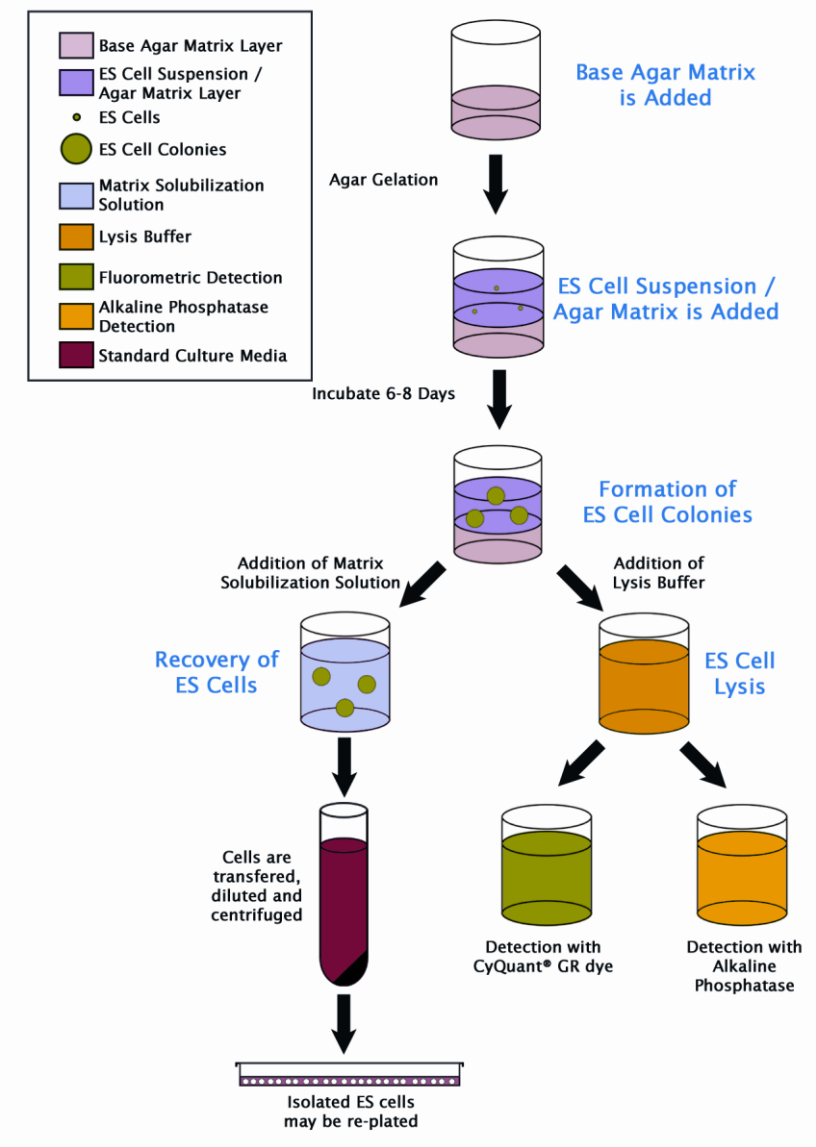
Several recent studies have revealed that stem cells from various types of self-renewing tissue could be cultured in suspension. For example, neuronal stem cells grow as neurospheres that do not depend on a particular substrate. Similarly, mammary stem cells or stem cells from skin can also grow in suspension using similar culture conditions. These cells differentiate into various cell lineages after exposure to extracellular matrix molecule (ECM) or serum. Additionally, various cancer stem cells have been successfully grown under these conditions. These results demonstrate that stem cells can survive and proliferate in anchorage-independent culture model, and clonogenic activity is a sensitive indicator of undifferentiated stem cells.

Traditionally, the colony formation assay is a common method to monitor anchorage-independent growth, which measures proliferation in a semisolid culture media after 3-4 weeks by manual counting of colonies. Standard assays are usually performed in 100-mm or 60 mm dishes, where cells are allowed to grow inside a semisolid culture media for 3-4 weeks before sizable colonies appear (> 40 cells/colony). This method is quite cumbersome, time-consuming, and difficult when testing a large number of samples. Additionally, the manual counting of colonies is highly subjective; with varying colony sizes, it's difficult to determine meaningful results.

Cell Biolabs' StemTag™ 96-Well Stem Cell Colony Formation Assay does **not** involve subjective manual counting of colonies or require a 3-4 week incubation period. Instead cells are incubated only 6-8 days in a proprietary semisolid agar media before being solubilized, lysed and detected by the patented CyQuant® GR Dye in a fluorescence plate reader or assayed for alkaline phosphatase activity colorimetrically (Figure 1). Alternatively, viable colony-forming cells (CFCs) can be easily recovered for further culturing and testing. This format provides a quantitative, high-throughput method to accurately measure colony formation, while the short incubation time makes it possible to assay transiently transfected cells.

The StemTag™ 96-Well Stem Cell Colony Formation Assay provides a stringent, anchorage-independent model for measuring stem cell colony formation, and allows for cell recovery. Each kit provides sufficient quantities to perform 96 tests in a microtiter plate. The assay may be modified for use in other plate formats (see Table 1 under Assay Protocol).

Assay Principle



Related Products

1. CBA-302: StemTAG™ Alkaline Phosphatase Complete Kit
2. CBA-320: CytoSelect™ 96-Well Hematopoietic Colony Forming Cell Assay
3. CBA-140: CytoSelect™ 96-Well Cell Transformation Assay (Cell Recovery Compatible)
4. CBA-130: CytoSelect™ 96-Well Cell Transformation Assay (Soft Agar Colony Formation)
5. CBA-310: MEF Feeder Cells
6. CBA-312: MEF Feeder Cells (Puromycin-resistant)

Kit Components

1. 10X CytoSelect™ Agar Matrix Solution (Part No. 114001): One sterile bottle – 10.0 mL
2. CytoSelect™ Matrix Diluent (Part No. 114002): One sterile bottle – 4.0 mL
3. 5X DMEM Solution (Part No. 113002): Three sterile tubes – 1.5 mL each
4. 10X Matrix Solubilization Solution (Part No. 114003): One sterile tube – 1.8 mL
5. CyQuant® GR Dye (Part No. 10105): One tube – 75 µL
6. Lysis Buffer (Part No. 132501): One bottle – 20.0 mL
7. StemTAG™ AP Activity Assay Substrate (Part No. C30004): One amber bottle – 5.0 mL
8. AP Stop Solution (Part No. 132502): One bottle – 20.0 mL
9. AP Activity Assay Standard (Part No. C30007): One tube – 1.0 mL of 5 mM p-Nitrophenol

Materials Not Supplied

1. Stem Cells and Culture Medium
2. 37ºC Incubator, 5% CO_2 Atmosphere
3. Light Microscope
4. 96-well Fluorometer
5. 96-well Sterile Microplate (flat bottom)
6. 37ºC and boiling water baths

Storage

Store all components at 4ºC.

Preparation of Reagents

- 2X DMEM/20% FBS Medium: In a sterile tube, dilute the provided 5X DMEM in sterile cell culture grade water to 2X containing 20% FBS. For example, to prepare a 5 mL solution, add 2 mL of 5X DMEM, 1 mL of FBS and 2 mL of sterile cell culture grade water. Additional supplements (e.g. LIF, bFGF, SCF) may also be added at a 2X formulation. Sterile filter the 2X media to 0.2 µm.
- 1X Matrix Solubilization Solution: Prepare a 1X Matrix Solubilization Solution by diluting the provided 10X stock 1:10 in sterile cell culture grade water. Sterile filter the 1X solution to 0.2 µm.
- 10X CytoSelect™ Agar Matrix Solution: Heat the Agar Matrix Solution bottle to 90-95ºC in a water bath for 30 minutes, or until agar matrix liquefies (microwaving is optional). Transfer the bottle to a 37ºC water bath for 20 minutes and maintain until needed.

Assay Protocol (must be under sterile conditions throughout)

The following assay protocol is written for a 96-well format. Refer to the below table for the appropriate dispensing volumes of other plate formats.

Culture Dish	96-well	48-well	24-well	12-well	6-well
Base Agar Matrix Layer (µL/well)	50	100	250	500	1000
Cell Suspension/Agar Matrix Layer (µL/well)	75	150	375	750	1500
Culture Media (µL/well)	50	100	250	500	1000
Lysis Buffer (µL/well)	125	250	625	1250	2500
1X Matrix Solubilization Solution (µL/well)	125	250	625	1250	2500

Table 1. Dispensing Volumes for Different Plate Formats

I. Preparation of Base Agar Matrix Layer

1. Heat the 10X CytoSelect™ Agar Matrix Solution to 90-95ºC in a water bath for 30 minutes, or until agar matrix liquefies (microwaving is optional). Transfer the bottle to a 37ºC water bath for 20 minutes and maintain until needed.
2. Warm the 2X DMEM/20% FBS medium (see Preparation of Reagents section) to 37ºC in a water bath. Allow at least 30 minutes for the temperature to equilibrate.
3. According to Table 2 (below), prepare the desired volume of Base Agar Matrix Layer in the following sequence:
    a. In a sterile tube, add the appropriate volume of 2X DMEM/20% FBS medium.
    b. Next, add the corresponding volume of sterile water. Mix well.
    c. Finally, add the corresponding volume of 10X CytoSelect™ Agar Matrix Solution. Mix well.

    Note: The 10X CytoSelect™ Agar Matrix Solution is slightly viscous; care should be taken in accurately pipetting the appropriate volume.

2X DMEM/20% FBS Medium (mL)	Sterile Water (mL)	10X CytoSelect™ Agar Matrix Solution (mL)	Total Volume of Base Agar Matrix Layer (mL)	**# of Tests in 96-well Plate (50 µL/test)**
2.5	2	0.5	5	**100**
1.25	1	0.25	2.5	**50**
0.5	0.4	0.1	1	**20**

Table 2: Preparation of Base Agar Matrix Layer

4. After mixing, maintain the Base Agar Matrix Layer at 37ºC to avoid gelation.

5. Dispense 50 µL of Base Agar Matrix Layer into each well of a 96-well sterile flat-bottom microplate (samples should be assayed in triplicate). Gently tap the plate a few times to ensure the Base Agar Matrix Layer evenly covers the wells.

   Notes:
   - *Work quickly with the layer to avoid gelation. Also, try to avoid adding air bubbles to the well.*
   - *To avoid fast and uneven evaporation that leads to aberrant results, we suggest not using the wells on the plate edge, or filling the edge wells with medium to reduce evaporation.*

6. Transfer the plate to 4ºC for 30 minutes to allow the Base Agar Matrix Layer to solidify.
7. Prior to adding the Cell Suspension/Agar Matrix Layer (Section II), allow the plate to warm to room temperature for 30 minutes.

II. Addition of Cell Suspension/Agar Matrix Layer

1. Heat the 10X CytoSelect™ Agar Matrix Solution to 90-95ºC in a water bath for 30 minutes, or until agar matrix liquefies (microwaving is optional). Transfer the bottle to a 37ºC water bath for 20 minutes and maintain until needed.
2. Warm the 2X DMEM/20% FBS medium (see Preparation of Reagents section) and CytoSelect™ Matrix Diluent to 37ºC in a water bath. Allow at least 30 minutes for the temperature to equilibrate.
3. Harvest and resuspend cells in culture medium at 0.1 - 1 x 10^6 cells/mL. Keep the cell suspension warm in a 37ºC water bath.
4. According to Table 3 (below), prepare the desired volume of Cell Suspension/Agar Matrix Layer in the following sequence:
   a. In a sterile tube, add the appropriate volume of 2X DMEM/20% FBS medium.
   b. Next, add the corresponding volume of CytoSelect™ Matrix Diluent. Mix well.
   c. Next, add the corresponding volume of 10X CytoSelect™ Agar Matrix Solution. Mix well.
   d. Finally, add the corresponding volume of cell suspension. Mix well.

   Note: The CytoSelect™ Matrix Diluent and 10X CytoSelect™ Agar Matrix Solution are slightly viscous; care should be taken in accurately pipetting the appropriate volumes.

2X DMEM/20% FBS Medium (mL)	CytoSelect™ Matrix Diluent (mL)	10X CytoSelect™ Agar Matrix Solution (mL)	Cell Suspension (mL)	Total Volume of Cell Suspension/ Agar Matrix Layer (mL)	**# of Tests in 96-well Plate (75 µL/test)**
3.5	2.75	0.75	0.5	7.5	**100**
1.75	1.375	0.375	0.25	3.75	**50**
0.875	0.688	0.188	0.125	1.875	**25**

Table 3: Preparation of Cell Suspension/Agar Matrix Layer

5. After mixing, incubate the Cell Suspension/Agar Matrix Layer at room temperature for 5 minutes.
6. Immediately dispense 75 µL of Cell Suspension/Agar Matrix Layer into each well of the 96-well plate, already containing the Base Agar Matrix Layer (Section I).

   Notes:
   - *Work quickly with the layer to avoid gelation, but gently pipette as not to disrupt the base layer integrity. Also, try to avoid adding air bubbles to the well.*
   - *Always include negative control wells that contain no cells in the Cell Suspension/Agar Matrix Layer.*
7. Transfer the plate to 4ºC for 20 minutes to allow the Cell Suspension/Agar Matrix Layer to solidify.
8. Allow the plate to warm to room temperature for 30 minutes.
9. Add 50 µL of culture medium containing cell growth activator(s) or inhibitor(s) to each well.
10. Incubate the cells for 6-8 days at 37ºC and 5% CO_2. Examine the colony formation under a light microscope.

III. Quantitation of Anchorage-Independent Growth (see section V if cell recovery/re-plating is desired)

1. Add 125 µL (for a 96-well plate) or 625 µL (for a 24-well plate) of Lysis Buffer to each well.
2. Pipette the entire volume of the well 15-20 times to mix thoroughly, solubilizing the agar matrix and lysing the cells completely.
3. Transfer 50 µL of the mixture to a 96-well plate suitable for fluorescence measurement. *Note: This same solution may be assayed for alkaline phosphatase activity (see step 3 of section IV below)*
4. Prepare sufficient PBS/CyQuant® GR dye solution for all samples by diluting the dye 1:200 in PBS (for example, add 5 µL dye to 995 µL of PBS).
5. Add 100 µL of PBS/CyQuant® GR dye solution to each well (already containing 50 µL of solution). Incubate the plate at room temperature for 30 minutes.
6. Pipette each well 7-10 times to ensure a homogeneous mixture.
7. Read the plate in a 96-well fluorometer using a 485/520 nm filter set.

IV. Quantitation of Alkaline Phosphatase Activity (see section V if cell recovery/re-plating is desired)

1. Add 125 µL (for a 96-well plate) or 625 µL (for a 24-well plate) of Lysis Buffer to each well.
2. Pipette the entire volume of the well 15-20 times to mix thoroughly, solubilizing the agar matrix and lysing the cells completely.
3. Transfer 50 µL of the mixture to a 96-well microtiter plate.
   Note: This same solution may be assayed for cell quantitation (see step 3 of section III above)
4. Add 50 µL of StemTAG™ AP Activity Assay Substrate to each well (already containing 50 µL of solution). Incubate the plate at 37ºC for 30 minutes.
5. To stop the reaction, add 50 µL of AP Stop Solution to each well.
6. Pipette each well 7-10 times to ensure a homogeneous mixture.
7. Measure the absorbance at 405 nm in a 96-well microtiter plate reader.

V. Cell Recovery and Re-plating

1. Add 125 µL (for a 96-well plate) or 625 µL (for a 24-well plate) of 1X Matrix Solubilization Solution to each well.
2. Pipette each well 10-12 times to mix thoroughly.
3. Transfer the entire mixture to at least 20 volumes of standard culture medium (for example, 1 mL would be transferred to 20 mL media).
4. Pipette the mixture vigorously 7-10 times.
5. Centrifuge the cell pellet and aspirate the media supernatant.
6. Resuspend the cell pellet in another 20 volumes of standard culture medium.
7. Repeat steps 4-6.
8. Resuspend the pellet and transfer to a tissue culture flask or dish.
9. Transfer to a cell culture incubator.

Cell Dose Curve (optional)

1. Heat the 10X CytoSelect™ Agar Matrix Solution to 90-95ºC in a water bath for 30 minutes, or until agar matrix liquefies (microwaving is optional). Transfer the bottle to a 37ºC water bath for 20 minutes and maintain until needed.
2. Warm the 2X DMEM/20% FBS medium (see Preparation of Reagents section) and CytoSelect™ Matrix Diluent to 37ºC in a water bath. Allow at least 30 minutes for the temperature to equilibrate.
3. Harvest and resuspend cells in culture medium at 1 - 5 x 10^6 cells/mL.
4. Prepare a serial 2-fold dilution in culture medium, including a blank without cells.
5. Transfer 50 µL of each dilution to a 96-well plate.

6. According to Table 4 (below), prepare the desired volume of Cell Dose Curve Solution in the following sequence:
    a. In a sterile tube, add the appropriate volume of 2X DMEM/20% FBS medium.
    b. Next, add the corresponding volume of sterile water. Mix well.
    c. Next, add the corresponding volume of CytoSelect™ Matrix Diluent. Mix well.
    d. Finally, add the corresponding volume of 10X CytoSelect™ Agar Matrix Solution. Mix well.

    Note: The CytoSelect™ Matrix Diluent and 10X CytoSelect™ Agar Matrix Solution are slightly viscous; care should be taken in accurately pipetting the appropriate volumes.

2X DMEM/20% FBS Medium (mL)	Sterile Water (mL)	CytoSelect™ Matrix Diluent (mL)	10X CytoSelect™ Agar Matrix Solution (mL)	**Total Volume of Cell Dose Curve Solution (mL)**
1.25	0.45	0.55	0.25	**2.5**
0.625	0.225	0.275	0.125	**1.25**

Table 4: Preparation of Cell Dose Curve Solution

7. Immediately dispense 125 µL of Cell Dose Curve Solution into the wells of the 96-well plate, already containing the cell serial dilution (from step 5).
8. Add 125 µL of Lysis Buffer to each well.
9. Pipette the entire volume of the well 15-20 times to mix thoroughly, lysing the cells completely.
10. Transfer 50 µL of the mixture to a 96-well plate suitable for fluorescence measurement.
11. Prepare sufficient PBS/CyQuant® GR dye solution for all samples by diluting the dye 1:200 in PBS (for example, add 5 µL dye to 995 µL of PBS).
12. Add 100 µL of PBS/CyQuant® GR dye solution to each well (already containing 50 µL of solution). Incubate the plate at room temperature for 30 minutes.
13. Pipette each well 7-10 times to ensure a homogeneous mixture.
14. Read the plate in a 96-well fluorometer using a 485/520 nm filter set.

Calculation of Anchorage-Independent Growth

1. Compare RFU values with the Cell Dose Curve and extrapolate the cell concentration.
2. Calculate the Total Cell Number/Well
   Total Cells/Well = cells/mL x 0.050 mL/well

For example: If you extrapolate your RFU value from your cell dose curve and determine you have 500,000 cells/mL in your sample.
Total Cells/Well = 500,000 cells/mL x 0.050 mL/well = 25,000 cells/well

Example of Results

The following figures demonstrate typical results with the StemTAG™ 96-well Stem Cell Colony Formation Assay. Fluorescence measurement was performed on SpectraMax Gemini XS Fluorometer (Molecular Device) with a 485/520 nm filter set and 530 nm cutoff. One should use the data below for reference only. This data should not be used to interpret actual results.

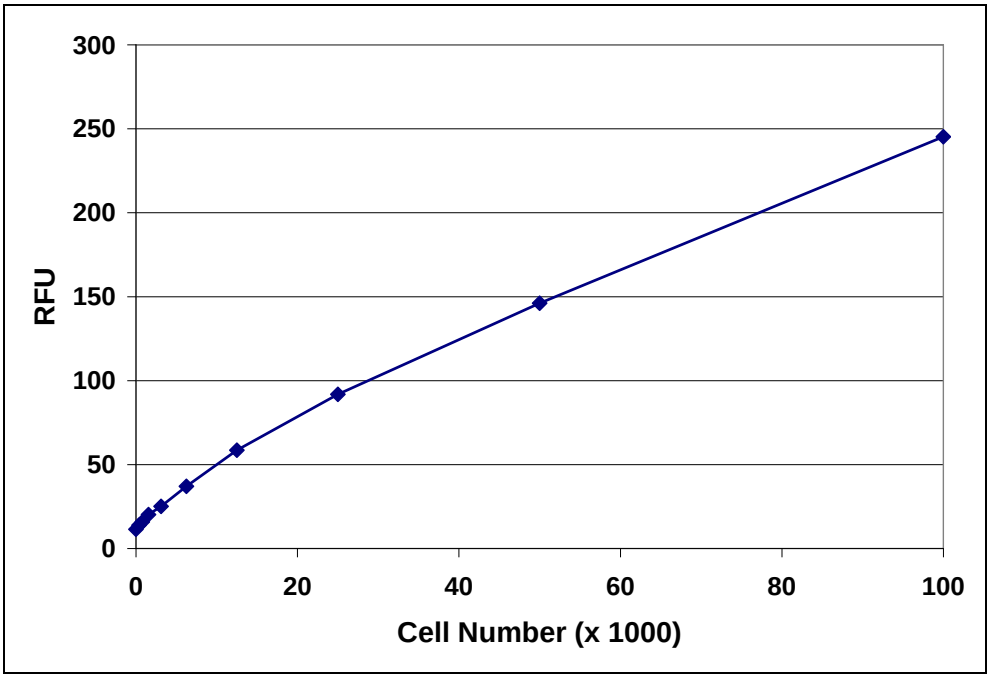


Figure 1: **ES-D3 Cell Dose Curve**. Murine embryonic stem cells (ES-D3) were resuspended at 12 x 10^6 cells/mL and titrated 1:2 in culture medium, followed by addition of Cell Dose Curve Solution, Matrix Solubilization Solution, Lysis Buffer, and Cyquant® GR Dye detection (as described in the Cell Dose Section). Results are shown by actual cell number in CyQuant Detection.

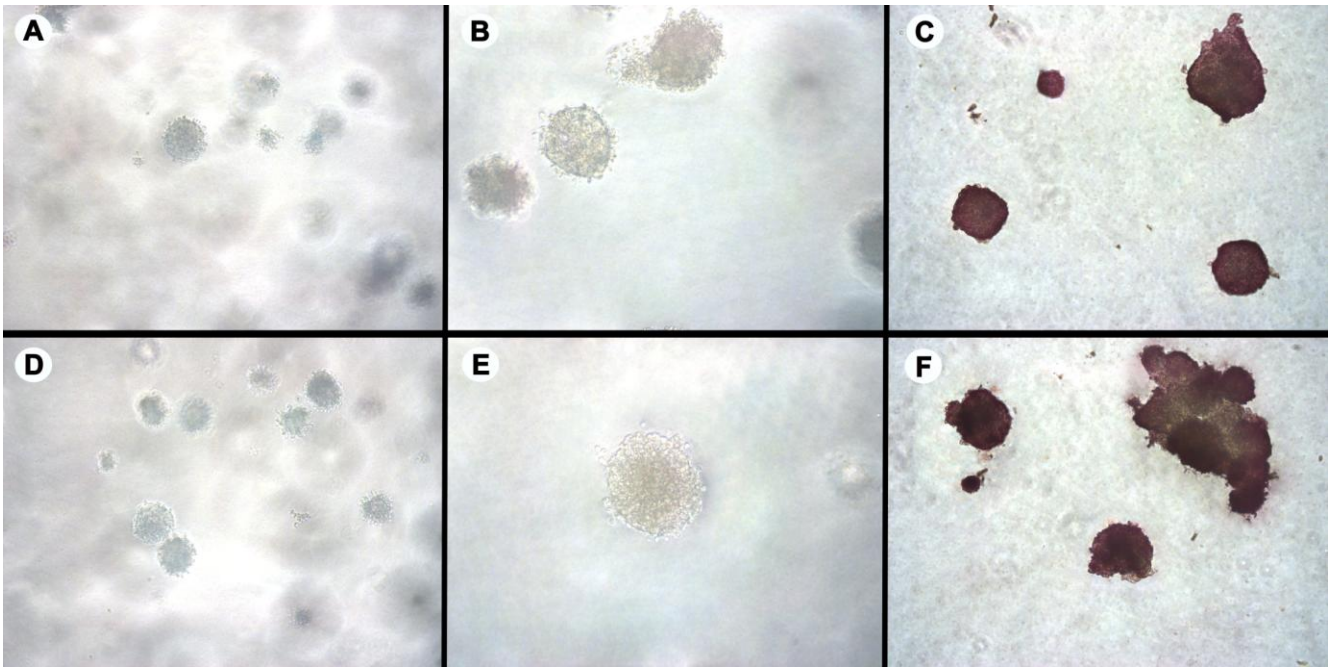


Figure 2: **ES-D3 Colony Formation**. Murine embryonic stem cells (ES-D3) were cultured for 7 days in the presence (D-F) or absence (A-C) of LIF, according to the assay protocol. Phase contrast images demonstrate colonies at 10X magnification (A, D), 20X magnification (B, E), and 20X magnification with AP staining (C, F).

References

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Recent Product Citations

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4. Shin, M. R. et al. (2014). Isocudraxanthone K induces growth inhibition and apoptosis in oral cancer cells via hypoxia inducible factor-1α. *Biomed Res Int.* **2014**:934691.

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