

Assays-in-a-Box™
by
Preferred Cell Systems™

CAMEO™-96

Standardized Methylcellulose Colony-Forming Cell (CFC) Proliferation and Differentiation Assay

Technical Manual
(Version 09-23)

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

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

No part of this instruction manual may be copied, duplicated or used
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1. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. *CAMEO™-96 is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)*
2. *CAMEO™-96 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 included in each literature packet.*

2. Introduction

CAMEO™-96 is an advanced, clonogenic assay that combines the traditional colony-forming cell (CFC) or colony-forming unit (CFU) assay with ATP bioluminescence technology to measure total colony cell proliferation in semi-solid, methylcellulose culture medium.

Although colony-formation is required cell proliferation, the traditional CFC assay does not and can not measure cell proliferation. The CFC assay is based on the fact that the cells produced in a colony must differentiate and mature in order for the colony to be identified as being derived from a stem or progenitor cell. Thus, the CFC assay is a differentiation assay. To measure cell proliferation, an endpoint, other than colony count, must be employed.

All mammalian cells require chemical energy in the form of intracellular adenosine triphosphate (iATP), which is also a biochemical indicator of viability, functionality and cell proliferation. The amount of iATP produced by a cell correlates directly with its functional status. The most sensitive non-radioactive readout to measure cell proliferation is iATP using a luciferin/luciferase bioluminescence signal detection system. This concept is used in a CAMEO™-96 assay to measure total colony cell proliferation, once the number of colonies has been counted.

To detect and measure stem and progenitor cells, growth factors are required to stimulate the cells in culture. The growth factor cocktail used defines which cell populations can be detected. CAMEO™-96 is available to detect individual cell populations based on the growth factor cocktail.

To measure total colony cell proliferation, it is recommended to first calibrate the luminescence plate reader and standardize the assay using the calibration controls and ATP standard included in the CAMEO™-96 kit, respectively. Assay standardization, in turn, allows the establishment of measurement assurance parameters (Section 14) that indicate to the user that the assay is functioning correctly prior to measuring any samples. This also allows the investigator to compare results over time. However, assay standardization has one other important aspect that is used in the CAMEO™-96 assay. It allows the CFC assay to be standardized by correlating the total colony counts with the amount of ATP produced.

Thus, CAMEO™-96 is the only CFC assay that can be quantitatively standardized, thereby allowing results to be directly compared for different samples at different time points.

3. Use and Availability

CAMEO™-96 can be used for virtually any research application that requires the detection and measurement of stem and progenitor cells of the lympho-hematopoietic system.

CAMEO™-96 can be used with the following tissues:

- Bone marrow
- Peripheral blood
- Umbilical cord blood
- Spleen
- Fetal liver
- Embryonic tissue (e.g. yolk sac)

CAMEO™-96 is available for the following species:

- Human
- Non-human primate
- Horse
- Pig
- Sheep
- Dog
- Rat
- Mouse

4. Colony Types Detected Using CAMEO™-96

Cell Type	Cell Population	Growth Factors/Cytokines
	Not specified	No growth factors or cytokines included
Stem Cells	HPP-SP 1	IL-3, IL-6, SCF, TPO, Flt3-L
	HPP-SP 2	EPO, GM-CSF, IL2, IL-3, IL-6, IL-7, SCF, TPO, Flt3-L
	CFC-GEMM 1	EPO, GM-CSF, IL-3, IL-6, SCF, TPO, Flt3-L
	CFC-GEMM 2	EPO, GM-CSF, IL-3, IL-6, SCF, TPO
	CFC-GEM 1	EPO, GM-CSF, IL-3, IL-6, SCF
	CFC-GEM 2	EPO, GM-CSF, IL-3, SCF
Progenitor Cells	BFU-E 1	EPO, IL-3, SCF
	GM-CFC 1	GM-CSF, IL-3, SCF
	Mk-CFC 1	TPO, IL-3, SCF
	T-CFC	IL-2
	B-CFC	IL-7

Abbreviations used. HPP-SP: High Proliferative Potential - Stem and Progenitor. CFC-GEMM: Colony-Forming Unit - Granulocyte, Erythroid, Macrophage, Megakaryocyte. CFC-GEM: Colony-Forming Unit - Granulocyte, Erythroid, Macrophage. BFU-E: Burst-Forming Unit - Erythroid. GM-CFC: Granulocyte-Macrophage Colony-Forming Cell. Mk-CFC: Megakaryocyte - Colony-Forming Cell. CFU-E: Colony-Forming Unit Erythroid. G-CFC: Granulocyte Colony-Forming Cell. M-CFC: Macrophage Colony-Forming Cell. B-CFC: B-lymphocyte Colony-Forming Cell. EPO: Erythropoietin. SCF: Stem Cell Factor. TPO: Thrombopoietin. CSF: Colony Stimulating Factor. IL: Interleukin.

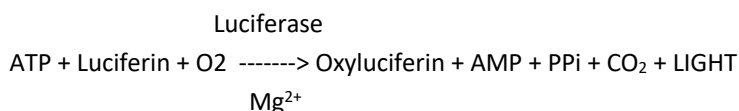
5. The Concept of ATP Bioluminescence Used in CAMEO™-96

CAMEO™-96 uses ATP bioluminescence to measure cell proliferation occurring in hematopoietic colonies. The fundamental concept underlying the assay is the measurement of the cell's chemical energy in the form of intracellular adenosine triphosphate (iATP). If a cell is producing iATP, it is demonstrating cellular and mitochondrial integrity and is therefore viable. When hematopoietic cells are

stimulated to proliferate, in culture with growth factors and/or cytokines, the iATP concentration increases several fold. The iATP concentration produced is directly dependent on:

- The proliferation potential (or primitiveness) of the cell population being detected.
- The concentration of the growth factor(s) and/or cytokine(s) used to stimulate the cells.
- The plated cell concentration.

To measure cell proliferation, a single-step addition of an ATP Enumeration Reagent is dispensed into each culture well, after colonies have been enumerated, and the contents mixed. The plate is incubated at room temperature in the dark for 10 minutes. During this time, the cells are lysed and the released iATP acts as a limiting substrate of a luciferin/luciferase reaction to produce bioluminescence in the form of light according to the following equation:



The bioluminescence emitted is detected and measured in a luminescence plate reader as relative luminescence units (RLU). The assay can be calibrated and standardized, and controls and standards are included for this purpose. Performing an ATP standard curve and controls is highly recommended, since it has the following advantages:

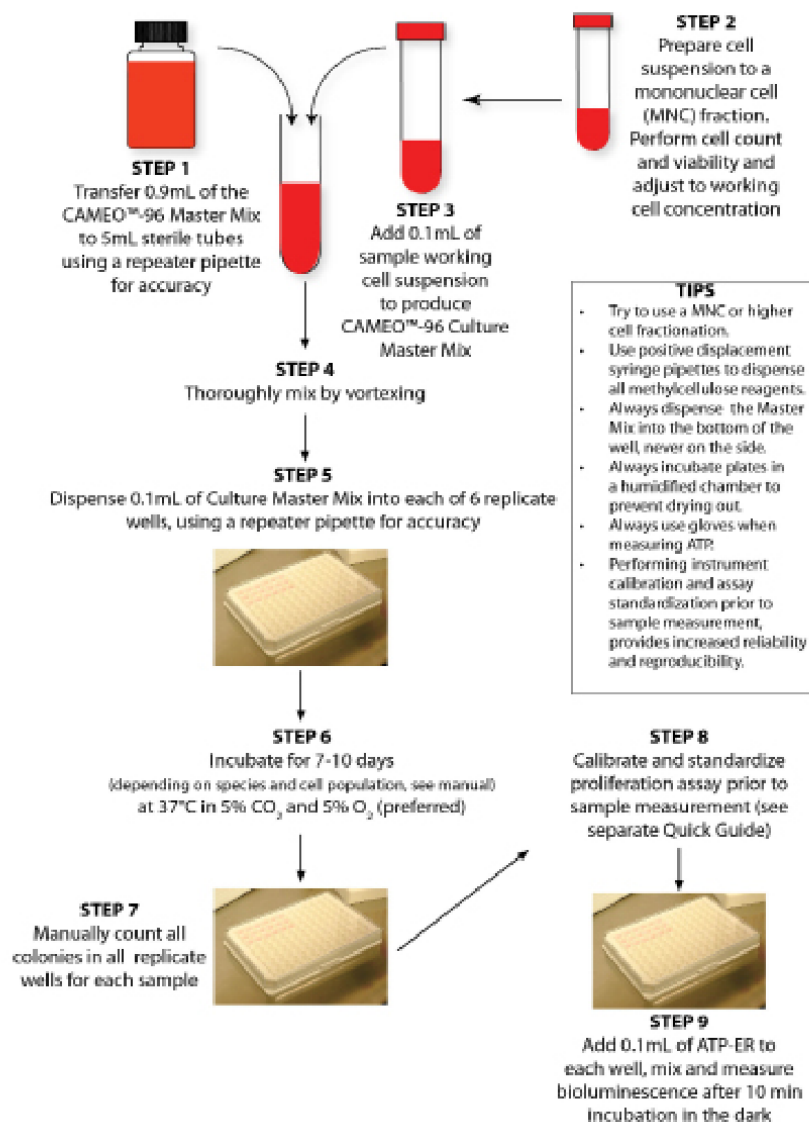
1. The controls calibrate the instrument and also ensure that the reagents are working correctly.
2. The ATP standard curve also ensures that the reagents are working correctly.
3. The ATP standard curve allows the luminometer output in Relative Luminescence Units (RLU) to be converted to standardized ATP concentrations (μM).
4. Performing the ATP standard curve allows results to be compared over time.
5. The results obtained from controls and standard curve should be compared with those provided in Section 12. These are the measurement assurance parameters that allow the investigator to ensure that the assay is working correctly prior to measuring samples. When the values from the controls and ATP standard curve are within the ranges provided in Section 12, the investigator can consider the results trustworthy.
6. The CFC assay is standardized by correlating the total number of colonies enumerated with the calculated ATP concentration derived from the ATP standard curve. Colony counts can then be expressed as standardized ATP concentrations (μM).

The ATP standard curve and controls need only be measured once, on the day samples are to be processed. Do not use previous results from an ATP standard curve and controls performed on a different day. This will cause erroneous results.

The ATP standard curve is used to convert sample RLU results into ATP concentrations by interpolation. This procedure can often be performed automatically by the instrument software. If the software does not allow this, it will be necessary to use third-party software to perform this operation.

6. QuickGuide to CAMEO™-96 (Figure 1)

QuickGuide to CAMEO™-96



QG010-001

7. Kit Contents and Storage Conditions

CAMEO™-96 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	CAMEO™-96 Master Mix for an individual cell population	-20°C until used
2	Medium (IMDM) for dilution of the ATP standard.	-20°C until used
3	ATP standard.	-20°C until used
4	ATP extra high, high and low controls.	-20°C until used
5	ATP Enumeration Reagent (ATP-ER)*	-20°C in the dark until used
6	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
7	White with clear growth surface, sterile, individually wrapped, 96-well plate for cell culture	Can be kept with other kit components
8	White with clear growth surface, non-sterile 96-well plate(s) for ATP standard curve determination.	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.

*The ATP-ER should not be thawed until needed and can be refrozen 11 cycles without significant loss of sensitivity. It can be kept at 2-8°C for 48h once thawed and is stable for 20 weeks when stored at -20°C. This reagent is light sensitive. Keep in the dark.

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 luminescence (ATP-ER) 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.

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

Equipment and Supplies

1. Laminar Flow Biohood
2. Plate luminometer (e.g. Berthold LB962 CentroLIA/pc; Molecular Devices, SpectraMaxL)
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% CO₂ (minimum requirement) and 5% O₂ (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. LymphoPrep).
4. 7-AAD, propidium iodide or trypan blue for viability assay.
5. LIVEGlo™ metabolic viability assay (Preferred Cell Systems™)

9. The CAMEO™-96 Protocol

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

Performing CAMEO™-96 is a 4-step process.

Step 1 – Cell preparation.

Step 2 – CAMEO™-96 cell culture master mix preparation, plating and incubation in the 96-well plate.

Step 3 - Colony enumeration

Step 4 – Luminescence measurement. It is recommended to perform an ATP dose response prior to sample luminescence measurements with conversion of RLUs to µM ATP.

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

STEP 1 – CELL PREPARATION

CAMEO™-96 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. Mouse cells do not usually need to be fractionated.
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.
3. **NOTE:** Do not base the working concentration on the TNC count or the number of viable cells as this will give erroneous results.
4. 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.
5. 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 CAMEO™-96 Culture Master Mix prepared.

TABLE 1

Recommended Cell Doses for Different Species, Cell Types, Cell Preparations and Cell States for CAMEO™-96

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

Species	Cell Type	Cell Preparation	Cell State	Working Cell Concentration Required (100 x Final Cells/Well)	Final Cell Dose / Well
	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
Non-human primate	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
Horse, Pig, Sheep	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
Dog	Bone marrow	MNC	Fresh/Frozen	0.5-0.75 x 10 ⁶	5,000-7,500
Rat	Bone Marrow	MNC	Fresh	0.5-0.75 x 10 ⁶	5,000-7,500
	Peripheral blood	MNC	Fresh	0.5-0.75 x 10 ⁶	5,000-7,500
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. CAMEO™-96 CELL CULTURE

- CAMEO™-96 Master Mixes are complete and ready-to-use.
 - Perform all procedures under a laminar flow, bio-hazard hood.
 - Wear protective clothing, including gloves for all operations.
1. Remove the CAMEO™-96 Master Mix that came with the assay kit 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. When thawed, mix the contents of the bottle by gentle inversion several times and leave to stand in the cold. Methylcellulose is more easily dispensed when cold.
 3. Label a sufficient number of 5mL tubes for the number of samples to be tested.
 4. The bottle of CAMEO™-96 methylcellulose master mix, included with the assay kit, contains a sufficient amount to perform 96 assays. It is recommended, however, that each sample be performed in a minimum of 6 replicate wells, each of which contains 0.1mL.
 5. To perform a 6-replicate well assay, use a calibrated, positive displacement syringe repeater pipette, to accurately dispense 0.9mL of the CAMEO™-96 Master Mix into each sample tube. **DO NOT** use a syringe and needle to dispense methylcellulose. It is extremely inaccurate and will lead to pipetting errors.
 6. 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.

7. Dispense 0.1mL of cells into each tube containing 0.81mL of the CAMEO™-96 Master Mix. The total volume will then be 1.0mL.
8. Mix the contents on a vortex mixer. Try not cause bubbles.
9. Remove the sterile, individually wrapped, 96-well plate from the assay kit box.
10. Using a calibrated, positive displacement, syringe repeater pipette, removed between 0.8-0.9mL of the culture master mix and dispense 0.1mL into the bottom and center of each replicate well of the white, 96-well plate.
11. Replace the lid after all of the samples have been plated and transfer the 96-well plate to a humidified container (see Section 10).
12. Transfer the humidified container to the incubator. The incubator should be fully humidified at 37°C and gassed with 5% CO₂ and, if possible, 5% O₂. This reduced oxygen tension improves plating efficiency by reducing oxygen toxicity caused by the production of free radicals.
13. Incubate the cells for the time shown in Table 2. The incubation times depend on the cell population being detected and the source of cells. It is good practice to monitor colony growth so that colonies do not grow into each other and make colony enumeration difficult.

TABLE 2

Species	Cell Type	Incubation Period (days)
Human	Bone marrow, normal and mobilized peripheral blood, umbilical cord blood	5-8
Non-human primate	Bone marrow, peripheral blood	5-8
Horse, Pig, Sheep	Bone marrow, peripheral blood	4-7
Dog, Rat	Bone marrow	4-5
Mouse	All	4-5

STEP 3 - COUNTING COLONIES

It is important to emphasize that the number of colonies is determined by the number of colony-forming cells present in the cell suspension. Therefore, the number of colonies that can be produced is “set in stone” very early in the culture. Increasing the time period does not increase the number of colonies; it only increases the size of the colonies and the state of differentiation and maturation of the cells that identify the colonies.

A colony is usually defined as an aggregate of 8 or more cells. This represents a minimum of 3 sequential divisions performed by the original colony-forming cell. There are three ways of counting colonies grown in methylcellulose.

1. *Count each individual center within a colony.* Colonies are rarely spherical with a single dark center indicating the center of proliferation. Most colonies are irregular in shape and contain many dark centers. Each dark center represents a single

“proliferation unit” (PU) in which the cells are actually proliferating. With time, these PUs grow together to form an irregular-shaped colony. Counting the individual PUs within a colony provides an indication of colony proliferation. However, this should not be misunderstood as a means to quantify cell proliferation. Although colony formation requires cell proliferation, the colony-forming assays is not a cell proliferation assay and does not measure cell proliferation.

2. *Count all PUs within a colony as 1 colony.* This is the normal manner in which colonies are counted. No consideration is made regarding whether the colony is spherical or irregular in shape. If it is a separate entity from another colony, it is counted as a single colony.
3. *Use a camera fitted to a microscope or an electronic colony counter* to image the colonies and save the image for later enumeration either to count the colonies manually or using image analysis software. This type of colony enumeration can be problematic since the walls of the well usually cast shadows around the outer rim of the well. In addition, the software may have to be “taught” how to count colonies. If this type of colony enumeration is to be performed, it is suggested to compare manual counting with electronic counting to ensure that the correct number of colonies are counted in both instances.

Important Considerations When Evaluating Colony-Forming Cell Results

1. The number of colonies counted provides no quantitative information on cell proliferation. Cell proliferation is inferred, since without cell proliferation, no colonies would be obtained.
2. The CFC/CFU assay detects cell differentiation since the colonies can only be identified by the ability of the cells to differentiate and mature.
3. Monitor the growth of colonies. Colonies should be counted at a time when they can be individually identified. Longer incubation times only increases the size of the colonies, which then grow together making it very difficult to identify individual colonies.
4. In general, the size of the colony is indicative of the primitiveness of the cell that produced the colony; that is, the larger the colony, the more primitive the cell.
5. Colony evaluation requires considerable time to learn. If performing a colony assay for the first time, ask a colleague or contact Preferred Cell Systems™ for advice.
- 6.

STEP 4 – BIOLUMINESCENCE MEASUREMENT

Please note the following important points:

1. *FOR ALL OF THE FOLLOWING STEPS, WEAR LABORATORY GLOVES. ATP is present on the skin and can cause erroneous results*
2. *PLEASE REFER TO SECTION 12 ON HOW TO SETUP THE PLATE LUMINOMETER. The instrument should be setup and prepared for use prior to any of the following steps being performed.*
3. *Please refer to Section 11 for recommendations and tips prior to starting this part of the procedure. In particular, please refer to Section 11 for important information on mixing components.*
4. *Remove the ATP Enumeration Reagent (ATP-ER) from the freezer and thaw at room temperature or in cold running water prior to analysis. Do not thaw the ATP-ER in a water bath or 37°C incubator.*
5. *If the assay is to be calibrated and standardized, remove the ATP standard, controls and reagents from the freezer and thaw to room temperature or in cold running water prior to analysis.*
6. *ATP standard curves performed on previous days or for previous experiments or studies must not be used since the ATP-ER intensity changes with time and lot number.*

7. Use the unwrapped, non-sterile, 96-well plate provided with the kit to perform the ATP standard dose response curve.

A. Calibrating and Standardizing the Assay

It is highly recommended to standardize the assay. Use the non-sterile, 96-well white plate provided with the assay kit for this purpose.

CAMEO™-96 includes the following to calibrate and standardize the ATP bioluminescence part of the assay to measure cell proliferation occurring in the colonies.

- IMDM medium: Used only for ATP standard serial dilution.
- ATP Standard at 10 μ M. Serially diluted to produce the ATP standard curve.
- Low ATP Calibration Control. Used for normal and extra high cell proliferation.
- High ATP Calibration Control. Used for normal cell proliferation.
- Extra High ATP Calibration Control. Used for extra high cell proliferation.

B. Deciding Which Calibration Controls to Use and ATP Standard Curve Range

PROTOCOL 1: At the time of colony enumeration, if the total colony count is less than 60 colonies, use the low and high calibration controls and perform an ATP standard curve from 0.01 μ M to 1 μ M. **See Page 24.**

PROTOCOL 2: At the time of colony enumeration, if the total colony count is greater than 60 colonies, use the low and extra high calibration controls and perform an ATP standard curve from 0.03 μ M to 3 μ M. **See Page 25.**

It is important that the sample ATP values are within the limits of the ATP standard curve, otherwise the interpolation of Relative Luminescence Unit (RLU) values from the luminescence plate reader into ATP concentrations will not be accurate. In some cases, cell proliferation could be greater than 3 μ M ATP. If ATP values from the samples are greater than 3 μ M, it is recommended to dilute the sample with additional medium so that the values are within the ATP standard curve range. This may require removing an aliquot from the replicate wells, transferring the aliquot to a new wells and diluting each aliquot with additional medium. The replicate wells would then be reread.

C. Sample Measurement

It is extremely important that when the ATP-ER is added to the methylcellulose cultures, the contents are mixed correctly. Follow the directions in Section 11 for mixing, but take care not to produce too many bubbles.

The addition of ATP-ER is performed in the same manner as the ATP Standard Curve.

1. If possible, place the sample plate(s) in a humidified incubator set at 22-23°C gassed with 5% CO₂ for 30min. Otherwise, allow the plate to come to room temperature for 30 min.
2. If only part of the 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 from the kit box, remove the backing and layer it 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 samples. (See Section 11, Adhesive Plate Covering Film).
3. Using a multichannel pipette (8- or 12-channel depending on the plate configuration), add 0.1mL of ATP-ER to each well of the first column (A1-H1) or row (A1-12). Mix the contents as described in Section 11.

4. Repeat this procedure for each column or row using new tips.
5. When ATP-ER has been added to all wells, replace the plastic cover and incubate for 10 min at room temperature in the dark to lyse the cells and stabilize the luminescence signal. Incubate the plate in the reader for the last 2 min to stabilize the plate.
6. Unused ATP-ER may be returned to the bottle and refrozen. See section 11 for ATP reagent storage conditions and stability.

D. Luminescence plate reader with automatic dispenser

The user may have a plate luminometer that allows reagents to be dispensed automatically, directly into the well. Preferred Cell Systems™ does not recommend using the automatic dispensers, since the contents of the well are not mixed sufficiently using this method.

E. Using a liquid handler

Although methylcellulose cannot be dispensed using a liquid handler, dispensing the ATP-ER and mixing the reagents can be performed using a liquid handler. If you intend to perform this part of the CAMEO™-96 procedure using a liquid handler, please contact Preferred Cell Systems™ for information on setting up the instrument. Extra ATP-ER is required when using a liquid handler.

10. Recommendations and Tips Prior To Using CAMEO™-96.

(i) Cell Suspension

- a) The preferred cell suspension is a mononuclear cell suspension (MNC).
- b) Extraneous ATP, red blood cells (which have high concentrations of ATP) and hemoglobin interfere with the ATP analysis. The cell suspension must have a hematocrit of 10% or less.
- c) If cells have been treated prior to cell culture, higher cell concentrations than those shown in Table 1 may be required.

(ii) Number of Replicates Performed

It is recommended to perform 6 replicates/sample to provide good statistical relevance. Although fewer replicates can be used, please remember that although this may save components in the short term, it 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) Plate Configuration

For 6 replicates/sample, culture should be plated in rows across the plate.

(iv) 96-Well Plates Provided

The reagents have been optimized to work with the 96-well plate(s) provided in the CAMEO™-96 kit. Please do not replace the plates included with the kit with those of another manufacturer. Cell growth and bioluminescence 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

A humidity chamber is recommended due to the small sample volume. 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”. This

phenomenon is observed when ATP values in the outside wells are lower than those in the inside wells. A humidity chamber can be assembled using plastic lunch boxes or other plasticware available from a supermarket or discount stores. Holes must 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.

(vi) Incubation Times

The incubation time may vary depending on cell type and species. Assay sensitivity might improve with longer incubation times, but usually at the expense of higher variability between wells. Once an optimal incubation time has been found, the same time period should be maintained for all future experiments so that results can be directly compared.

11. Recommendations and Tips Prior To Measuring Bioluminescence

- *Always wear laboratory (e.g. latex) gloves during this operation to avoid ATP contamination from skin.*
- *DO NOT wipe the pipette tip with tissue etc as this will wick the reagent from the tip and cause an erroneous ATP standard curve and false sample results.*
- *Always change pipette tips after each use.*
- *Each day bioluminescence is measured, a standard curve MUST be performed. The ATP-ER decays with time. A new ATP standard curve must be performed to ensure accurate conversion of the RLU values to ATP concentrations so that results can be compared.*
- *CAMEO™-96 includes solid white plates for both cell culture and the ATP standard curve and controls. Do not use different plates for the assay. Doing so will result in inaccurate results and invalidation of the assay kit warranty. Extra plates can be purchased from Preferred Cell Systems™.*

Bioluminescence Assay Kit Components

- Prior to measuring bioluminescence, remove the ATP standard, 1 set of ATP controls and the ATP-Enumeration Reagent (ATP-ER) from the freezer and thaw at room temperature or at 22 - 23°C.
- Sufficient ATP standard, controls and ATP-ER are supplied to perform 2 standard curves and controls/assay kit. Additional ATP standards and controls can be obtained from Preferred Cell Systems™.
- If thawing more than one bottle of ATP-ER for analysis, mix the contents of the bottles together before dispensing into reagent reservoir.
- ATP-ER can be refrozen up to 11 cycles without significant loss of sensitivity. Thawed ATP-ER can be kept at 2-8°C, in the dark, for 48h or is stable at -20°C for 20 weeks.

Reconstitution of Lyophilized Monitoring Reagent (if included)

- Thaw the ATP Enumeration Reagent Buffer at room temperature, in cold running water, or at 2-8°C overnight
- Do not use any form of heat to thaw this reagent.
- Allow the lyophilized ATP-ER substrate (brown glass bottle) to come to room temperature.
- Remove the closures from both bottles.
- Carefully pour the entire contents of the buffer bottle into the lyophilized ATP-ER substrate bottle. Swirl gently or invert slowly to mix. Do not shake.

- Allow the ATP-ER mix to reconstitute for 10 minutes at room temperature.
- Reconstituted ATP-ER is stable for 8 hours at room temperature, 48 hours at 2-8°C, or 20 weeks at -20°C.
- ATP-ER can be refrozen up to 11 cycles without significant loss of sensitivity.

Volumes of Luminescence Kit Components Required

- Each vial of ATP standard contains enough volume to perform one or two ATP standard dose responses.
- The amount of ATP-ER added to each well is 0.10mL. Therefore:
Total amount of ATP-ER (μl) required = 0.1mL x (number of wells used + 24 (background, ATP dose response wells and ATP controls)).

ATP Standard Curve

Depending on the size of the kit purchased, non-sterile, 96-well plates have been included to perform an ATP standard curve prior to processing the sample cultures. Performing an ATP standard curve and controls on each day samples are processed is an essential part of the assay because it has 4 functions:

- It tests whether the instrument is working properly and calibrates it.
- It ensures that the reagents are working correctly.
- It calibrates and standardizes the assay and allows the assay system to be validated, if required.
- It allows the output of the plate luminometer, in relative luminescence units (RLU), to be converted to ATP concentrations, thereby standardizing the procedure so that intra- and inter-laboratory experiments can be compared.

Adhesive Plate Covering Film

To help keep the plate(s) sterile, adhesive, air permeable, sterile films are provided so that the part of the plate that is not being used can be covered and kept sterile until required. If using the adhesive film provided, the plate cover should be removed in a laminar air-flow hood and replaced with the film to ensure sterility.

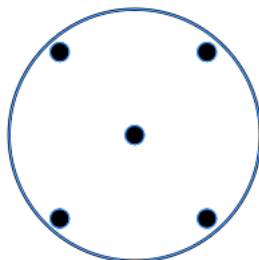
Mixing the Contents of the 96-well Plate

Mixing the contents of the wells after adding ATP-ER is one of the most important procedures of the assay. It is even more important for CAMEO™-96 since the ATP-ER reagents, which contains a lysis buffer, must be mixed with the cells to ensure proper release of the intracellular ATP. If insufficient mixing occurs, the ATP concentrations measured will be too low. If too much mixing is performed, ATPases will be released, which will also result in a low ATP concentration.

It is recommended that the addition of ATP-ER is performed using a multi-channel pipette to achieve consistency and reduce variability. Addition of the reagent and mixing should be performed in the following manner:

1. Take up the required amount of reagent and add it to the well without inserting the tip into the well contents.
2. Starting from the center of the well, aspirate and dispense the contents twice without removing the pipette tip from the contents of the well.
3. Move the pipette tip to one corner of the well. Aspirate and dispense the contents twice without removing the tip from the contents of the well.
4. Repeat this operation as shown in Figure 4 for each corner of the well.
5. Try not to cause excessive bubbles in the culture and DO NOT over mix since this can result in drastically reduced luminescence values.
6. This procedure effectively and optimally mixes the contents well.

Figure 2. Positions of pipette tip for mixing the well contents



12. Luminescence Plate Reader Setup and Conversion of RLU Values to ATP Values Using the ATP Standard Curve

It is very important that the luminescence or multimode plate reader is setup correctly, otherwise false results could occur. Preferred Cell Systems™ has provided a separate document to help the investigator setup their instrument and perform the calculations in order to convert Relative Luminescence Units (RLU) into ATP concentrations using the ATP standard curve. It is strongly recommended that the investigator consult this document prior to performing any ATP bioluminescence assay. This document can be downloaded with this manual.

13. Standardizing the CAMEO™-96 CFC Assay

To standardize colony counts, it is necessary to perform the ATP standard curve.

1. Ensure that the luminescence or multimode plate reader is setup correctly to measure bioluminescence. See Section 12 and consult the separate document that can be downloaded with this technical manual.
2. Perform CAMEO™-96 calibration and standardization as described in Step 4 of Section 9, The CAMEO™-96 Protocol.
3. Convert all RLU values into ATP concentrations (μM) as described in Section 12 and the separate document that can be downloaded with this technical manual.
4. Plot total colony counts on the Y-axis and ATP concentrations on the X-axis. The ATP concentration will increase with the increased total colony counts; i.e. there should be a direct correlation between these two parameters allowing a linear regression to be performed.
5. Interpolate the number of colonies into standardized ATP concentrations (μM).
6. CFC colony counts can now be reported as standardized ATP concentration equivalents.

14. CAMEO™-96 Assay Measurement Assurance and Validation Parameters

If CAMEO™-96 has been calibrated and standardized, ATP bioluminescence technology allows the User's results to be compared to the measurement assurance parameters shown in Table 3. For each control, ATP standard dose and the log-log linear regression curve fit parameters provided, the User's results must lie within the ranges provided. If this is the case, then the following are applicable:

1. The user has performed and passed the integrated proficiency test.
2. The instrument and assay readout reagents are working correctly.
3. The user can continue to process and measure samples.
4. The user can trust results of the assay.

IMPORTANT. If the User's results DO NOT comply with those in the table, DO NOT measure the samples. Perform a repeat of the controls and ATP standard curve. If the results still do not comply with those in the Table 3, contact Preferred Cell Systems for help.

Table 3: ATP Controls and Standard Curve Measurement Assurance Parameters

Expected Parameter	Observed Value	Mean \pm 15% ^(*)	Min / Max	%CV (where applicable)
0.01 μ M ATP	0.0099 μ M ATP	0.00972 - 0.0114	0.009 - 0.01	2.34%
0.03 μ M ATP	0.029 μ M ATP	0.285 - 0.0336	0.028 - 0.03	1.67%
0.05 μ M ATP	0.0497 μ M ATP	0.0486 - 0.0571	0.048 - 0.051	1.57%
0.01 μ M ATP	0.1026 μ M ATP	0.1003 - 0.118	0.099 - 0.107	1.96%
0.3 μ M ATP	0.317 μ M ATP	0.310 - 0.364	0.302 - 0.325	1.51%
0.5 μ M ATP	0.5023 μ M ATP	0.491 - 0.578	0.491 - 0.515	1.19%
1.0 μ M ATP	1.048 μ M ATP	1.024 - 1.205	0.977 - 1.117	3.7%
3.0 μ M ATP	2.722 μ M ATP	2.661 - 3.130	2.633 - 2.934	2.09%
Intercept	6.533	6.386 - 7.513	5.86 - 6.7	1.84%
Slope	0.9656	0.944 - 1.110	0.947 - 0.988	1.21%
r ² goodness of fit)	0.9993	-	0.998 - 1	0.05%
R (correlation coefficient)	1	-	0.999 - 1	0.02%
Low control, (0.05 μ M ATP)	0.0487 μ M ATP	0.0476 - 0.0560	0.042 - 0.063	6.79%
High control 0.7 μ M ATP	0.725	0.710 - 0.836	0.655 - 0.904	5.35%
Extra high control (1.75 μ M ATP)	1.756	1.717 - 2.019	1.61 - 2.198	5.24%
The above values represent results from 71 control and ATP standard curve studies performed from January 2016 to June 2018				

(*) 15% represents the acceptable range of values for FDA Bioanalytical Method Validation Guidelines

Samples Values:

- Lowest ATP value indicating unsustainable cell proliferation for hematopoietic cells: $\sim 0.04 \mu\text{M}$

Please note that human B-cells, especially cryopreserved cells, may exhibit very low ATP values. It is important to compare the stimulated B-cells with their background (no growth factors added) to determine B-cell activity.

- ATP value below which cells are not metabolically viable: $\sim 0.01 \mu\text{M}$.
- All samples values must lie on the ATP standard curve for accurate RLU to ATP conversion. If ATP values are greater than $3 \mu\text{M}$, the replicate samples should be diluted with IMDM and re-measured. Take the dilution value into account when estimating the true ATP concentration. Alternatively, repeat the culture and ATP measurement using fewer cells.

Assay Validation Parameters

HALO® exhibits the following validation parameters:

- Assay ATP linearity \Rightarrow 4 logs
- Assay ATP sensitivity: $\sim 0.001 \mu\text{M}$
- Assay cell sensitivity: 20-25 cells/well (depending on cell type and purity)
- Accuracy (% correct outcomes): $\sim 95\%$
- Sensitivity and specificity detected by Receiver Operator Characteristics (ROC) curve fit and detected as area under the curve (AUC): 0.73 - 0.752 (lowest possible value, 0.5; highest possible value, 1).
- Precision (Reliability and Reproducibility) $\leq 15\%$. At lower limit of quantification (LLOQ): 20%
- Robustness (intra- and inter-laboratory comparison): $\sim 95\%$.

15. Troubleshooting

If Calibration and Standardization Results Do Not Conform to Measurement Assurance Parameters (Section 12)

If the investigator has elected to calibrate and standardize the assay using the ATP controls and standard supplied with the kit, the results should be within the ranges provided in Section 12. If the values obtained conform to the measurement assurance parameters, the investigator can continue the assay to process and measure the samples with the assurance that the results can be trusted.

If any of the values obtained during calibration and standardization do not conform or are not within the ranges provided in Section 12, the user should repeat the calibration and standardization. Often discrepancies occur due to pipetting and/or dilution errors. Accurate and careful dilution of the ATP stock solution is important. It is also possible that if pipettes have not been professionally calibrated, errors can occur. These will also be picked up during this phase of the assay. Finally, if the ATP-ER has not been handled or stored correctly, it will decay, leading to erroneous results. Please contact Preferred Cell Systems™ to obtain new ATP-ER.

High Coefficients of Variation (%CV)

Coefficients of variation (%CV) should be $\leq 15\%$. The percent coefficient of variation is calculated as standard deviation/mean $\times 100$. High %CVs are usually an indication of incorrect dilutions or pipetting error. Although outliers can be obtained, these being observed for the more primitive stem cells than for the more mature proliferating cells, large variations between replicates should not be obtained. Please consider the following:

- Accurate reagent dispensing and mixing are of prime importance. Since the volumes dispensed are small it is imperative to use instruments that have been properly calibrated to avoid pipetting error.
- Insufficient mixing of components prior to cell plating and insufficient mixing during the addition of luminescence reagents to cultures in the 96-well plate can also lead to high CVs. Use repeater pipettes. Use calibrated or self-calibrating electronic pipettes or dispensers to add and mix the luminescence reagents.
- If the luminometer requires determining the “gain” empirically, it is possible that this parameter has not been optimally set and will result in an incorrect signal to noise ratio. Once the optimal “gain” has been set for the instrument, it should not be changed.

Low RLU Values

Performing an ATP dose response prior to sample measurement can help detect problems prior to sample measurement. If low RLU values occur, this could be due to the following reasons.

- *Reagent decay:* The ATP-ER decays with time, even when frozen. This can lead to low bioluminescence. Once thawed the reagent can be refrozen up to 11 cycles without significant loss of sensitivity. Do not use the reagent after expiry date has elapsed. As a rule of thumb, the RLU value for the lowest ATP standard should be 10 times greater than that of the background value.
- *Inadequate cell growth:* Cells did not exhibit sufficiently high viability. Measure cell viability prior to using cells. A cell viability lower than 85% should not be used. Viabilities lower than 85% can be an indication that the sample was not processed in a time-sensitive manner or that the processing procedures were not standardized and controlled.
- *Reagent deterioration:* Reagents arrived thawed, at room temperature or greater or were not stored correctly.
- *Inadequate incubator conditions:* Maintaining a correct humidified gaseous atmosphere in the incubator is essential (See Culture Plate Drying Out).
- *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 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 96-well plates cannot be viewed under a microscope. If contamination occurs it will usually be seen by the difference in color of the cultures, if the medium contains an indicator, e.g. phenol red. Contaminated cultures will usually be bright yellow in color and probably cloudy in appearance. Cell cultures that demonstrate high proliferation will 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.

Luminescence Reagent Mixing.

The luminescence reagent has to be added and thoroughly mixed with the culture components. The ATP-ER lyses the cells and releases intracellular ATP. If mixing is not adequate, only a proportion of the cells will be lysed and the RLU values will be low. Conversely, too much mixing can lead to ATP degradation and low luminescence readings.

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. Please refer to Section 9 (v) for instructions on how to build a humidity chamber.

Ordering Information

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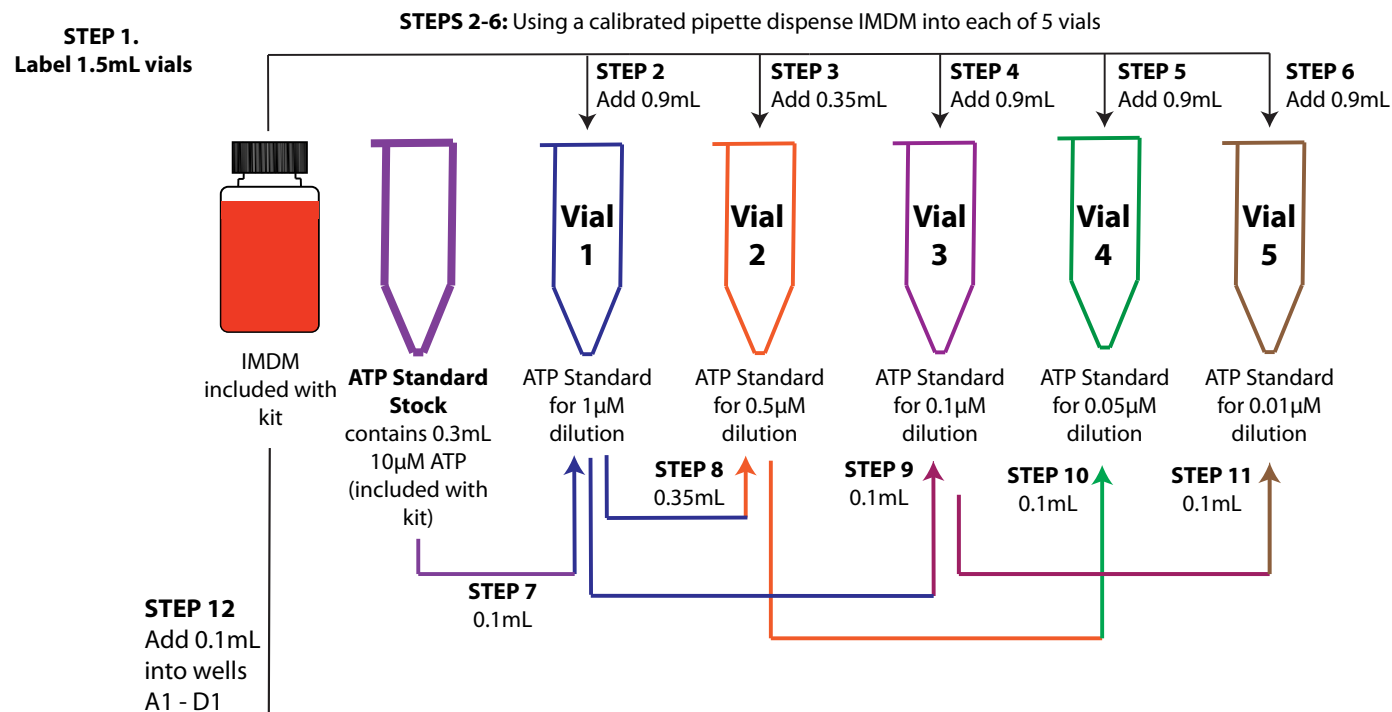
CAMEO™-96 was designed and developed by HemoGenix®, Inc.

Patents pertaining to CAMEO™-96: 7,354,729, 7,354,730, 7,666,615, 7,709,258, 7,883,861, 7,700,354.

Preferred Cell Systems™

Calibration and Standardization Protocol of an ATP Bioluminescence Assay

PROTOCOL 1: ATP Standard Curve from 0.01 μ M to 1 μ M For Samples with Known or Expected Normal Cell Proliferation



Reagents & Materials Needed

- 1.5mL vials or similar (not included)
- IMDM (included)
- ATP Standard (included)
- ATP Controls (included)
- Non-sterile, 96-well plate (included)

TIPS

- > Use calibrated pipettes throughout.
- > Vortex thoroughly between each dilution.
- > Change tips between each dilution.
- > Follow color coding.

Change pipette tips for each well

	1	2	3	4	5	6	7	8	9	10	11	12
A	○	4	2	LC	○	○	○	○	○	○	○	○
B	○	4	2	LC	○	○	○	○	○	○	○	○
C	○	4	2	LC	○	○	○	○	○	○	○	○
D	○	4	2	LC	○	○	○	○	○	○	○	○
E	5	3	1	HC	○	○	○	○	○	○	○	○
F	5	3	1	HC	○	○	○	○	○	○	○	○
G	5	3	1	HC	○	○	○	○	○	○	○	○
H	5	3	1	HC	○	○	○	○	○	○	○	○



STEP 18: LOW CONTROL (LC, included with kit)

Vortex and lightly centrifuge to remove liquid from cap
Add 0.1mL from low control to wells A4-D4



STEP 19: HIGH CONTROL (HC, included with kit)

Vortex and lightly centrifuge to remove liquid from cap
Add 0.1mL from high control to wells E4-H4

Follow Color Coding

STEP 13: Add 0.1ml from **Vial 5** into wells E1-H1

STEP 14: Add 0.1mL from **Vial 4** into wells A2-D2

STEP 15: Add 0.1mL from **Vial 3** into wells E2-H2

STEP 16: Add 0.1mL from **Vial 2** into wells A3-D3

STEP 17: Add 0.1mL from **Vial 1** into wells E3-H3

STEP 20: Add ATP-ER to reservoir and using a multichannel pipette, dispense 0.1mL into each replicate well

STEP 21: Mix replicate wells as described for Figure 2 in this manual. Change tips for each new addition of ATP-ER

STEP 22: Transfer 96-well plate to luminescence plate reader

STEP 23: Incubate in the dark for 2 minutes and measure luminescence

Calibration and Standardization Protocol of an ATP Bioluminescence Assay

PROTOCOL 2: ATP Standard Curve from 0.03 μ M - 3 μ M For Samples with Known or Expected High Cell Proliferation

