Product Manual

CytoSelect™ 96-Well Cell Transformation Assay (Cell Recovery Compatible, Fluorometric)

Catalog Number

CBA-140	96 assays
CBA-140-5	5 x 96 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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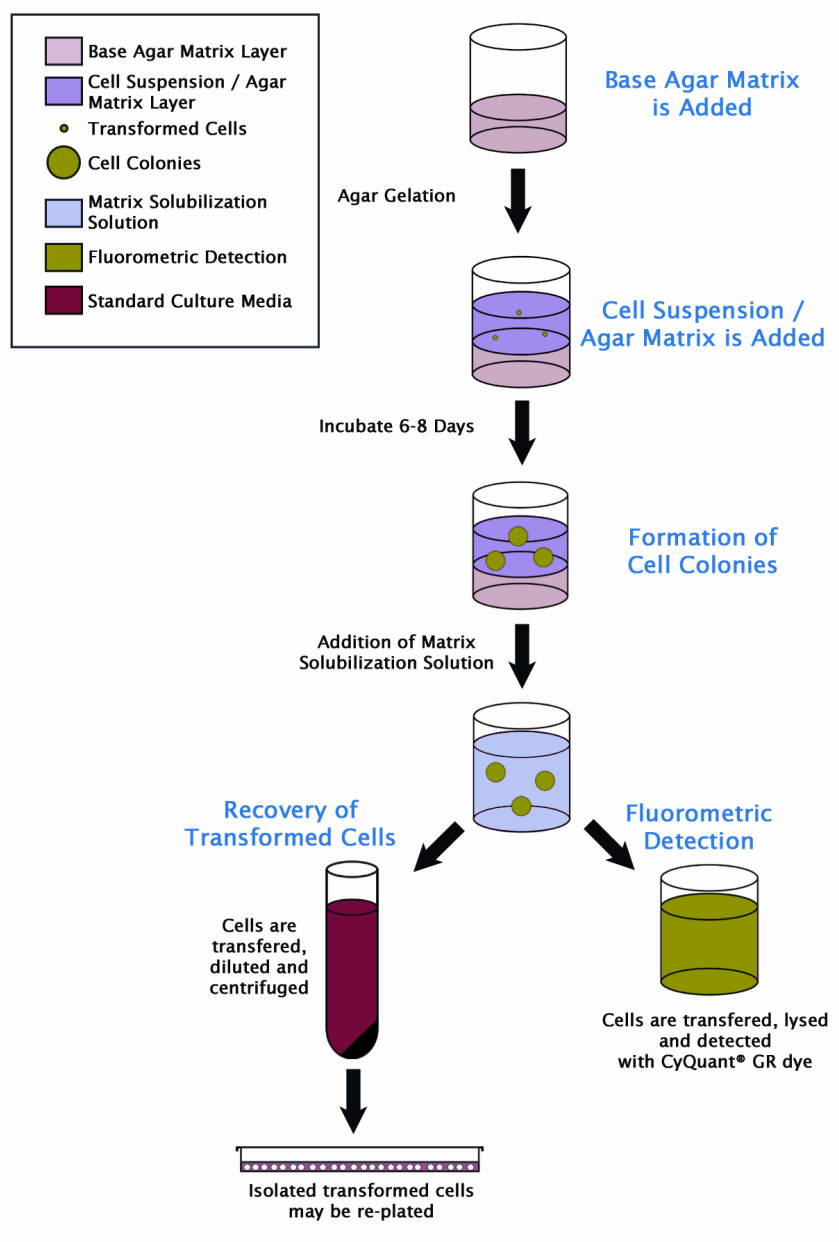
Introduction

Neoplastic transformation occurs via a series of genetic and epigenetic alterations that yield a cell population that is capable of proliferating independently of both external and internal signals that normally restrain growth. For example, transformed cells show reduced requirements for extracellular growth promoting factors, are not restricted by cell-cell contact, and are often immortal. Anchorage-independent growth is one of the hallmarks of transformation, which is considered the most accurate and stringent in vitro assay for detecting malignant transformation of cells.

Traditionally, the soft agar 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 soft agar 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. 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' CytoSelect™ Cell Transformation 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 (see Assay Principle below). **Alternatively, viable transformed cells can be easily recovered for further culturing and testing, such as in protein/DNA array analysis and cancer vaccine development.** This format provides a quantitative, high-throughput method to accurately measure cell transformation, while the short incubation time makes it possible to assay cells transiently transfected with oncogenes or siRNA.

The CytoSelect™ Cell Transformation Kit (Cell Recovery Compatible) provides a robust system for measuring in vitro drug sensitivity, screening oncogenes and cell transformation inhibitors, and allows for transformed cell recovery. Each kit provides sufficient quantities to perform 96, 48, 24, 12, or 6 tests in a 96, 48, 24, 12, or 6-well plate, respectively.



Related Products

1. CBA-100-C: CytoSelect™ 24-Well Cell Migration and Invasion Assay (8 µm, Colorimetric)
2. CBA-106-C: CytoSelect™ 96-Well Cell Migration and Invasion Assay (8µm, Fluorometric)
3. CBA-112: CytoSelect™ 96-Well Cell Invasion Assay (Basement Membrane, Fluorometric)
4. CBA-135: CytoSelect™ 96-Well Cell Transformation Assay (Cell Recovery, Colorimetric)
5. CBA-130: CytoSelect™ 96-Well Cell Transformation Assay (Soft Agar Colony Formation)

Kit Components

1. 10X CytoSelect™ Agar Matrix Solution (Part No. 114001): One 10 mL sterile bottle

2. CytoSelect™ Matrix Diluent (Part No. 114002): One 4 mL sterile bottle
3. 5X DMEM Medium (Part No. 113005): One 5 mL bottle
4. 10X Matrix Solubilization Solution (Part No. 114003): One 1.8 mL sterile tube
5. 4X Lysis Buffer (Part No. 10404): One 10 mL bottle
6. CyQuant® GR Dye (Part No. 10105): One 75 µL tube

Materials Not Supplied

1. Cells and Culture Medium
2. 37ºC Incubator, 5% CO_2 Atmosphere
3. Light Microscope
4. 96-well Fluorometer
5. 37ºC and boiling water baths
6. (Optional) Positive Control cells such as NIH 3T3 (Ras G12V)

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. Sterile filter the 2X media to 0.2 µm.

  Note: You may substitute your own medium in place of the DMEM we provide, but ensure that it is at a 2X concentration.

- 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)

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
1X Matrix Solubilization Solution (µL/well)	125	250	625	1250	2500

Table 1. Dispensing Volumes of 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 (under sterile conditions)

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 (skip to section IV if cell recovery/re-plating is desired)

1. Add 125 µL of 1X Matrix Solubilization Solution to each well.
2. Pipette the entire volume of the well 10-12 times to mix thoroughly and solubilize the agar matrix completely.
3. Transfer 150 µL of the mixture to a 96-well plate suitable for fluorescence measurement.
4. Prepare sufficient 4X Lysis Buffer/CyQuant® GR dye solution for all samples by diluting the dye 1:75 in 4X Lysis Buffer (for example, add 5 µL dye to 370 µL of 4X Lysis Buffer).
5. Add 50 µL of 4X Lysis Buffer/CyQuant® GR dye solution to each well (already containing 150 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. Cell Recovery and Re-plating (under sterile conditions)

1. Add 125 µL 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 1X Matrix Solubilization Solution to each well. Pipette each well 10-12 times to mix thoroughly.
9. Transfer 150 µL of the mixture to a 96-well plate suitable for fluorescence measurement.
10. Prepare sufficient 4X Lysis Buffer/CyQuant® GR dye solution for all samples by diluting the dye 1:75 in 4X Lysis Buffer (for example, add 5 µL dye to 370 µL of 4X Lysis Buffer).
11. Add 50 µL of 4X Lysis Buffer/CyQuant® GR dye solution to each well (already containing 150 µL of solution). Incubate the plate at room temperature for 30 minutes.

12. Pipette each well 7-10 times to ensure a homogeneous mixture.
13. Read the plate in a 96-well fluorometer using a 485/520 nm filter set.

Example of Results

The following figures demonstrate typical results with the CytoSelect™ 96-well Cell Transformation Assay Kit. One should use the data below for reference only. This data should not be used to interpret actual results.

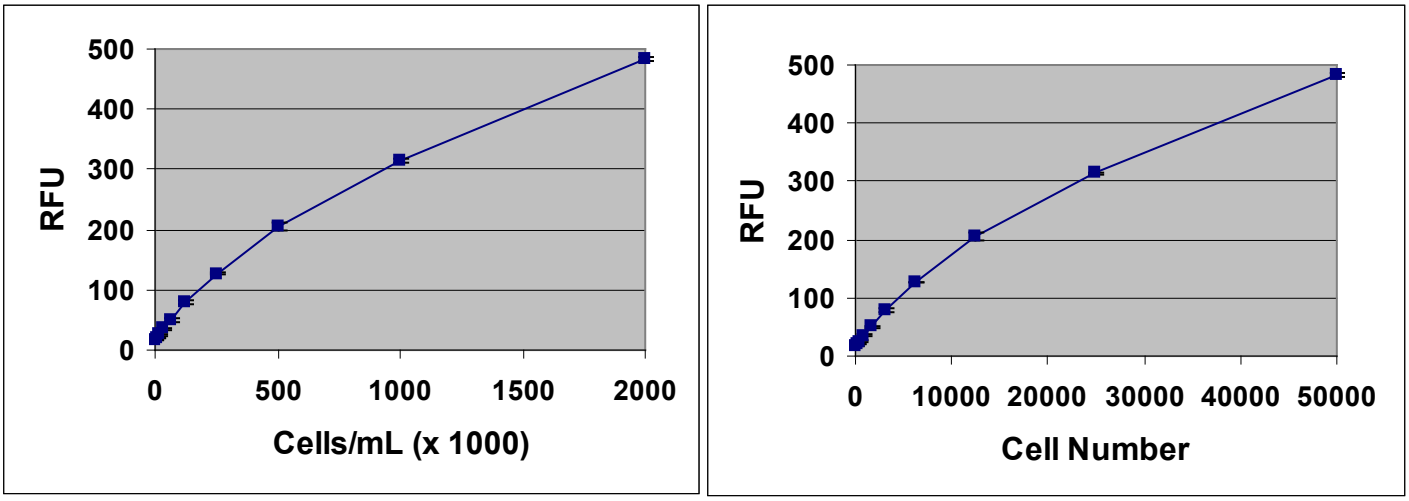


Figure 1. HeLa Cell Dose Curve. Cervical carcinoma HeLa cells were resuspended at 2 x 10^6 cells/mL and titrated 1:2 in culture medium, followed by addition of Cell Dose Curve Solution, Matrix Solubilization Solution, Lysis Buffer, and Cyquant® GR Dye detection (as described in the Cell Dose Section).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

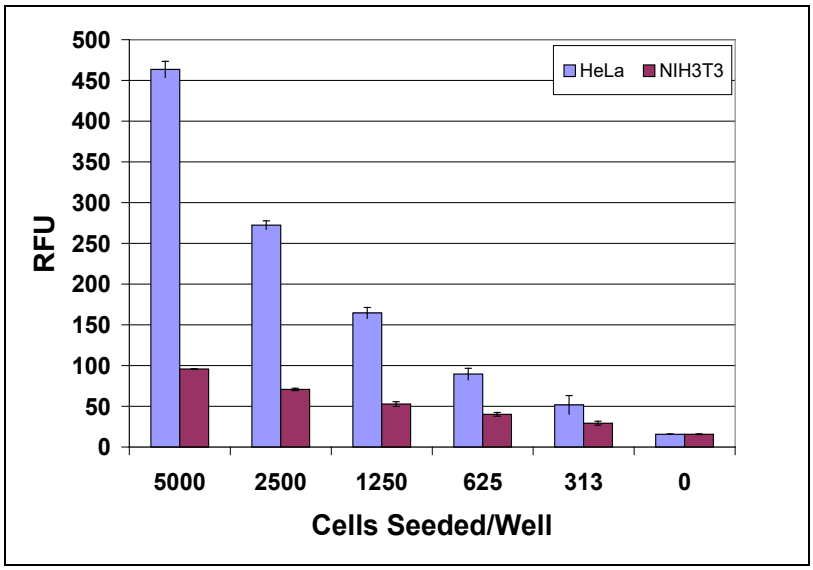


Figure 2. Anchorage-Independent Growth of HeLa and NIH3T3 Cells. HeLa and NIH3T3 cells were seeded at various concentrations and cultured for 6 days. Cell transformation was determined according to the assay protocol.

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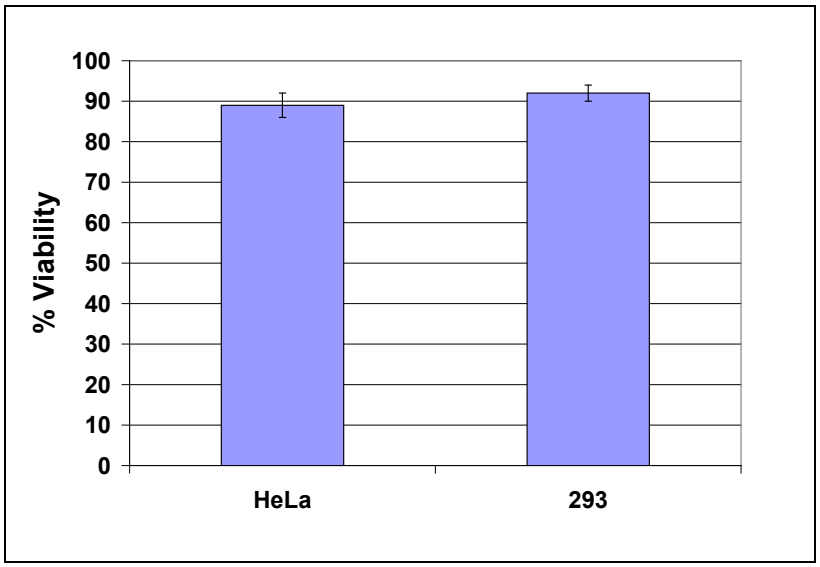

Figure 3. Cell Viability of Recovered Cells. HeLa and 293 cells were cultured for 6 days according to the assay protocol. Cells were recovered and the cell viability was determined by trypan blue exclusion.

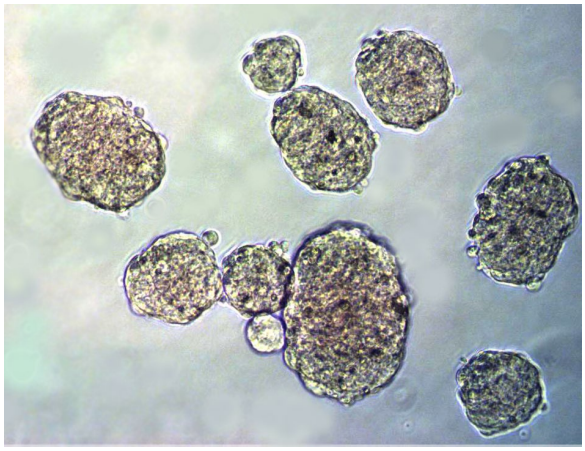

Figure 4: **HeLa Colony Formation**. HeLa cells were cultured for 10 days according to the assay protocol.

Calculation of Anchorage-Independent Growth

1. Compare RFU values with the Cell Dose Curve and extrapolate the cell concentration.
2. Calculate the Total Transformed Cell Number/Well
   Total Transformed 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 Transformed Cells/Well = 500,000 cells/mL x 0.050 mL/well = 25,000 cells/well

References

1. Shin SI, Freedman VH, Risser R, and Pollack R. (1975) *Proc Natl Acad Sci U S A.* 72:4435-9.

2. Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW and Weinberg RA. (1999) *Nature* 400:464-8.

Recent Product Citations

1. Paul, M. et al. (2022). Nitric-Oxide Synthase trafficking inducer (NOSTRIN) is an emerging negative regulator of colon cancer progression. *BMC Cancer*. **22**(1):594. doi: 10.1186/s12885-022-09670-6.
2. Kondo, M. et al. (2021). Safety and efficacy of human juvenile chondrocyte-derived cell sheets for osteochondral defect treatment. *NPJ Regen Med*. **6**(1):65. doi: 10.1038/s41536-021-00173-9.
3. van der Toorn, M. et al. (2018). The biological effects of long-term exposure of human bronchial epithelial cells to total particulate matter from a candidate modified-risk tobacco product. *Toxicol In Vitro*. **50**:95-108. doi: 10.1016/j.tiv.2018.02.019.
4. Montalbano, M. et al. (2016). Modeling of hepatocytes proliferation isolated from proximal and distal zones from human hepatocellular carcinoma lesion. *PLoS One.* **11**:e0153613.

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