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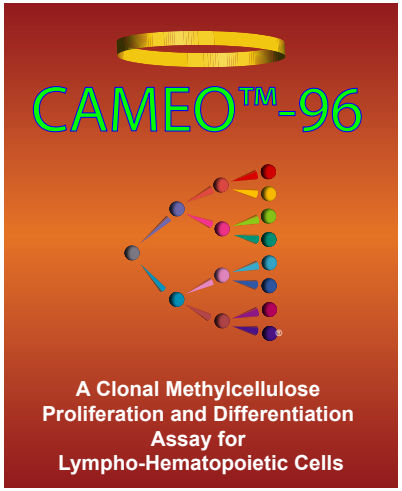

CAMEO™-96

A Methylcellulose, Clonal Proliferation and Differentiation Assay for Hematopoietic Cell and Standardization Assay for the Colony-Forming Cell / Unit (CFC/CFU) Assay

Instruction Manual

(Version 5-2013)

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

HemoGenix®, Inc

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1. Introduction

CAMEO™-96 (originally called HALO®-96 MeC) was the first non-subjective, instrument-based colony-forming cell (CFC) assay ever developed. Completely rebuilt from the ground up, CAMEO™-96 is an advanced assay that combines the "classic" CFC differentiation assay with bioluminomics™ technology to measure cell proliferation in colonies grown in semi-solid, methylcellulose culture medium.

The CFC assay is a differentiation assay because the hematopoietic cells of colonies that grow in methylcellulose must first differentiate and mature in order to identify the type of colony being produced when viewed under a dissection or inverted microscope. Although the proliferation of the cells producing the colony occurs prior to the initiation of the differentiation process, the CFC assay cannot be used to directly measure cell proliferation, since proliferation and differentiation require different readouts to be measured.

CAMEO™-96 is a clonogenic hematopoietic assay platform in which stem, progenitor or precursor cells can be grown as colonies in methylcellulose. The colonies are counted under an inverted microscope in a similar manner to the CFC assay. However, after counting (and if necessary differentiating the colonies into colony types), the proliferation of all cells in the culture is measured by releasing the intracellular ATP (iATP) and measuring its concentration using a luciferin/luciferase bioluminescence signal detection system. Since iATP is a biochemical marker that increases proportionately in response to (a) the cell population being measured, (b) the type and concentration of the growth factor/cytokines used to stimulate the cells and (c) the cell dose, it is a ideal marker for cell proliferation.

CAMEO™-96 is one of a family of assays developed by HemoGenix® that uses bioluminomics™ technology. Bioluminomics™ incorporates the calibration, standardization and, if necessary, the validation of the assay being used. Since the proliferation process of the cultured cells is a standardized measurement, CAMEO™-96 can be used to "back-calibrate" and standardize the subjective CFC/CFU stage of the assay so that total colony numbers can be expressed as ATP concentration equivalents. Bioluminomics™ ensures that the results obtained are reliable and reproducible, not only within one laboratory, but between laboratories.

CAMEO™-96 is a research assay since it can be used to measure both proliferation and differentiation of the same cells cultured under exactly the same conditions. It is not recommended to use CAMEO™-96 in place of a colony-forming unit (CFU) for hematopoietic cellular therapy applications. However, CAMEO™-96 can be used to as a stepping stone to more advanced cellular therapy assays, due to the correlation between the CFC/CFU assay and bioluminomics™ technology (see Section 11). For CFC/CFU equivalent assays, HemoGenix® has developed HALO®-96 PCA^{EQ}.

2. Intended Use

CAMEO™-96 is for basic research applications where the proliferation and/or differentiation of cells cultured in methylcellulose medium under clonogenic conditions is required. CAMEO™-96 can also be used to convert total colony counts into equivalent ATP concentrations (µM)

CAMEO™-96 can be used to test hematopoietic stem, progenitor and precursor cells from:

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

derived from the following species:

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

CAMEO™-96 is available for multiple, single cell populations and can be used to detect and measure purified populations.

CAMEO™-96 is also available without growth factors/cytokines. This provides flexibility and allows the investigator to use their own growth factors and/or cytokines.

IMPORTANT:
CAMEO™-96 is for research use only and has not been approved for clinical diagnostic use.

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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 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 8 (vii) for instructions on how to build a humidity chamber.

3. The Concept and Principle of Bioluminomics™ Assays

CAMEO™-96 is a bioluminomics™ assay that also includes manual, subjective counting of colonies. The fundamental concept underlying bioluminomics™ is the measurement of the cell's chemical energy in the form of intracellular ATP (iATP). If a cell is producing iATP, it is demonstrating cellular and mitochondrial integrity and is therefore viable. When cells are stimulated to proliferate, which is the case when hematopoietic cells are cultured in the presence of growth factor 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 types and concentration of the growth factor(s) and/or cytokine(s) used to stimulate the cells.
- The plated cell concentration.

Lympho-hematopoietic cells are incubated in the CAMEO™-96 Master Mix(s) provided with this kit for a specific period of time. When the culture period has elapsed, a single ATP Enumeration Reagent is dispensed into each culture well 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:

$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{LIGHT}$$

The bioluminescence emitted is detected and measured in a plate luminometer as relative luminescence units (RLU). To calibrate and standardize the assay, an ATP standard and high and low controls are provided. **Performing the ATP standard curve and controls is the most important part of CAMEO™-96. Failing to perform the ATP standard curve and controls can invalidate the results.** The ATP standard curve and controls must be performed prior to processing the samples for the following reasons:

1. Performing an ATP standard curve calibrates and standardizes the assay.
2. The controls ensure 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.

The ATP standard curve and controls are measured once, on the day samples are to be processed. DO NOT use results from an ATP standard curve or controls performed on one day for samples processed on another day.

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 luminometer software. If the software does not allow this, it will be necessary to use third-party software to perform this operation.

4. Overview of the CAMEO™-96 Procedure

Using CAMEO™-96 is a 4 step process.

Step 1 – Cell Preparation
Cells are not provided with the CAMEO™-96 assay kits. Cells are prepared either by using a user-defined, pre-validated protocol to obtain a single cell suspension or procedures that are suggested in this manual. A dye exclusion viability and/or metabolic viability and nucleated cell count should be performed on all samples.

Step 2 – Cell Culture
CAMEO™-96 contains a Master Mix for detection of a specific hematopoietic cell population derived from the species of choice. Alternatively, the assay kit may have been obtained without growth factors/cytokines, in which case the investigator can add their own growth factors and/or cytokines. In addition to the CAMEO™-96 Master Mix, medium is also provided for the dilution of the ATP standard. Sufficient CAMEO™-96 Master Mix volume is provided to culture samples in the 96-well plate included with the kit. Since this is a kit for basic research, the user can perform as many replicate wells/sample as needed. For statistical purposes, HemoGenix® recommends 6 replicates/sample. Using a positive displacement syringe pipette, sufficient CAMEO™-96 Master Mix is removed and dispensed into sterile 5ml plastic tubes. This is followed by adding the cell suspension adjusted to the correct working concentrating. The cell volume added is 10% of the total volume required. The contents of the tube(s) are mixed on a vortex mixer and 0.1ml dispensed into wells of the 96-well plate using a positive displacement syringe pipette. The plates are transferred to a 37°C, humidified incubator gassed with 5% CO_2 and preferably 5% O_2.

Step 3 - Colony Counting
After incubation, the colonies in all replicate wells from all samples are counted and, if required, differentiated into colony types, prior to proceeding to Step 4.

Step 4 – Bioluminescence measurement
To measure bioluminescence, the iATP is released from the cells. This is accomplished by the addition of 0.1ml of the ATP Enumeration Reagent (ATP-ER) using a multichannel pipette. The ATP-ER also contains luciferin and luciferase, the reagents used to produce bioluminescence. After mixing and a 10 min incubation period, the bioluminescence is measured in a plate luminometer. Prior to sample luminescence measurements, the instrument is calibrated and the assay standardized by performing an ATP standard curve with controls (provided with the kit).

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13. 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 associated with the methylcellulose CFC/CFU assay as well as incorrect dilutions or pipetting error. Outliers can be obtained resulting in large variations between replicates. 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 plating the culture master mix 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. Never use syringes with needles to dispense reagents. 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.
- Perform a minimum of 6 replicates per point.

Low RLU Values
Performing an ATP standard curve 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 adding the cells to the master mix. 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 view under a

12. CAMEO™-96 ATP Bioluminescence Assay Parameters

To ensure that cell proliferation and CFC/CFU standardization results are trustworthy and can be used to measure samples correctly, the ATP standard curve and controls must conform to the parameters below. If this is not the case, please check dilutions, pipetting and ensure that the pipettes are professionally calibrated prior to use.

Assay Characteristics

When performing the ATP standard curve, the following parameters should be obtained:

- Log-log linear regression goodness of fit (r^2) = > 0.995
- Log-log linear regression correlation coefficient (r) = > 0.997
- Log-log linear regression slope = 0.937 ± 15% (slope range: 0.796 - 1.07)

- Low ATP control = 0.05µM ± 15% (allowable range: 0.043µM - 0.058µM)
- High ATP control for ATP standard curves up to 1µM = 0.7µM ± 15% (allowable range: 0.595µM - 0.805µM)
- Extra High ATP control for ATP standard curves up to 3µM = 1.75µM ± 15% (allowable range: 1.488µM - 2.013µM).

For samples:

- Lowest ATP value indicating unsustainable stem cell proliferation: ~0.04µM
- ATP value below which cells are not metabolically viable: ~0.01µM.

If these parameters are not attainable, please refer to Troubleshooting (Section 12) or contact HemoGenix for technical assistance.

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5. Kit Contents

CAMEO™-96 kits contain the following components:

1. CAMEO™-96 Master Mix to detect the cell population of choice.
2. Medium (IMDM) for ATP standard dilution and background.
3. ATP standard.
4. ATP high and low controls.
5. ATP Enumeration Reagent (ATP-ER)*
6. Adhesive Plate Covering—a sterile foil to protect and keep unused wells sterile.
7. Sterile, 96-well plates for stem cell culture.
8. Non-sterile 96-well plates for ATP standard curve determination.
9. Instruction manual.

Exact volumes of the 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 times 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. The ATP-ER must not be used past the expiration date.**

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 HemoGenix®.

This kit contains a reagent for measuring luminescence (ATP-ER) that decays with time. HemoGenix® suggests that this kit be used before the expiry date of this reagent. HemoGenix® 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 reagents can be purchased from HemoGenix®. Please contact HemoGenix®.

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.

6. Equipment, Supplies and Reagents required, but not Provided

Equipment and Supplies

1. Laminar Flow Biohood
2. Plate luminometer (e.g.; Promega GloMax®-96, Molecular Devices, SpectraMaxL; TECAN, GENios)
3. Sterile, capped, plastic tubes (5ml, 10ml, 50ml)
4. Positive displacement (preferably electronic) syringe pipette (e.g. Eppendorf Repeater® Stream)
5. Single channel pipettes, preferably electronic (e.g. ViaFow or Rainin EDP pipettes for variable volumes between 1µl and 1000µl).
6. 8 or 12-channel pipette, preferably electronic (e.g. ViaFlow or Rainin EDP pipettes for fixed or variable volumes between 10µl and 100µl).
7. Reservoir for 8- or 12 channel pipette
8. Sterile pipette tips.
9. Vortex mixer.
10. Tissue culture incubator, humidified at 37°C with 5% CO_2 (minimum requirement) and 5% O_2 (preferable).
11. 1.5ml plastic vials (5 for each ATP dose response).
12. Hemocytometer or electronic cell counter to determine cell concentration.
13. Flow cytometer or hemocytometer for determining viability.

Reagents

1. Sterile Phosphate Buffered Saline (PBS)
2. Iscove's Modified Dulbecco's Medium (IMDM)
3. DNase (Sigma-Aldrich, Catalog No. D4513-1VL)
4. Density-gradient medium (e.g. NycoPrep 1.077, Axis-Shield).
5. ACK Lysis buffer (Cat. No. K-Lysis-100, HemoGenix®, Inc)
6. 7-AAD, propidium iodide, trypan blue, acridine orange or other dye exclusion viability assay.

Kits

ProliferSTEM™ (HemoGenix®, Inc), for metabolic viability.

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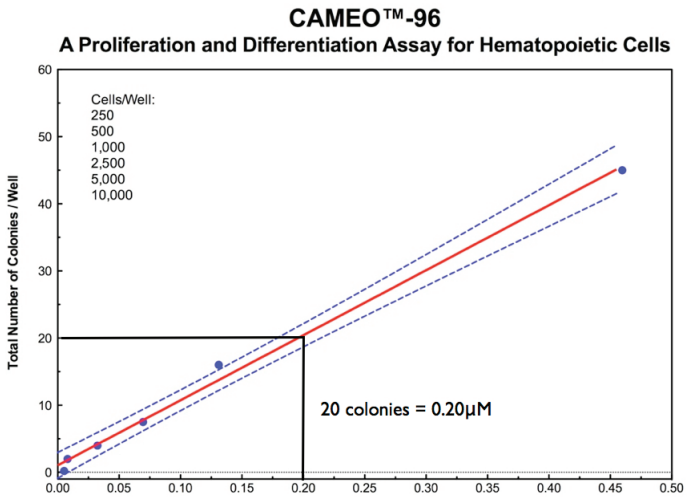
11. Standardizing the Colony-Forming Cell Assay

Due to the lack of standards and controls, the "classic" colony forming cell or unit (CFC/CFU) assay cannot be calibrated, standardized or validated according to regulatory requirements. However, since CAMEO™-96 incorporates bioluminomics™ technology, that allows cell proliferation to be quantitatively measured using an ATP standard curve and controls, it is possible to use this capability to back-calibrate and standardize the CFC/CFU assay and express total colony counts as ATP equivalent concentrations. This is performed as follows:

1. Perform a cell dose response using CAMEO™-96 from 500 to 7,500 or 10,000 cells/well.
2. Include unknown samples is the experiment or study.
3. After the incubation time has elapsed, count the total number of colonies in each well for all cell dose samples and unknowns.
4. Process the plate and measure the ATP concentration.
5. Plot the total colonies against the ATP concentration for all cell doses as shown in the Fig. 3.
6. Perform a linear regression analysis. A regression line similar to the one shown should be obtained.
7. It is now possible either manually or using statistical software to interpolate the ATP equivalent concentration from the total colony count for a sample.

Since the ATP measurement has been calibrated and standardized against an external ATP standard and controls, the colony forming assay, by virtue of the correlation between the two parameters (colony counts and ATP concentration) has also been calibrated and standardized.

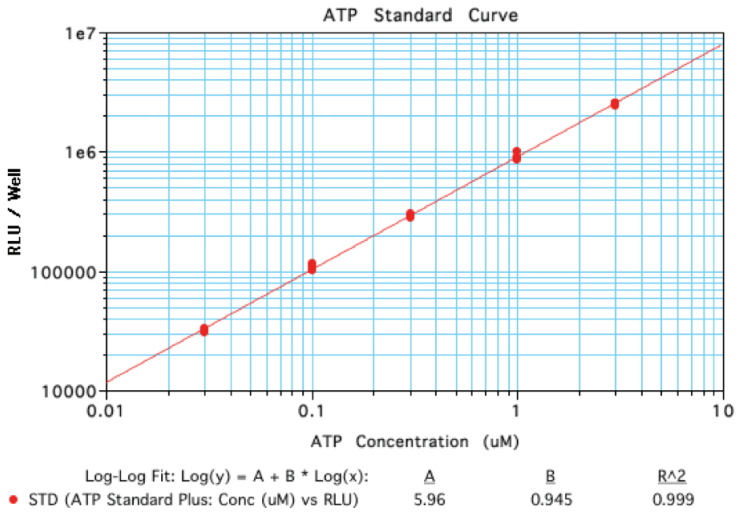
Figure 3



- GraphPad Prism version 5.0d
- TableCurve 2D from Systat Software, Inc.
- OriginLab version 8.1or higher from Origin Software.

For technical assistance using these third-party software packages, please contact HemoGenix®.

Figure 2. Typical ATP Standard Dose Response Curve Using Molecular Devices SoftMax Pro Software



7. The CAMEO™-96 Protocol

PLEASE READ THE FOLLOWING PROTOCOL CAREFULLY.
SEE SECTION 8 BEFORE PERFORMING AN ASSAY

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 counting to detect differentiation ability/potential.
Step 4 – Luminescence measurement. An ATP dose response is performed 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 assay kits can be obtained for multiple cell populations derived from several different tissues for up to 8 different species. For human, primate, horse, pig, sheep, dog and rat, it is essential that the erythrocyte content is as low as possible, since erythrocytes can interfere with the assay when present at high concentrations and cause false positive results. For human tissue preparations, the hematocrit should be below 10%. In addition, for best results, a mononuclear cell (MNC) preparation should be used that has low granulocyte and platelet concentrations.

- All peripheral blood samples, regardless of species, must be treated to reduce the red blood cell content. This can be performed using one of the methods described below.
- With the exception of peripheral blood and spleen cells, murine hematopoietic tissues do not normally need to be subjected to fractionation.
- For human, primate, horse, pig, sheep, dog and rat bone marrow and peripheral blood, red blood cell reduction is essential. Umbilical cord blood should be treated in the same manner as peripheral blood.

A. Umbilical cord blood

A mononuclear cell (MNC) suspension is required for best results. It is recommended to deplete human umbilical cord blood of red blood cells using a current Hetastarch® protocol. Additionally, cell lysis or density gradient fractionation can be used per the manufacture's protocol. The hematocrit of the test sample should be 10% or less so that it does not interfere with the assay. If the hematocrit is reduced as suggested, the final hematocrit in culture will be 0.1% or less. The small number of enucleated and nucleated erythrocytes (present in human cord blood) present should not interfere with the assay. However, if depletion is not complete or it appears that erythrocytes are present in a high concentration, it is recommended that a density gradient centrifugation or cold lysis is performed. Please see below for details.

B. Peripheral blood or bone marrow cells

If normal peripheral blood or bone marrow aspirates are used, it is essential that the red blood cell concentration be reduced. If human mobilized peripheral blood samples are used, they are usually produced during apheresis of the patient. They normally contain a very low level of red blood cells and are essentially a MNC fraction. However, if the red blood cell content is high, the sample must be further processed.

C. Isolation of hematopoietic subpopulations

Providing sufficient cells are available, subpopulations of stem cells or other lympho-hematopoietic cell populations can be isolated and purified for use in the CAMEO™-96 assay. HemoGenix® recommends using magnetic cell isolation procedures (e.g. Miltenyi Biotech), since these allow standardized and rapid isolation of stem cell populations with substantial purity, viability and yield. Please see table of recommended cell concentrations to determine the optimal, final cell concentration to use in CAMEO™-96.

D. Thawing of Cells and Procedures to Reduce Red Blood Cell Content and Produce a Mononuclear Cell Suspension

(i) Thawing of Cells

If cells are cryopreserved as a total nucleated cell product, they will contain red blood cells, granulocytes and other cell populations. When the cells are thawed, granulocytes and other cell components will rupture and release DNA. Large amounts of released DNA will clump together encasing cells. If the cell preparation originally cryopreserved was a MNC or similar fraction, the chances of clumping will be low. However to reduce or alleviate the possibility of clumping during cell thawing, it is recommended that DNase be added to the cell suspension. The following procedure is used for small aliquots of cells only (1-1.5ml).

1. Thawing of the vial contents is initiated in a 37°C water bath, by swirling the vial for approx. 1 min.
2. When a small ball of ice still remains in the vial (1-2 min), remove the vial from the water bath, sterilized the outside of the vial by spraying with 70% ethanol and carefully unscrew the vial lid.
3. It is possible that clumping can occur at this stage, in which case, add DNase to the total volume in the vial to achieve a concentration of 6µg/ml before proceeding to the next step.
4. Using a 1ml pipette, gently mix the contents of the vial and transfer to a 50ml tube containing 20ml of thaw medium. Up to 3 vials of the same cells can be added to this 20ml of thaw medium. However, clumping can also occur at this stage. In this case, DNase at a final concentration of 6µg/ml should be added before proceeding to the next step.
5. Gently mix the cells by swirling the contents of the tube. Do not use repeat pipetting to mix the cells. This could cause further rupture of cells and the release of DNA resulting in increased clumping.
6. Centrifuge the cells at 300 x g for 10 min at room temperature and discard the supernatant after centrifugation.
7. Resuspend the cells in 1ml of culture medium. If necessary, add 6µg/ml DNase.

c. The third set of measurements to be performed will be those of the samples. Setup the software to perform the following calculations:

1. Group or sample designation
2. Sample number
3. Well number
4. RLU/well
5. ATP values/well (calculated from the ATP standard dose response curve)
6. Calculated mean ATP values
7. Standard deviation of calculated ATP values
8. % CV of calculated ATP values.

Most, if not all, the calculations and results can be obtained automatically directly from the luminometer without any further manipulation. By automatically converting the RLU values into ATP concentrations (µM) directly from the ATP standard curve, results from the samples can be graphically displayed via the software.

Most software packages can export the results to MS Excel either directly or via text files.

(iii) Instrument Setup for Luminometers without Software Analysis Capabilities

Many plate luminometers do not come with analysis software. Instead, the data is either automatically exported or has to be manually exported to a Microsoft Excel file for calculation and analysis. Excel has functions to perform the necessary calculations for interpolating RLU values into ATP concentrations using the ATP standard curve. The basic Excel procedure is as follows:

1. Column 1: Make a column for the calculated ATP concentrations used for the ATP standard curve.
2. Column 2: Copy the RLU values for the standard curve.
3. Column 3. Transform the RLU values into log RLU values using the LOG function.
4. Column 4. Transform the ATP values in column 1 into log ATP values.
5. Column 5. Using the Excel TREND function, perform a Trend analysis for the log RLU values in Column 3.
6. Column 6. Transform the log values back into actual values using the Excel ANTI-LOG function.
7. Column 7. Perform a TREND function for the log ATP values.
8. Column 8. Transform the log trend ATP values back into actual ATP values using the Excel ANTILOG function.
9. Column 9. Copy the sample RLU values.
10. Column 10. Transform the sample RLU values into LOG RLU values.
11. Column 11. Using the Excel TREND function, perform a trend analysis for the sample.
12. Column 12. Convert the calculated sample values back into ATP concentrations.

(iv) Using Third-Party Software

Instead of using Microsoft Excel, third party software can also be used. In this case, the raw data in the Excel file must be copied and pasted or copied into the clipboard and imported into the software program. It is important that the third-party software can either perform a log-log linear regression analysis on the raw data or can transform the data into log values. The following software has been tested to perform the necessary calculations and graphs:

and whether it can perform the necessary calculations so that the procedure can be automated.

a. The first measurement to be performed will be to detect the background (Bkg) luminescence in wells A1 – D1. Setup the software to produce the following results:
   1. Well numbers
   2. RLU/well
   3. Mean RLU
   4. RLU Standard Deviation (St. Dev)
   5. RLU Percent Coefficient of Variation (%CV)

b. The second set of measurements to be performed will be the ATP standard curve. Setup the software to give the following information:
   1. Group or sample designation
   2. ATP standard dose response values (these are the calculated values of the ATP concentrations used for the dose response)
   3. Well numbers
   4. RLU/well
   5. Mean RLU (optional)
   6. Standard deviation of Mean RLU (optional)
   7. %CV of Mean RLU (optional)
   8. Predicted ATP concentration/well calculated by interpolating the RLU values from the ATP standard curve into ATP concentrations actually obtained. This should be performed automatically by the luminometer software. This is actually a back calculation of the ATP doses used to generate curve. The calculated ATP concentrations should correspond to the expected ATP values.
   9. Mean predicted ATP
   10. Standard deviation of mean predicted ATP
   11. %CV of mean predicted ATP.

The software should be capable of performing a log-log linear regression curve fit according to the equation:

$$\log Y = A + B * \log X$$

where A is the Y-intercept and B is the slope of the dose response curve. Do not use the equation Y = A + B*X as this will normally produce negative values for the lowest ATP dose. In addition, converting the X- and Y-axes to log is not equivalent to the curve fit shown above.

Figure 2 shows a typical ATP standard dose response using SoftMax Pro software that controls a Molecular Devices Lmax luminometer. The curve fit is for a 5-point ATP dose response ranging from 0.03µM to 3µM. If the log-log linear regression curve fit is performed as stated above, then the curve fit parameters should fall within the following guidelines obtained for 93 individual ATP dose response curves:

- Goodness of fit (r^2) = 0.999 ± 0.001 (%CV = 0.071)
- Y-Intercept (A) = 6.71 ± 0.63 (%CV = 9.37)
- Slope (B) = 0.969 ± 0.18 (%CV = 1.9). This is slightly different to the value given in Section 11.

(Values are the Mean ± 1 Standard Deviation)

(ii) Reduction or Removal of Red Blood Cells

The red blood cell content must be reduced to 10% or less for assay cell suspensions used in CAMEO™-96. This is because red blood cells contain high levels of ATP that will cause false positive results. If the sample to be assayed is fresh, the red blood cells can be subjected to a cold lysis procedure. Lysis should not be used to remove red blood cells from a cryopreserved sample. Density gradient centrifugation is recommended for cryopreserved samples.

Red Blood Cell Cold Lysis

To perform a cold lysis, ACK Lysis Buffer (Cat. No. K-Lysis-100, HemoGenix®) is recommended. The procedure is performed as follows:

1. Refrigerate the ACK Lysis Buffer.
2. Prepare an ice water bath in a 200ml beaker.
3. Transfer the sample to a centrifuge tube.
4. Add 10 parts of the cold ACK Lysis Buffer to 1 part cell suspension. For 1ml of cell suspension, add 10ml ACK Lysis Buffer.
5. Place the tube in the ice water bath and set a lab timer for 5 min.
6. At 2.5 min, invert the tube once and replace in the ice water bath.
7. At 5 min, remove the tube. There should be a cherry red clear solution. If lysis has not taken place, a cloudy cell suspension will still be observed. Replace the tube in the ice water bath for another 2.5 min. Continue this for a maximum of 10 min.
8. Centrifuge the tube at 400 x g for 5 min and discard the supernatant after centrifugation.
9. Resuspend the cells in IMDM to the original cell volume.
10. Perform a cell and differential count and viability assay.
11. The white blood cell recovery should be at least 95% and the viability must be greater than 85%.

Density Gradient Centrifugation

Red blood cells and other contaminating cells can be removed by density gradient centrifugation. It should be emphasized that when the red blood cell content is high, even density gradient centrifugation may not provide optimal depletion. In most cases, however, density gradient centrifugation will produce a high quality MNC fraction and will also increase the viability of the cell sample.

HemoGenix® does not recommend using a Ficoll density gradient centrifugation procedure. This is because all Ficoll-derived reagents are toxic to cells. It is recommended to use NycoPrep 1.077, which is non-toxic and can also be added to cultures without any deleterious effects. It is essential that the density gradient centrifugation reagent is brought to room temperature prior to use. The procedure for density gradient centrifugation using NycoPrep is as follows:

1. Dilute the sample to be fractionated with an equal volume of Dulbecco's phosphate buffered saline (dPBS) and mix gently by inversion.
2. For samples 3ml or less use a 15ml conical plastic tube for separation. For samples greater than 3ml, use a 50ml conical, plastic tube for separation.
3. For samples of 3ml diluted to 6ml with dPBS, dispense 5ml of the density gradient reagent into the tube. For samples greater than 3ml, dispense 15ml of the density gradient reagent into a 50ml tube.
4. Using a sterile, serological pipette, dispense the diluted sample gently on top of the density gradient reagent by holding the tube at approx. 45° and using

a Pipette Aid on slow delivery. The cleaner the interface between the density gradient reagent and cell suspension, the better the separation will be. Do not allow the cells to mix with the reagent.

5. Centrifuge for 10 min at 1,000 x g or 20 min at 600 x g at room temperature with NO brake.
6. After centrifugation, remove the tube(s) gently and carefully aspirate the top layer above the MNC interface leaving approx. ¼" above the interface. Discard the supernatant.
7. Harvest the MNCs from the interface and transfer the cells to another sterile tube. It is best to harvest the cells using a manual 1ml pipette. Do not remove cells below the interface.
8. Add approx. 10-20 ml dPBS, mix gently and centrifuge the cells for 10 min at 300 x g at room temperature.
9. Aspirate the supernatant after centrifugation taking care not to aspirate the cell pellet.
10. Add 1-2ml of IMDM and resuspend the cells, breaking up any clumps using a 1ml manual pipette.
11. Perform a nucleated cell count and viability. The cell viability must be greater than 85%. Using cells with a viability lower than 85% will produce results with low proliferation ability.

Fractionating the MNCs by density gradient centrifugation will usually dramatically improve viability.

Density Gradient Centrifugation Troubleshooting

- Specimens that are not fresh or greater than 24 hours old, may not separate cleanly. These specimens may need to have the procedure performed twice.
- Fibrin strands can appear after separation. They are attached to the mononuclear cell layer. Care should be taken in this situation.
- Aspirate the supernatant using a serological pipette and save to a sterile tube. Do not discard the supernatant until a clean transfer has been made. Try to avoid the fibrin strands.
- If fibrin strands get caught up with the cells, transfer entire supernatant to a sterile tube and centrifuge for 10 minutes @ 300 x g. The resulting cells will have some residual platelets; however, most of the platelets will be removed with the supernatant after centrifugation.
- Bone marrow often has fat globules and bone spicules; these can get caught in the cell interface. The fat will rise to the top during the second spin and the spicules can be "picked out" using a manual 1ml pipette.
- If the cell separation is not clean and distinct, transfer the supernatant to a sterile tube using a serological pipette and reserve until the cell count is performed. If the cell count is low, centrifuge the supernatant to recover additional cells.
- Specimens that have a high red cell concentrations can be separated again or treated with ACK lysis buffer.
- Removing excess density gradient reagent below the interface can result in granulocyte and platelet contamination of the MNCs.
- In some cases, a trade-off of higher cell recovery with increased platelet contamination may be necessary. In these cases, cells can be centrifuged for 10 minutes at 400 x g instead of 300 x g, with no detrimental effect on the cells.

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10. Luminometer Setup and Conversion of RLU Values to ATP Concentrations

The readout from all plate luminometers is Relative Luminescence Units (RLU). The term "relative" is used because luminometers from different manufacturers produce different RLU ranges. The RLU range may be from 0-100 for one instrument and 1-1,000,000 for another. A RLU value is a non-standardized unit of measurement. It is therefore difficult to compare results from one experiment to another. The term "bioluminomics™" includes not only the procedure for measuring cell proliferation or inhibition by virtue of the iATP concentration, but also the procedures for standardizing the assays. This is done by interpolating the RLU values into ATP concentrations using the ATP standard dose response curve and a log-log linear regression least squares analysis.

(i) Luminometer Setup

Multiparameter instruments, i.e. those that can detect absorbance, fluorescence and luminescence, often need to be manually set for both the integration time and the "gain". Dedicated instruments, i.e. those that only detect luminescence, usually only have to be set for the "integration time". It is therefore necessary to first know whether the instrument is a multiparameter or multipurpose instrument and whether "integration time" and "gain" need to be set. The instrument instruction manual will provide this information. If the "gain" has to be set, the instruction manual will explain how the correct "gain" is established. Once the "integration time" and "gain" are set, they should not be changed.

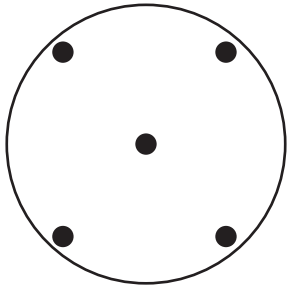
a. First set the integration time to 2 seconds.
b. Next, set the "gain". This must be determined empirically and is best performed when the ATP standard curve is measured. The gain should be adjusted so that the percent coefficients of variation (%CV) for the mean of the replicates are the lowest value. These values should be about 5% or less.
c. The measurement temperature of the instrument should be set to between 22°C and 24°C or turned off.
d. Most luminometers are equipped with a plate shaking protocol. It is not necessary to use the plate shaker mode.
e. Do not use injectors if the instrument is so equipped.

(ii) Instrument Setup for Luminometers with Software Analysis Capabilities

The luminometer is usually controlled by software installed on a computer using a serial or USB interface cable. The software for some luminometers comes with extensive analysis capabilities. This allows all the calculations to be programmed and performed by the luminometer software. If the software does not include analysis capabilities, the results are usually exported directly to a Microsoft Excel file for calculation and analysis.

Before using any luminometer, ensure that you are familiar with the software that controls the instrument. For luminometer software that has analysis capabilities, setting up the software properly prior to any measurements can save considerable time and produce an optimized report. It may be necessary to contact the instrument manufacturer to determine whether the software can provide the information below

Figure 1. Positions of pipette tip for mixing the well contents



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E. 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 cytometer 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 ProliferSTEM™ (HemoGenix®, Inc) as a metabolic viability assay.
2. Determine the cell concentration using either a hemocytometer or electronic cell/particle counter.
3. Adjust the cell suspension concentration to that recommended in Table 1.
   Note *that 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 or lower cell concentrations may be required.
4. Prepare the total volume of cell suspension required using IMDM or PBS. 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 Concentrations

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
	Umbilical cord blood	MNC	Fresh/ 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
	Umbilical cord blood	$CD34^+$	Fresh/ Frozen	0.1-5 x 10^5	100-5,000
Non-human primate, horse, pig, sheep, dog, rat	Bone marrow	MNC	Fresh/ Frozen	0.5-0.75 x 10^6	5,000-7,500
Non-human primate, horse, pig, sheep, dog, rat	Peripheral blood	MNC	Fresh/ 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
Mouse	Bone marrow	Total	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
	Spleen	MNC	Fresh	0.75 - 1 x 10^6	7,500 - 10,000
	Fetal liver	Total	Fresh	0.5-0.75 x 10^6	5,000-7,500

STEP 2. CAMEO™-96 Cell Culture

Please refer to Section 8 for recommendations and tips prior to beginning this stage of the procedure.

Perform all cell culture under sterile conditions in a biosafety cabinet.

The CAMEO™-96 kit contains a pre-mixed master mix for the cell population to be tested. Only the cells need to be added to prepare the Culture Master Mix. This allows the assay to be performed rapidly and with a minimum of manipulation.

1. Transfer the frozen pre-mixed bottles of Master Mix to a 37ºC incubator or water bath or allow the bottles to thaw at room temperature.
2. When thawed, mix the contents of the bottle of Master Mix thoroughly using a vortex mixer.
3. Prepare the cell suspension as required.
4. Table 1 shows the recommended cell concentrations required for different tissues. Calculate the working cell concentration required for each sample accordingly. NOTE: the concentration of the cell suspension in cells/ml is 100 x the concentration for cells/well. For example: 5,000 cells/well requires a working cell suspension concentration of 500,000 cells/ml.
5. Prepare and label one or more 5ml sterile, plastic tubes for each sample to be tested.
6. For dispensing the CAMEO™-96 methylcellulose Master Mix it is recommended to use a calibrated, positive displacement (preferably electronic) syringe pipette. DO NOT use a syringe and needle since this method of dispensing methylcellulose is inaccurate and will lead to high coefficients of variation.
7. For 6 replicate wells/sample, dispense 0.9ml of the CAMEO™-96 methylcellulose Master Mix into each tube. Ensure that the Master Mix is dispensed to the bottom of the tube, rather than on the sides of the tube.
8. Using calibrated and preferably electronic pipettes, dispense 0.1ml of the adjusted cell suspension into each tube. Cap the tube.
9. Mix the contents of each tube thoroughly using a vortex mixer. This is now the CAMEO™-96 Culture Master Mix.
10. Remove the sterile, 96-well plate from the plastic covering under the hood.
11. Using calibrated and preferably electronic positive displacement syringe pipette, take up as much of the Culture Master Mix as possible without

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 96-well Plate

Besides mixing the contents of the tubes after cells have been added prior to plating the CAMEO™-96 Culture Master Mix, mixing the contents of the wells after adding ATP-ER is probably the most important procedure. 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 and aspirate and dispense the contents twice without removing the tip from the contents of the well.
4. Repeat this operation as shown in Figure 1 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.

9. 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 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 white plates with a transparent growth surface 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 HemoGenix®.

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.
- Enough ATP standard and monitoring reagent is supplied to perform 4 standard curves and controls for each sterile plate provided.
- 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 -20ºC 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 2-8ºC 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 2-8ºC, or 20 weeks at -20ºC.
- 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

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including bubbles.

12. Prior to dispensing into the first well, discard the first amount back into the tube. This ensures that the tip of the syringe is completely full and that the correct amount (0.1ml) will be dispensed into the well.
13. Carefully dispense 0.1ml of the Culture Master Mix into the bottom of each well according to the configuration given in Section 9. For each 96-well plate, 16 samples can be performed. It is best to dispense each sample into wells across the plate, e.g. A1 - A6, B1 - B6 etc. or A1-A6 and A7-A12. The configuration of the plate will, in part, depend on how the plate luminometer reads the plate.
14. After dispensing all of the sample wells, replace the lid on the 96-well plate and transfer the culture plate to a humidity chamber to ensure high humidity during incubation (See section 8 (vii)). **NOTE**: It is not necessary to use the whole plate at once. Sterile adhesive foils are included so that unused wells can remain sterile for later use.
15. Place the humidity chamber into a fully humidified incubator set at 37˚C and gassed with 5% CO_2 and, if available, 5% O_2.
16. The cell incubation time will depend on the species and cell population being determined. In general, animal cells will be incubated for approx. 7 days, but should be monitored after 5-6 days in culture to ensure that the colonies remain separate and do not grow together so that they cannot be counted. Similarly, human cells, should also be monitored after about day 8-9 to ensure that the colonies remain separate and do not grow together, making enumeration difficult. IMPORTANT. Once an incubation period has been elected, it should be used continually and should not be changed. If the incubation period is changed, it will not be possible to compare results between different samples setup on different days. It is therefore important to maintain the elected incubation period.

STEP 3 - COUNTING COLONIES

Counting and enumeration of colonies must be performed prior to luminescence measurement, since ATP measurement involves lysing the cells to release the ATP

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. Most colonies are irregular in shape and contain many dark centers. Each dark center represents a single "proliferation unit" (PU). With time, these PUs grow together to form an irregular-shaped colony. Counting the individual centers within a colony provides a more accurate assessment and a better correlation with the ATP concentration obtained.
2. Counting 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 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 cause a shadow

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.

The colony counts can be expressed as colonies/number of cells plated.

STEP 4 – LUMINESCENCE MEASUREMENT

FOR ALL OF THE FOLLOWING STEPS, WEAR LABORATORY GLOVES
ATP is present on the skin and can cause erroneous results

IMPORTANT: PLEASE REFER TO SECTION 10 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.

IMPORTANT. Please refer to Section 9 for recommendations and tips prior to starting this part of the procedure. In particular, please refer to Section 9 for important information on mixing components.

PLEASE NOTE: ATP standard curves performed on previous days or for previous experiments or studies should not be used since the ATP-ER intensity changes with time and lot number.

Remove the ATP standard, controls and reagents from the freezer and thaw to room temperature prior to analysis.

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

A. ATP Standard Curve for Use with Samples that are Expected to Exhibit High Proliferation.

It is important that the sample ATP values measured are within the range of the ATP dose response curve. This is, in part, determined by the number of cells plated, since the ATP concentration correlates directly with the cell dose. For samples expected to produce high ATP concentrations (high cell proliferation), it is recommended to perform an ATP standard curve from 0.03µM to 3µM. For example, samples from human cell cultures incubated for 10 days, may exhibit ATP values greater than 1µM. Since the sample values should lie within the ATP standard curve range, it would be necessary to perform an ATP standard curve from 0.03µM to 3µM. If a sample is found to exhibit an iATP concentration greater than 3µM, the sample will have to be diluted so that the iATP falls within the ATP standard curve dose range. The dilution would then be taken into account when calculating the ATP value of the sample. Perform the ATP standard curve as follows:

1. Label 5 vials (e.g. 1.5ml volume) for the ATP dose response consisting of the following ATP concentrations:
   Label the ATP Standard (containing 0.3ml) supplied as Vial #1 (3µM)
   Vial #2: 1µM
   Vial #3: 0.3µM
   Vial #4: 0.1µM

(vii) 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". 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 HemoGenix® for further information about assembling and using humidity chambers.

(viii) Incubation Times

The culture time will depend on the species, cell population and cell dose used. The 96-well plates provided have a transparent growth surface. The plates can be removed at any time from the incubator to monitor colony growth. Do not culture the cells so that the colonies cannot be counted because of confluency. The cells should be cultured until the colony type can be identified and the colonies have grown so that they can be discreetly counted individually.

8. Recommendation and Tips Prior to Using CAMEO™-96.

(i) Cell Suspension

a. The preferred cell suspension is a mononuclear cell suspension (MNC) of human umbilical cord blood, human, primate, horse, pig, sheep, dog and rat bone marrow and peripheral blood. Murine peripheral blood should also be a MNC preparation. However, murine bone marrow can usually be used without any fractionation.

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) Thawing of HALO® Master Mix

a. Prior to using the CAMEO™-96 Master Mix, remove the bottle from the kit box and thaw either at 37ºC or at room temperature.

b. The CAMEO™-96 Master Mix may be aliquotted into 5ml tubes after mixing and frozen until the expiration date.

c. CAMEO™-96 Master Mix is stable at 2-8°C for 1 month after thawing.

(iii) Dispensing the CAMEO™-96 Master Mix and Culture Master Mix

Methylcellulose is notoriously difficult to dispense accurately. It is recommended to use positive displacement (preferably electronic) syringe pipettes to dispense both the Master Mix and Culture Mast Mix (which contains cells).

(iv) Number of Replicates Performed

CAMEO™-96 has been designed for samples to be tested using 6 replicates/sample. 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.

(v) Plate Configuration

Performing 6 replicates/well means that the samples replicates are plated across the plate, for example from A1 to A6, A7 to A12 or B1 to B6. In this manner 16 samples can be tested on a single plate. If samples are tested across the plate, for example from A1 to A6 and then from A7 to A12, use a 12-channel pipette to dispense and mix the ATP-ER. If it is preferred to dispense the samples from A1 to A6 followed by B1 to B6 etc., then it is suggested to use an 8-channel pipette. Only attached sufficient pipette tips for the number of samples to be processed.

(vi) 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 HemoGenix® if required.

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Vial #5: 0.03µM

2. Now dispense:
   0.40ml IMDM medium to vial #2
   0.90ml IMDM medium to vial #3
   0.90ml IMDM medium to vial #4
   0.90ml IMDM medium to vial #5
3. Dispense 0.7ml of the IMDM medium into Vial #1. Mix.
4. Dispense 0.20ml from vial #1 to vial #2. Mix. This concentration is 1µM.
5. Dispense 0.10ml from vial #2 to vial #4. Mix. This concentration is 0.1µM.
6. Dispense 0.10ml from vial #1 to vial #3. Mix. This concentration is 0.3µM.
7. Dispense 0.10ml from vial #3 to vial #5. Mix. This concentration is 0.03µM.
8. Dispense 0.10ml of the supplied medium alone into wells A1, B1, C1 and D1 on the non-sterile luminescence plate(s) provided. NOTE: This is to measure background luminescence. *PLEASE NOTE that it is important to use the same medium to measure background luminescence as used for the ATP standard curve and cell cultures. Using different media will cause discrepancies in the results.*
9. Dispense 0.10ml from ATP dilution in vial #5 to wells E1, F1, G1, and H1.
10. Dispense 0.10ml from ATP dilution in vial #4 to wells A2, B2, C2, and D2.
11. Dispense 0.10ml from ATP dilution in vial #3 to wells E2, F2, G2, and H2.
12. Dispense 0.10ml from ATP dilution in vial #2 to wells A3, B3, C3, and D3.
13. Dispense 0.10ml from ATP dilution in vial #1 to wells E3, F3, G3 and H3.
14. Mix the vials containing the low and extra high controls provided.
15. Dispense 0.1ml from the ATP low control to wells A4, B4, C4, D4.
16. Dispense 0.1ml from the ATP extra high control into wells E4, F4, G4, H4.
17. Add the required amount of ATP-ER to a non-sterile reagent reservoir.
18. Using a multichannel pipette, add 0.10ml to each well of the first column (A1-H1).
19. Mix the contents as directed in Section 9 by repeated pipetting and discard the tips.
20. Change tips for each new addition of ATP-ER.

B. ATP Standard Curve for Samples that Exhibit Low Proliferation

1. Prepare and label 5 vials (e.g. 1.5ml volume) for the ATP dose response consisting of the following ATP concentrations:
   Vial #1: 1µM
   Vial #2: 0.5µM
   Vial #3: 0.1µM
   Vial #4: 0.05µM
   Vial #5: 0.01µM
2. Now dispense:
   0.90ml of the medium to vial #1.
   0.35ml of the medium to vial #2
   0.90ml of the medium to vial #3
   0.90ml of the medium to vial #4
   0.90ml of the medium to vial #5
3. Remove 0.1ml of the supplied stock ATP solution (at 10µM) and transfer it to vial #1. Mix by vortexing. This ATP concentration is 1µM.
4. Dispense 0.35ml from vial #1 to vial #2. Mix. This concentration is 0.5µM.
5. Dispense 0.10ml from vial #2 to vial #4. Mix. This concentration is 0.05µM.

6. Dispense 0.10ml from vial #1 to vial #3. Mix. This concentration is 0.1µM.
7. Dispense 0.10ml from vial #3 to vial #5. Mix. This concentration is 0.01µM.
8. Dispense 0.10ml of the supplied medium alone into wells A1, B1, C1 and D1 on the non-sterile luminescence plate(s) provided. NOTE: This is to measure background luminescence.
9. Dispense 0.10ml from ATP dilution in vial #5 to wells E1, F1, G1, and H1.
10. Dispense 0.10ml from ATP dilution in vial #4 to wells A2, B2, C2, and D2.
11. Dispense 0.10ml from ATP dilution in vial #3 to wells E2, F2, G2, and H2.
12. Dispense 0.10ml from ATP dilution in vial #2 to wells A3, B3, C3, and D3.
13. Dispense 0.10ml from ATP dilution in vial #1 to wells E3, F3, G3 and H3.
14. Mix the vials containing the low and high controls provided.
15. Dispense 0.1ml from the ATP low control to wells A4, B4, C4, D4.
16. Dispense 0.1ml from the ATP high control into wells E4, F4, G4, H4.
17. Add the required amount of ATP-ER to a non-sterile reagent reservoir.
18. Using a multichannel pipette, add 0.10ml to each well of the first column (A1-H1).
19. Mix the contents as directed in Section 9 by repeated pipetting and discard the tips.
20. Change tips for each new addition of ATP-ER.

IMPORTANT: Mixing the contents of each well properly is a very important procedure. PLEASE REFER TO SECTION 9 ON HOW TO MIX THE WELL CONTENTS CORRECTLY. THIS PROCEDURE IS ALWAYS USED WHEN MIXING REAGENTS FOR BIOLUMINESCENCE MEASUREMENT IS REQUIRED.

21. Add 0.10ml of ATP-ER to each well of the other columns, mixing the contents as described in Section 9.
22. Repeat the procedure for each new column.
23. Place the ATP plate in the luminometer and incubate 2 min before initiating measurement.

NOTE: The non-sterile 96-well plate(s) is/are used for all ATP standard curves and controls. Continue using empty wells on the plate for additional ATP standard curves.

C. Sample Measurement

IMPORTANT. It is very important that the ATP-ER is mixed with the methylcellulose using the procedure described in Section 9. If the cells are not mixed correctly with the ATP-ER, the cells may not be lysed and lower ATP concentrations will be measured.

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

1. If possible, place the sample plate(s) in a humidified incubator set at 22-23˚C gassed with 5% CO_2 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 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 9, Adhesive Plate Covering Film).
3. Using a multichannel pipette (8- or 12-channel depending on the plate configuration), add 0.10ml of ATP-ER to each well of the first column (A1-H1) or row (A1-12). Mix the contents as described in Section 9.
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.
6. Incubate the plate in the reader for the last 2 min to stabilize the plate.
7. Unused ATP-ER may be returned to the bottle and refrozen. See section 9 for ATP reagent storage conditions and stability.

D. Using a plate luminometer with automatic dispenser

The user may have a plate luminometer that allows reagents to be dispensed automatically directly into the well. Please do not use the automatic dispensers, since the contents of the well will not mixed sufficiently using this method.

E. Using a liquid handler

It is not recommended to use a liquid handler for CAMEO™-96.