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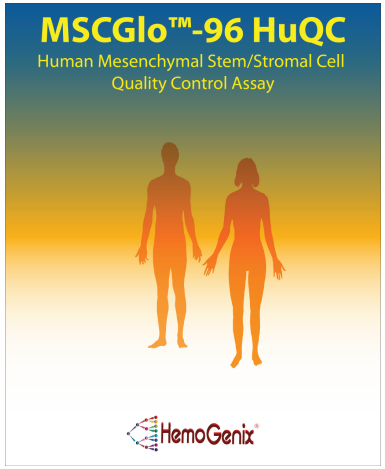

MSCGlo™-96 HuQC

for Mesenchymal Stem/Stromal Cell Quality Control

Assay Manual

(Version 10.13)
This manual should be read in its entirety prior to using this product

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

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1. INTRODUCTION

A stem cell system is comprised of multiple cell populations, each exhibiting different degrees of primitiveness or "stemness" within a single compartment. Stem cells proliferate. Only when they attain a specific maturity do they pass through a state of determination to enter one or other lineage to differentiate and mature to become a lineage-specific functional end cell.

Stem cell quality control is the ability of a stem cell population to demonstrate proliferation ability. The quality of the stem cell population being measured is therefore the ability of that population to proliferate at a particular cell concentration. The cell concentration used must be on the linear part of the cell dose response. Only then will the proliferation ability be similar at any cell dose elected.

Stem cell quality should not be considered the same as stem cell potency. The two parameters, are measured using different assays and requirements. However, stem cell quality and potency correlate with each other and must be used together to define the release criteria for a stem cell product.

MSCGlo™-96 HuQC has been designed specifically for measuring the quality of mesenchymal stem/stromal cells (MSC). It incorporates high performance MSCGro™ medium and bioluminomics™ technology. Bioluminomics™ is the instrument-based, non-subjective measurement of iATP concentrations using a luciferin/luciferase bioluminescence signal detection system, the most sensitive and accurate, non-radioactive readout available. Bioluminomics™ technology encompasses the ability to calibrate, standardize and, if required, fully validate the assay. This technology ensures that the results obtained are trustworthy, reliable and reproducible, not only within one laboratory, but between laboratories.

Manual, subjective assays such as the colony-forming unit - fibroblast (CFU-F) assay should not be used to determine MSC quality. The assay lacks quantitative output, standards and controls that are required to measure MSC quality.

MSCGlo™-96 HuQC should be used to determine the proliferation ability (quality) of MSC products. By using a standardized assay at all stages of the production and manufacturing process, a standardized and consistently good quality product can be obtained. Prior to using the MSC product for any clinical procedure, MSC potency determination is recommended. MSCGlo™-96 PQR should be used for this purpose. It allows both the potency and quality of the MSC product to be determined in a single assay. It also allows the investigator to define acceptance/rejection limits to ascertain release criteria.

2. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. **MSCGlo™-96 HuQC is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)**
2. **MSCGlo™-96 HuQC 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.**

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- **Hall KM, Harper H, Rich IN**. Hematopoietic stem cell potency for cellular therapeutic transplantation. In: Hematopoietic Stem Cells, Ed.: RP Camacho. ISBN 978-953-307-746-8. (In preparation).

- *Contamination*: Cells cultured in 96-well plates cannot be view under a microscope. If contamination occurs it will usually be seen by the difference in color of the cultures. Contaminated cultures will usually be bright yellow in color and probably cloudy in appearance. Cell cultures that demonstrate high proliferation will 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.

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

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3. INTENDED USE

MSCGlo™-96 HuQC is a quality control assay intended to measure the proliferation status or ability (quality) of human mesenchymal stem/stromal cells (MSC) during growth, expansion and processing procedures to produce and manufacture a cell product for cellular therapeutic use. By using a standardized and validated assay for this purpose, it is then possible to ensure that product quality is well characterized at the end of the process.

MSCGlo™-96 HuQC is used to quantify the proliferation status or ability (quality) of human MSCs derived from, but not limited to:

- Human bone marrow
- Human umbilical cord blood
- Whorton's Jelly
- Human adipose tissue
- Human induced pluripotent stem cells (iPS)

MSCGlo™-96 HuQC is available with MSCGro™ Medium with the following catalog numbers:

Catalog No.	MSCGro™ Medium	No. of Plates/kit
KLMC-LSQC-1	Low serum	1
KLMC-LSQC-2	Low serum	2
KLMC-LSQC-4	Low serum	4
KLMC-SFQC-1	Serum free	1
KLMC-SFQC-2	Serum free	2
KLMC-SFQC-4	Serum free	4
KLMC-HMQC-1	Humanized	1
KLMC-HMQC-2	Humanized	2
KLMC-HMQC-4	Humanized	4

MSCGlo™-96 HuQC is for research use only and must not be used as a diagnostic test or for clinical applications.

4. The CONCEPT and PRINCIPLE of BIOLUMINOMICS™ ASSAYS

MSCGlo™-96 HuQC is a bioluminomics™ assay. 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.

Mesenchymal stem/stromal cells are incubated in the MSCGro™ Medium 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 MSCGlo™-96 HuQC. 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.

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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 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 calibrate 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 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 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.

12. MSCGlo™-96 HuQC Readout Characteristics and Validation Parameters

MSCGlo™-96 HuQC bioluminomics™ technology allows the assay to be calibrated and standardized. Inclusion of an ATP standard and controls are requirements for assay validation.

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).

Assay Validation Parameters

- Assay ATP linearity => 4 logs
- Assay cell linearity: 1,000 - >25,000 cells/well
- Assay ATP sensitivity: ~ 0.001µM
- Assay cell sensitivity: 20-25 cells/well (depending on purity and growth factor cocktail)
- Accuracy (% correct outcomes): ~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) =< 15%. At lower limit of quantification (LLOQ): 20%
- Robustness (intra- and inter-laboratory): ~95%.
- High throughput capability (Z-Factor): >0.76 (lowest possible value, 0.5; highest possible value, 1).

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5. OVERVIEW OF THE MSCGlo™-96 HuQC PROCEDURE

The MSCGlo™-96 HuQC Kit Platform comes with 1, 2 or 4 plates using 96-well clear bottomed plates. The cells can be observed during culture using an inverted microscope.

Each kit contains 1 or more non-sterile plates and sufficient MSCGro™ medium to perform ATP standard dose response curves including high and low ATP controls.

There are 3 steps in using the MSCGlo™96 HuQC Kit.

Step 1 – Cell Preparation

Cells are not provided with MSCGlo™-96 HuQC. The user should prepare the MSCs for assay according to the user's own protocol. A viability and cell count should be performed after each growth, expansion or processing step. In addition, it is recommended to characterize the presence of MSCs using specific MSC biomarkers. Such biomarkers are usually cellular antigens or receptors detected by flow cytometry.

Step 2 – Cell Culture

MSCGlo™-96 HuQC has been designed for MSCs that have been passaged and/or expanded in large vessels, bio-reactors or on 3D-scaffolds. There are two ways of measuring MSC quality.

1. Mesenchymal stem cells can be passaged and expanded using MSCGro™ medium or the user's own medium formulation. When MSC are removed after each passage, an aliquot is then suspended in MSCGro™ medium and the proliferation status measured using MSCGlo™-96 HuQC.
2. Alternatively, MSC can be suspended in MSCGro™ medium and cultured directly in the sterile 96-well plate(s) provided.

Step 3 – 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), which also contains luciferin and luciferase. The ATP-ER is added using a multi-channel pipette. After a short 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).

6. KIT CONTENTS and STORAGE

MSCGlo™-96 HuQC 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	MSCGro™ Medium—a high performance MSC growth medium available as low serum, serum-free or humanized formulations. The MSCGro™ medium should be used to adjust, dilute and grow the MSC for culture as well as for the background control and dilution of the ATP standard concentrations.	4°C - 8°C until used
2	ATP standard.	-20°C until used
3	ATP "extra high", high and low controls.	-20°C until used
4	ATP Enumeration Reagent (ATP-ER)*	-20°C in the dark until used
5	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
6	Sterile, 96-well plates for stem cell culture	Can be kept with other kit components
7	Non-sterile 96-well plates for ATP standard curve determination.	Can be kept with other kit components
8	Assay manual.	Can be kept with other kit components

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 2-8ºC for 48h once thawed and is stable for 20 weeks when stored at -20ºC. 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®.

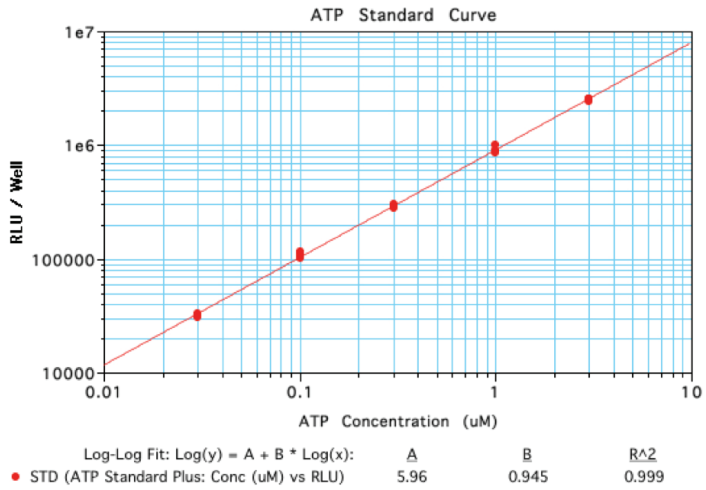
Good laboratory practices and universal protective precautions should be undertaken at all times when handling the kit components as well as cells and tissues. Material safety data sheets (MSDS) are included in each literature packet.

calculations and graphs:

- 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 3. Typical ATP Standard Dose Response Curve Using SoftMax Pro Software



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

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7. EQUIPMENT, SUPPLIES AND REAGENTS REQUIRED, BUT NOT PROVIDED

Equipment and Supplies

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

Reagents

1. Sterile Phosphate Buffered Saline (PBS)
2. MSCGro™ Medium (extra medium can be ordered from HemoGenix®).
3. Density-gradient medium (e.g. NycoPrep 1.077, Axis-Shield).
4. 7-AAD, propidium iodide, trypan blue or other dye exclusion viability assay.
5. Trypsin/EDTA or Accutase® to release cells from the adherent growth surface.

8. The MSCGlo™-96 HuQC PROTOCOL

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

Performing MSCGlo™-96 HuQC is a 3-step process.

Step 1 – Cell preparation.
Step 2 – MSC expansion or culture in 96-well plate.
Step 3 – 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

MSCGlo™-96 HuQC is intended only for human cells. Preparation of the cells depends on the source of cells being tested.

Cell Viability

Cell viability can be determined either manually using virtually any dye exclusion stain and a hemacytometer or can be determined by flow cytometry using 7-AAD. A viability of 85% or greater is recommended for optimal results.

Cell Concentrations

The assay shows linearity between 1000 cells/well and approx. 20,000 cells/well. The working cell suspension concentration (cells/ml) must be 10x the concentration of the final cell concentration/well. The cell suspension (cells/ml) is prepared in MSCGro™ medium provided. 0.1ml of this suspension is dispensed per well.

STEP 2. Cell Culture for MSCGlo™-96 HuQC

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

MSC Expansion prior to Direct ATP Measurement.

Mesenchymal stem/stromal cells are often passaged and expanded in flask cultures, bio-reactors or even 3D-culture systems. MSC quality would then be measured during expansion or passaging of the cells or after large-scale expansion. The initial tissue source is usually started in small flask cultures. Each time the cells are passaged for expansion, an aliquot of the cells can be analyzed by measuring intracellular ATP. MSCs are adherent cells and require an established trypsin, EDTA or Accutase® protocol to release the cells from the growth surface of the flask.

Once the cells have been removed, perform a cell count. To measure MSC proliferation ability at the time of harvest, only a single cell dose is required. A final concentration of 5,000 cells/well is usually sufficient, but can be increased to 7,500 if necessary. Alternatively a cell dose response can also be performed. Dispense 100µl of each cell concentration into 6 replicate wells of the sterile 96-well plate(s) provided. The

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manufacturer to determine whether the software can provide the information below 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 3 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)

11. LUMINOMETER SETUP AND CONVERSION OF RLU VALUES TO ATP VALUES USING THE ATP STANDARD DOSE RESPONSE CURVE

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

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intracellular ATP concentration, as a measure of proliferation status, can then either be measured immediately, or the cells can be incubated in MSCGro™ medium for 24h and then measured.

MSC Culture in 96-Well Plates

Another method of MSC propagation involves plating the newly acquired or passaged MSCs directly in the sterile 96-well plates provided with the kit. Whichever method is used to grow MSCs, stringent control of culture time periods prior to measuring bioluminescence is important when comparing different batches of MSCs.

If required, a time growth curve for MSC can also be performed. The time growth curve will provide information on the MSC expansion kinetics, which can then be compared with different MSC batches to help determine better procedures.

To culture MSC in the sterile 96-well plates provided, perform the following steps:

Remove twice the number of tubes from the kit that correspond with the number of samples to be assayed. Each sample will be assayed at 2 different cell concentrations.

1. Prepare cells according to the user-defined procedures (STEP 1).
2. Remove the MSCGro™ medium from the kit and warm to 37°C in an incubator or water bath.
3. Determine the cell concentration and viability of the original suspension.
4. Prepare a single dilution in MSCGro™ medium that is 10 fold greater in concentration than the required final dilution in the well. For example, if the final dilution is to be 5,000 cells/well, prepare a dilution that is 50,000 cells/ml. Prepare 1ml of this working concentration in MSCGro™ medium for each cell concentration used. If preparing several cell concentrations, perform a serial dilution so that 1ml remains for each cell concentration.
5. Using a calibrated pipette, preferably an electronic pipette, dispense 0.1ml into each of 6 wells of the sterile 96-well plate. This reduces the cell concentration 10 fold so that the final cell concentration in each replicate well will be the desired final cell dose. When dispensing 6 wells, dispense in rows, i.e. A1-A6, B1-B6 etc. This will allow 16 samples to be tested on a single plate. NOTE: It is not necessary to use the whole plate at the same time. See Step 3, Sample Measurement.
6. Incubate the cells at 37°C in a fully humidified atmosphere containing 5% CO_2 and, if possible, 5% O_2.
7. After 24hr, the MSC will have attached to the growth surface of the plate. Gently swirl the plate to suspend the non-adherent cells in the media. Using a manual pipette or vacuum apparatus, remove 50-75% of the medium from each well being careful not to touch the bottom of the well.
8. Dispense another 0.1ml of pre-warmed (37°C) fresh MSCGro™ medium to each well and return plate to the incubator.
9. Monitor the MSC growth using an inverted microscope.
10. When the cells have grown to approx 70%-80% confluency, the proliferation status can be measured.
11. If performing a growth curve, 6 replicate wells should be prepared for every day of the study that will be measured.

PLEASE NOTE: The cell concentrations and incubation times are suggested and may

have to be varied depending on the state and type of the human tissue source. Once optimized, these parameters should remain constant for all samples tested so that, if necessary, a direct comparison between samples can be performed.

STEP 3 – BIOLUMINESCENCE 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.

See Figure 1 for the ATP standard curve and control plate configuration.

A. "Extra High" ATP Standard Curve
Mesenchymal stem/stromal cells usually exhibit a high iATP concentration. For this reason, it is recommended to use an "extra high" ATP standard curve range so that sample ATP values fall within this range. If the ATP concentrations of the MSCs are high than 3µM, the sample will have to be diluted and rerun. The dilution would then be taken into account when the ATP concentrations are calculated. If the MSCs exhibit a low ATP or lower than 0.03µM, a low ATP standard curve should be used. A plate configuration similar to that shown in Fig. 1 should be used.

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
   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

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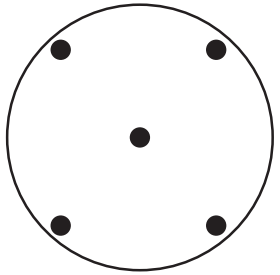
Figure 2. Positions of pipette tip for mixing the well contents



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 HALO® 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 2 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.

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 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. Low ATP Standard Curve Preparation for Umbilical Cord Blood, Bone Marrow or Normal Peripheral Blood

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.

18. Add 0.10ml of ATP-ER to each well of the other columns, mixing the contents as described in Section 10.
19. Repeat the procedure for each new column.
20. 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.

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10. 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.

MSCGlo™-96 HuQC 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 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

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.

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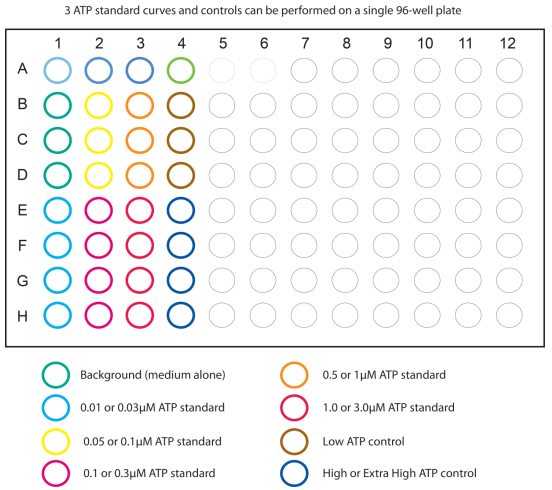
Figure 1

96-Well Plate Configuration for ATP Standard Curve and Controls



C. Sample Measurement

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 10, 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 10.
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 10 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. HemoGenix® 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

MSCGlo™-96 HuQC can be performed in high throughput mode. If you intend to perform any part of the MSCGlo™-96 HuQC procedure using a liquid handler, please contact HemoGenix® for information on setting up the instrument. Extra ATP-ER is required when using a liquid handler.

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9. RECOMMENDATIONS AND TIPS PRIOR TO USING THE HALO® KIT CELL CULTURE MIXES.

(i) Cell Suspension

- Extraneous ATP, red blood cells (which have high concentrations of ATP) and hemoglobin interfere with the ATP analysis. Most MSCs preparations will not have any red blood cells. However, if RBCs contaminate the preparation, they should be removed so that the hematocrit is less than 10%.
- If cells have been treated prior to cell culture, higher cell concentrations than those shown in Table 1 may be required.

(ii) Dispensing the Culture Master Mix

Once the cell suspension has been added to produce the Culture Master Mix, we strongly recommend using electronic pipettes to dispense the Culture Master Mix into individual wells for greater accuracy. Mix frequently during dispensing.

(iii) Number of Replicates Performed

It is recommended that a minimum of 6 replicates/sample be performed using MSCGlo™-96 HuQC. 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. (For quality control purposes, extra samples may not be available to perform an additional experiment. For these reasons, performing 6 replicates/sample should be maintained).

(iv) Plate Configuration

If 6 replicates wells/sample are used, the replicates should be plated in rows across the 96-well plate as this will provide the best use of the whole plate. In this way, 16 samples can be performed, but do not have to be performed all at the same time.

(v) 96-Well Plates Provided

The reagents have been optimized to work with the 96-well plate(s) provided in the MSCGlo™-96 HuQC 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.

(vi) 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