MultiCellGro™

Hematopoietic, Multi-Stem, Multi-Lineage Fluorescence Proliferation Assay with Phenotypic Analysis Capability

Technical Manual

(Version 5-17)

This manual should be read in its entirety prior to using this product

For *In Vitro* Research Use Only.
Not for clinical diagnostic use.

Preferred Cell Systems™

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1. Limitations of the Assay and Precautions

1. *MultiCellGro™ is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)*
2. *MultiCellGro™ is for research use only and has not been approved for clinical diagnostic use.*
3. *Reagents and supplies in this kit are STERILE. Perform all procedures under sterile conditions, except where indicated.*
4. *This kit should not be used beyond the expiration date on the kit label.*
5. *Do not mix or substitute reagents or other kit contents from other kit lots or sources.*
6. *Always use professionally calibrated and, preferably, electronic pipettes for all dispensing procedures. Small discrepancies in pipetting can lead to large pipetting errors. Although electronic pipettes self-calibrate themselves, they still need to be professionally calibrated on a regular basis.*
7. *Good laboratory practices and universal protective precautions should be undertaken at all times when handling the kit components as well as human cells and tissues. Material safety data sheets (MSDS) are available for download from this website.*

2. Introduction

MultiCellGro™ is a 96-well plate assay that uses Suspension Expansion Culture™ (SEC™) Technology containing a proprietary cocktail of growth factors, cytokines and supplements to stimulate primitive hematopoietic stem cells into mature stem cells and progenitor cells from multiple hematopoietic lineages. Total cell proliferation is measured using a non-destructive fluorescence signal that is measured in a fluorescence plate reader. After measuring cell proliferation, phenotypic analysis of the culture cells can be performed directly in each well or cells can be pooled from replicate wells and flow cytometry performed in a tube assay.

MultiCellGro™ incorporates a fluorescence readout that measures the activity of a constitutive live-cell protease that is present in intact viable cells. The fluorogenic peptide substrate enters cells and is cleaved by the protease activity to generate a fluorescence emission signal at 505nm after excitation between 380 and 400nm.

3. Use and Availability

MultiCellGro™ can be used for virtually any application requiring the clonal culture of hematopoietic cells where multiple colony types need to be enumerated in a single culture dish. MultiCellGro™ is particularly useful in the cell processing laboratory, where stem, progenitor and precursor cells can be detected simultaneously.

AllColonies™ can be used with cells from the following tissue sources:

- Embryonic tissue
- Fetal tissue
- Spleen
- Bone marrow
- Peripheral blood
- Cord blood

AllColonies™ is available for cells derived from the following species:

- Human
- Mouse

The MultiCellGro™ Reagent contained in the assay kit is available as a serum-free or low serum formulation.

4. Cell Types Produced in MultiCellGro™ Cultures

Cell types produced are dependent upon a number of factors. These include, but are not limited to:

- Fractionation or purification of the tissue being used.
- The quality of cells after fractionation and/or purification.
- Metabolic and functional status of the tissue at the time of use.
- Tissue source
- Species

Cell types might include, but are not limited to:

- Primitive stem cells
- Mature stem cells
- Erythropoietic and erythroid cells
- Myelomonocytic cells, granulocytes, monocytes and macrophages
- Megakaryocytes
- T-cell lineage

- B-cell lineage

5. Principle of the MultiCellGro™ Assay

MultiCellGro™ is a fluorescence *in vitro* cell viability/proliferation assay. It incorporates a fluorogenic, glycyl-phenylalanyl-aminofluorocoumarin (GF-AFC) peptide substrate that can enter cells. In intact viable cells, a conserved and constitutive, live-cell protease cleaves the substrate and generates a fluorescence signal at 505nm that is proportional to the number of living cells. If cells loose their membrane integrity, the live-cell protease becomes inactive. The reaction is measured in a fluorescence plate reader using an excitation of 380-400nm. After adding 0.1ml of the prepared MultiCellGro™ reagent to the wells and mixing briefly, the plates are incubated at 37°C for at least 30 minutes, but no longer than 3 hours. Optimal fluorescence is usually achieved after about 2 hours incubation. The plate can be removed from the incubator at different times to follow the development of the fluorescence signal in real-time. It is recommended to include a background control without cells and subtract the background fluorescence from the sample being measured.

The MultiCellGro™ reagent is non-destructive, i.e. it does not lyse cells. Therefore, cells can also be labeled with fluorophore-conjugated antibodies that excite and emit fluorescence at different wavelengths in order to detect specific cell populations. In addition, MultiCellGro™ can also be multiplex with other Preferred Cell Systems™ ATP bioluminescence assays (e.g. MultiCellGro™, ImmunoGlo™, MSCGlo™) to provide an extremely powerful and informative assay system.

Advantages of using MultiCellGro™

- Ready-to-use assay.
- Non-subjective, instrument-based and quantitative.
- Determines cell proliferation, viability and cell number.
- Multiplexes with flow cytometric protocols using other fluorescent labels.
- Multiplexes with Preferred Cell Systems™ ATP bioluminescence assays.
- Greater sensitivity than colorimetric/absorbance assays.
- Learn in 1 day and easy to use.

6. Kit Contents and Storage Conditions

MultiCellGro™ assay kits contain reagents that have been frozen and stored at -80°C prior to shipment. The kit is shipped either with dry ice or blue ice. The following components are included:

Item	Component	Storage
1	MultiCellGro™ Base Medium	-20°C until used
2	MultiCellGro™ Reagent	-20°C until used
3	GF-AFC Substrate	-20°C until used. Protect from light.
4	MultiCellGro™ Buffer, used to dilute GF-AFC substrate	-20°C until used. Protect from light.
5	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
6	Sterile, black, individually wrapped, 96-well plate for cell culture	Can be kept with other kit components

Exact volumes of kit reagents and supplies are provided on a separate sheet included with this assay kit.

Please Note. The assay kit is shipped on dry ice (solid carbon dioxide). It is possible that when the kit arrives, bottles of Master Mix may be yellow or orange in color. This is not contamination. Carbon dioxide can leak into the bottles causing a pH change. Prior to thawing the Reagent, gently unscrew the lid, but do not remove the lid. Unscrewing the lid will release the carbon dioxide. When thawed, the Reagent should be at the correct pH.

IMPORTANT

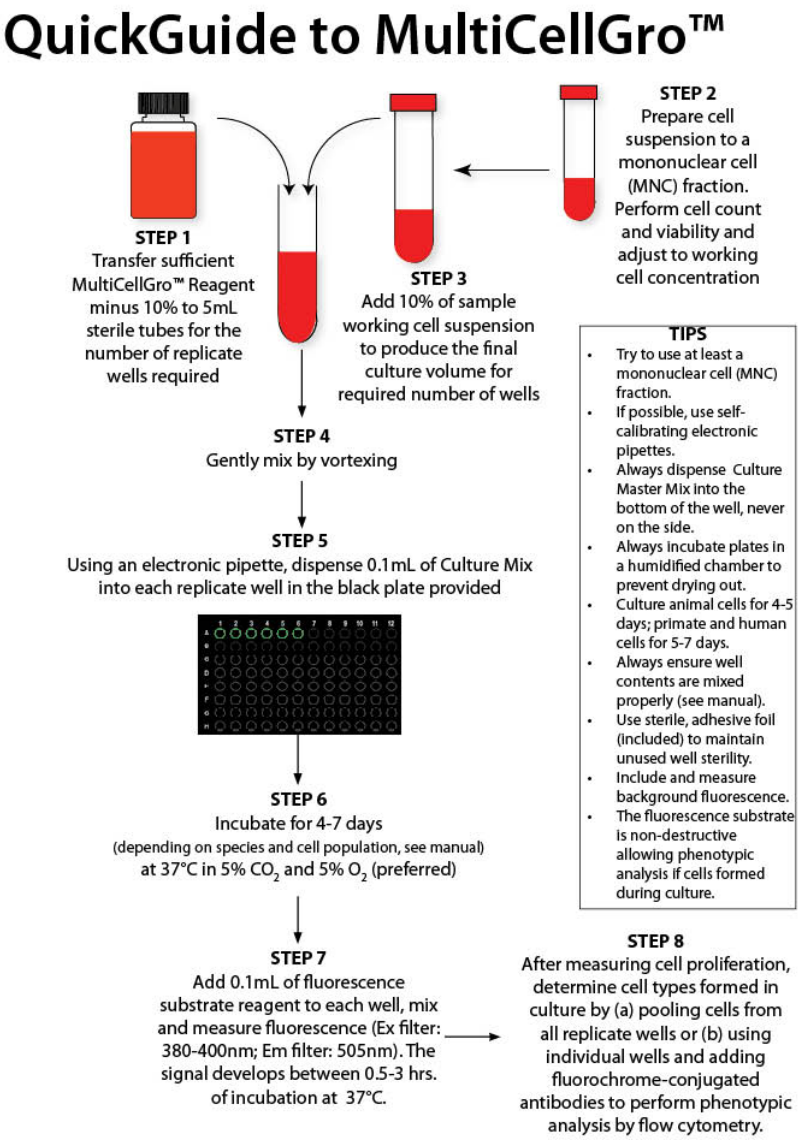
All kit components are quality controlled and optimized so that they work together. Please do not replace kit

components with those of a different product. This will invalidate the warranty provided by Preferred Cell Systems™.

This kit contains a reagent for measuring fluorescence that decays with time. Preferred Cell Systems™ recommends that this kit be used before the expiry date of this reagent. Preferred Cell Systems™ does not take responsibility for the quality of reagents beyond their expiry date. If the kit cannot be used prior to the expiry date of this reagent, fresh reagent can be obtained from Preferred Cell Systems™.

Good laboratory practices and universal protective precautions should be undertaken at all times when handling the kit components as well as cells and tissues. Material safety data sheets (MSDS) are included in each literature packet.

7. QuickGuide to MultiCellGro™



8. Equipment, Supplies and Reagents Required, but not Provided

Equipment and Supplies

1. Laminar Flow Biohood
2. Fluorescence plate reader
3. Sterile plastic tubes (5ml, 10ml, 50ml)
4. Single channel pipettes, preferably electronic (e.g. Rainin EDP pipettes for variable volumes between 1µl and 1000µl).
5. 8 or 12-channel pipette, preferably electronic (e.g. Rainin EDP pipettes for fixed or variable volumes between 10µl and 100µl).
6. Reservoir for 8- or 12 channel pipette
7. Sterile pipette tips.
8. Vortex mixer.
9. Tissue culture incubator, humidified at 37°C with 5% CO_2 (minimum requirement) and 5% O_2 (preferable).
10. 1.5ml plastic vials (5 for each ATP dose response).
11. Hemocytometer or electronic cell counter to determine cell concentration.
12. Flow cytometer or hemocytometer for determining viability.

Reagents

1. HemoGro™ Hematopoietic Growth Medium for cell suspensions and dilutions (Preferred Cell Systems™)
2. Iscove's Modified Dulbecco's Medium (IMDM)
3. Density-gradient medium (e.g. NycoPrep 1.077, Axis-Shield).
4. 7-AAD, propidium iodide or trypan blue for viability assay.
5. LIVEGlo™ metabolic viability assay (Preferred Cell Systems™)

9. The MultiCellGro™ Protocol

PLEASE READ THE FOLLOWING PROTOCOL CAREFULLY
SEE SECTION 10 BEFORE PERFORMING THE PROTOCOL

Performing MultiCellGro™ is a 4-step process.

Step 1 – Cell preparation.
Step 2 – MultiCellGro™ cell culture preparation, plating and incubation in the 96-well plate.
Step 3 – Fluorescence measurement.
Step 4 - Phenotypic analysis of cells by flow cytometry.

Step 1 and Step 2 must be performed in a laminar flow biohazard hood

STEP 1 – Cell Preparation

MultiCellGro™ can be performed using tissues with the following purity:

1. Total nucleated cell (TNC) fraction usually produced by red blood cell reduction. The TNC fraction is often used for human bone marrow and umbilical cord blood. The concentration of red blood cells in this preparation may be 30% or higher. Although the TNC fraction can be used, it is not recommended due to (a) dilution of primitive stem and progenitor cells, (b) underestimation of primitive stem and progenitor cells, and (c) interference of high red blood cell concentrations with the ATP readout resulting in an unsatisfactory high ATP readout.
2. Mononuclear cell (MNC) fraction is the preparation of choice for human, large animals and rats. This fraction can be prepared by density gradient centrifugation
3. Purified stem and progenitor cell populations prepared by cell sorting or magnetic bead separation.

Cell Viability, Cell Counting and Cell Culture Suspension Preparation

1. For dye exclusion viability methods, use trypan blue and a hemocytometer or automated method, flow cytometry using 7-AAD or another vital stain.
   Note *that dye exclusion viability methods detect membrane integrity. They do not detect cellular and mitochondrial integrity and therefore metabolic viability.*
   A viability of 85% or greater should be obtained when using dye exclusion viability methods only. It is recommended not to use cell suspensions with a viability of less than 85% since these cells will not be able to sustain proliferation ability. Use LIVEGlo™ (Preferred Cell Systems™) as a metabolic viability assay.
2. Determine the cell concentration using either a hemocytometer or electronic cell/particle counter. **NOTE**: Do not base the working concentration on the TNC count or the number of viable cells as this will give erroneous results.
3. Adjust the cell suspension concentration to that recommended in Table 1.
   Note *the working cell concentration per ml is 100 x the final cell concentration per well.* If cells have been treated prior to cell culture, higher cell concentrations may be required.
4. Prepare the total volume of cell suspension required using HemoGro™ or IMDM. The volume of the adjusted cell suspension required will be 10% of the total volume of MultiCellGro™ Culture Master Mix prepared.

TABLE 1
Recommended Cell Doses for Different Species, Cell Types, Cell Preparations and Cell States for MultiCellGro™

Species	Cell Type	Cell Preparation	Cell State	Working Cell Concentration Required (100 x Final Cells/Well)	Final Cell Dose / Well
Human	Bone marrow	MNC	Fresh/Frozen	0.5-0.75 x 10^6	5,000-7,500
	Peripheral blood	MNC	Fresh/Frozen	0.5-0.75 x 10^6	5,000-7,500
	Mobilized peripheral blood	MNC	Fresh/Frozen	0.5-0.75 x 10^6	5,000-7,500
	Umbilical cord blood	MNC	Fresh/Frozen	0.5-0.75 x 10^6	5,000-7,500
	Umbilical cord blood	MNC	Frozen	0.5-0.75 x 10^6	5,000-7,500
	Bone marrow	$CD34^+$	Fresh	0.1-1 x 10^5	100-1,000
	Mobilized peripheral blood*	$CD34^+$	Fresh/Frozen	0.1-5 x 10^5	100-5,000
	Umbilical cord blood	$CD34^+$	Fresh/Frozen	0.1-5 x 10^5	100-5,000
Mouse	Bone Marrow	MNC	Fresh	0.5-0.75 x 10^6	5,000-7,500
	Spleen	MNC	Fresh	0.5-1 x 10^6	5,000-10,000
	Fetal liver	MNC	Fresh	0.5-0.75 x 10^6	5,000-7,500

STEP 2. MultiCellGro™ Cell Culture Preparation

- ***The MultiCellGro™ Reagent is complete and ready-to-use.***
- ***Perform all procedures under a laminar flow, bio-hazzard hood.***
- ***Wear protective clothing, including gloves for all operations.***

The MultiCellGro™ Methodology

1. Remove the MultiCellGro™ Reagent from the freezer and thaw either at room temperature or in a beaker of cold water. Do not thaw in a 37°C water bath or incubator.
2. Label sufficient 5mL tubes for the number of samples to be tested.
3. Sufficient Reagent is supplied for 96-wells. Using a calibrated pipette, preferably an electronic pipette with repeat function, dispense a volume of the Reagent minus 10%, that will allow 0.1mL to be dispensed into the required number of replicate wells. See Table 2. For example, if 4 replicate wells are to be prepared, dispense 0.405mL of the MultiCellGro™ Reagent into each tube. This would be followed by 0.045mL of cells to produce a total volume of 0.45mL.

TABLE 2

Number of Replicate Wells Required	Volume of MultiCellGro™ Master Mix	Volume of Cells (10% of final volume)	Total Volume
2	0.315mL	0.035mL	0.35mL
4	0.405mL	0.045mL	0.45mL
6	0.585mL	0.065mL	0.65mL

4. Adjust the cell concentration to the working cell concentration shown in Table 1. The working cell concentration should be 100 fold higher than the final cell concentration in the well. Thus, if the final cell concentration is to be 5,000 cells/well, the working cell concentration should be 100 times 5,000 or 500,000 (5×10^5) cells/mL.
5. Dispense the required volume of cells into each tube containing the MultiCellGro™ Reagent.
6. Mix the contents gently on a vortex mixer. Do not cause bubbles.
7. Remove the sterile, black, wrapped, 96-well plate from the assay kit box.
8. Using a calibrated pipette, preferably an electronic pipette with repeat function, dispense 0.1mL into each of the required number of replicate wells of the 96-well plate.
9. After replacing the lid, transfer the 96-well plate to a humidified container (see Section 10).
10. Transfer the humidified container to the incubator. The incubator should be fully humidified at 37ºC and gassed with 5% CO_2 and, if possible, 5% O_2. This reduced oxygen tension improves plating efficiency by reducing oxygen toxicity cause by the producing of free radicals.
11. Incubate the cells for the time shown in Table 3.

TABLE 3

Species	Cell Source	Incubation Period (days)
Human	Bone marrow, normal and mobilized peripheral blood, umbilical cord blood	5-8
Mouse	All	4

STEP 3 – Fluorescence Reagent Preparation and Measurement

1. Remove the MultiCellGro™ Base Medium, substrate buffer and GF-AFC substrate from the freezer and thaw at 37°C in a water bath.
2. Vortex the GF-AFC substrate to ensure homogeneity and briefly centrifuge to recover the complete volume.
3. Transfer all the GF-AFC substrate to the buffer container. This produces a 2 fold concentrate. Mix by vortexing until thoroughly dissolved. The concentrate may appear "milky" but this is normal and will dissolve with vortexing. The prepared fluorescence substrate should be used within 24 hours if stored at room temperature. At 4°C, the substrate can be stored for 7 days.
4. Remove the sample plate(s) and allow to equilibrate to room temperature.
5. If only part of the culture plate has been used, transfer the plate to a bio-safety hood and remove the lid under sterile conditions. Take a sterile adhesive plate coverfoil provided with the kit and remove the backing foil. Layer the adhesive side on the plate to over the top of the plate. Using a sharp knife or scalpel, cut away the foil that covers the wells to be processed. The unused, empty wells will now remain sterile for the next experiment.
6. Dispense 0.1ml of the MultiCellGro™ Base Medium provided with the kit into empty replicate wells. 4-6 wells should be sufficient. This will provide the background fluorescence that is subtracted from the sample fluorescence.
7. Accurately dispense 0.1ml of the prepared culture cell suspensions into each of the required number of replicate wells.
8. Dispense 0.1ml of the MultiCellGro™ fluorescence substrate into each of the sample wells and mix the contents with the same tip. If a large number of samples are to be processed, pour the substrate into a reservoir and use a multichannel pipette to dispense 0.1ml into each well. Mix with the same tip.

9. After dispensing, change the pipette tip(s).
10. Repeat this procedure for each column or row using new tips.
11. When the fluorescence substrate has been dispensed into all sample wells, replace the plastic lid.
12. Incubate the plate(s) at 37°C for a minimum of 30 minutes and a maximum of 3 hours. The plate(s) can be removed from the incubator to measure the fluorescence at any time during this period and then replace the plate(s) back into the incubator. A 2 hours incubation is usually sufficient to obtain optimum sensitivity.
13. Measure the background fluorescence at 505nm prior to measuring the sample fluorescence. **Please note** that it may be necessary to adjust the instrument gain. (The "gain" is the applied photomultiplier tube energy).
14. Subtract the background fluorescence from the fluorescence obtained from the samples to yield the corrected fluorescence.

STEP 4. Phenotypic Analysis by Flow Cytometry

MultiCellGro™ is a non-destructive assay, i.e. it does not lyse the cells. As a result, the cells growing in culture can be analyzed phenotypically by flow cytometry after cell proliferation has been measured.

The cells from replicate wells can be removed and pooled together prior to the addition of fluorochrome-conjugated antibodies.

Alternatively, fluorochrome-conjugated antibodies can be added directly to each well so that phenotypic analysis can be performed on individual cell cultures using a multiwell sampler. The antibody markers should not emit fluorescence at the same wavelength as the MultiCellGro™ reagent (505nm).

10. Recommendations and Tips Prior to Using the MultiCellGro™

(i) Background Controls
If the culture medium contains serum, background fluorescence may result. It is recommended to always include a background control of the same culture medium, but without cells. Other controls may be needed depending on the type of experiment being conducted.

(ii) Number of Replicates Performed
The number of replicates/sample is arbitrary. For statistical purposes, 6 replicates/sample are recommended. Please remember that using fewer replicates may save components in the short term, but may also cause inconclusive results. If outliers are encountered, which may have to be removed from the analysis, the consequence could be that extra experiments would be required resulting in extra time and costs.

(iii) 96-Well Plates Provided
The reagents have been optimized to work with the black, 96-well plate(s) provided. Other plates can be used. However, cell growth and fluorescence output can be seriously affected and the assay kit warranty will be void. Additional plates can be purchased from Preferred Cell Systems™ if required.

(v) Humidity Chamber
If cell incubation time are greater than 3 days, a humidity chamber is recommended due sample volume evaporation. Even fully humidified incubators do not keep the humidity level high enough to keep the sample from evaporating. This usually results in so-called "edge effects". A humidity chamber can be assembled using plastic lunch boxes or other plastic ware available from a supermarket or discount stores. Holes should be made in the lid to enable adequate gas exchange. Disposable serological pipettes are cut to an appropriate length to fill the bottom of the container. Distilled/deionized water is poured into the container to just below the level of the pipettes. This allows for adequate water to keep the humidity high without the plates sitting in water. Please contact Preferred Cell Systems™ for further information about assembling and using humidity chambers.

11. Troubleshooting

High Coefficients of Variation (%CV)
Coefficients of variation (%CV) should be =< 15%. The percent coefficient of variation is calculated as standard deviation/ mean x 100. High %CVs are usually an indication of incorrect dilutions or pipetting error. Although outliers can be obtained, large variations between replicates should not be obtained. Please consider the following:

- Accurate reagent dispensing and mixing are of prime importance. Small volumes are dispensed and the use of instruments that have not been calibrated correctly or have not be calibrated for a long period of time, can lead to high CVs.
- Insufficient mixing of components prior to and during plating should be performed. Use repeater pipettes where possible. Use calibrated or self-calibrating electronic pipettes or dispensers to add and mix reagents.
- Perform a minimum of 6 replicates per point.

Inadequate Cell Culture
- *Inadequate cell growth:* Cells did not exhibit sufficiently high viability. Measure cell viability prior to adding the cells to the master mix. Ensure that the viability is high prior to culture. If using dye exclusion viability, cells should exhibit approx. 85% viability.
- *Reagent deterioration:* Reagents arrived thawed, at room temperature or greater or were not stored correctly as indicated in Section 6 of this manual.
- *Inadequate incubator conditions:* Maintaining a correct humidified gaseous atmosphere in the incubator is essential (See Section 9 (iv) and below).
- *Carbon dioxide concentration is inadequate*. Ensure that the carbon dioxide concentration in the incubator is correct using a Fyrite gas analyzer.
- *Use low oxygen tension*. Using an oxygen concentration of about 5% reduces oxygen toxicity due to free radical production and increases plating efficiency. Check that the incubator oxygen concentration is correct using a Fyrite gas analyzer.
- *Low humidity*. Plates dry out (see below) and cell growth declines.
- *Contamination*: Cells cultured in clear 96-well plates can be viewed under a microscope. If contamination occurs it will usually be seen by the difference in color of the cultures. Contaminated cultures will usually be bright yellow in color and probably cloudy in appearance. Cell cultures that demonstrate high proliferation will also usually appear orange to light orange, but will not be cloudy. If only "spot" contamination occurs, this is usually due to pipette or repeater tips coming in contact with materials other than the reagents. Contamination will usually lead to outlier RLU values.

Culture Plates Drying Out
- Due to the relatively small culture volume (0.1ml), drying out of the culture wells, particularly around the outside of the plate, can be a problem. These are called "edge effects". An incubator with insufficient humidity will cause this problem. To ensure that this does not occur, the incubator water reservoir should be full and the humidity in the chamber checked using a hygrometer.
  If drying out continues, use of a humidity chamber is recommended.

Ordering Information
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MultiCellGro™ was designed and developed by Preferred Cell Systems™ and contains reagents produced by Promega Corporation.

Preferred Cell Systems™