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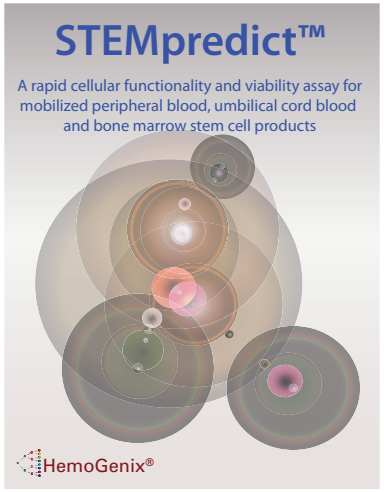

STEMpredict™

A rapid 3 day assay to predict umbilical cord blood bankability and optimization of mobilized peripheral blood collection

Instruction Manual

(Version 1-2014)

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Not for clinical diagnostic use.

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1. INTRODUCTION

STEMpredict™ is a specialized HALO® assay designed to rapidly determine umbilical cord blood bankability, that is, if cord blood units can be permanently stored, or to optimize peripheral blood collection during patient mobilization. STEMpredict™ determines if hematopoietic stem cells have the capability to enter the exponential phase of proliferation and growth. The assay takes just 3 days to perform. STEMpredict™ can be used in a low and medium throughput mode with 96-well plates or in high-throughput screening mode for large numbers of cord blood units using 384-well plates and a liquid handler.

Stem cell proliferation is measured by virtue of the fact that when stimulated with growth factors and/or cytokines, changes in intracellular ATP (iATP) concentration correlate directly with the proliferation status of the target cells. STEMpredict™ measures these changes that can be compared to a background control (no growth factors). The ratio between the iATP concentration for stimulated stem cells and the background provides the required information on stem cell proliferation and growth. Since iATP is a marker for cellular and mitochondrial integrity, STEMpredict™ is also a rapid stem cell metabolic viability assay.

STEMpredict™ is one of several HALO® assays designed specifically for hematopoietic stem cell therapy applications. All assays incorporate Bioluminomics™ technology that allows assay calibration, standardization and validation, thereby ensuring accuracy, sensitivity, reliability and reproducibility. This, in turn leads to trustworthy results than can be compared directly between samples over time and between different laboratories.

STEMpredict™ also incorporates Suspension Expansion Culture™ (SEC™) Technology. SEC™ technology (no methylcellulose) that improves accuracy and sensitivity and significantly shorter culture times.

Although STEMpredict™ is an advanced cell therapy assay for hematopoietic stem cells, it is fast to learn and easy to use.

2. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. **STEMpredict™ has not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)**
2. **STEMpredict™ 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.**

15. REFERENCES

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- *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.

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

Culture Plates Drying Out
- Due to the relatively small culture volume (0.1ml), drying out of the culture wells, particularly around the outside of the plate, can be a problem. These are called "edge effects". An incubator with insufficient humidity will cause this problem. To ensure that this does not occur, the incubator water reservoir should be full and the humidity in the chamber checked using a hygrometer.
- If drying out continues, use of a humidity chamber is recommended. Please refer to Section 9 (vii) for instructions on how to build a humidity chamber.

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3. INTENDED USE

STEMpredict™ is intended for hematopoietic stem cell therapy processing laboratories. It is used to predict umbilical cord blood bankability, i.e. whether a cord blood unit can be permanently stored or to optimize collections of peripheral blood during mobilization of a patient.

STEMpredict™ measures the metabolic viability, cell functionality and growth of stem cells in a sample that might ultimately be used for patient transplantation purposes. STEMpredict™ rapidly determines whether a stem cell product will exhibit sufficient proliferation ability for the cells to be processed and/or stored for future use. The assay is standardized and can be validated.

STEMpredict™ can be used for human stem cells derived from:
- Umbilical cord blood
- Mobilized peripheral blood

STEMpredict™ incorporates Suspension Expansion Culture™ (SEC™) technology. Suspension expansion culture™ technology has several advantages, including:
- Ease and accuracy of dispensing reagents.
- Allows cell-cell interaction.
- Provides greater assay sensitivity.
- Shorter cell incubation times.
- Coefficients of variation =<15%.
- Intra- and inter-laboratory comparability.

STEMpredict™ Assays Available

Catalog Nos.	Multiwell Plate Type	No. of Samples/Kit	No. of Plates
K2-SP-1	96-well plate	8	1
K2-SP-2	96-well plate	16	2
K2-SP-4	96-well plate	32	4
K3-SP-1*	384-well plate	32	1
K3-SP-2*	384-well plate	64	2
K3-SP-3*	384-well plate	128	4

* 384-well plate kits are for high throughput screening and require a liquid handler (robot) for dispensing. A separate manual is available for these kits.

4. The CONCEPT and PRINCIPLE of BIOLUMINOMICS™ ASSAYS

STEMpredict™ 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.

Hematopoietic stem cells are incubated in the STEMpredict™ 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 STEMpredict™. 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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14. 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.

13. STEMpredict™ Assay Characteristics and Validation Parameters

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)
- 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 14).

Assay Validation Parameters

- Assay ATP linearity => 5 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 STEMpredict™ PROCEDURE

STEMpredict™ detects the viability, functionality and stimulated growth of hematopoietic stem cells as they begin to proliferate in culture. This growth is compared to control cells of the same sample that are not stimulated. The ratio between the stem cells and the control at 3 days of culture provides an indication that the stem cells demonstrate the growth capability.

There are 3 steps to using STEMpredict™.

Step 1 – Cell Preparation

Cells are not provided with STEMpredict™ assay kits. Cells are prepared using a user-defined, pre-validated protocol to obtain a single cell suspension. A dye exclusion viability assay is initially performed as well as a cell count.

Step 2 – Cell Culture

Two Master Mixes are provided. The first is the Control Master Mix (CMM) which contains no growth factors or cytokines. The second is the Stem Cell Master Mix (SCMM) which contains a growth factor/cytokine cocktail to stimulate hematopoietic stem cells. In addition, medium is also provided for the dilution of the ATP standard. Two tubes are labeled for each sample tested. One tube will contain 0.72ml of the CMM, while the other will contain 0.72ml of the SCMM. The cell suspension is adjusted to the correct concentration and 0.08ml is added to each of the tubes. The contents of each tube is mixed by vortexing and 0.1ml is dispensed into each of 6 replicate wells of the sterile 96-well plate provided. The cells are cultured for 3 days in a fully humidified incubator at 37°C in an atmosphere containing 5% CO_2 and preferably, 5% O_2.

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 assay standardized by performing an ATP standard curve with controls (provided with the kit).

6. KIT CONTENTS and STORAGE

STEMpredict™ 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	STEMpredict™ Stem Cell Culture Master Mix (SCMM).	-20°C until used
3	STEMpredict™ Control Master Mix (CMM) to detect background cell growth.	-20°C until used
4	Medium (IMDM) for dilution of the ATP standard.	-20°C until used
5	ATP standard.	-20°C until used
6	ATP extra high, high and low controls.	-20°C until used
7	ATP Enumeration Reagent (ATP-ER)*	-20°C in the dark until used
8	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
9	Sterile, 96-well plates for stem cell culture	Can be kept with other kit components
10	Non-sterile 96-well plates for ATP standard curve determination.	Can be kept with other kit components
11	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 2-8°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.

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12. INTERPRETATION OF RESULTS

To predict umbilical cord blood bankability or to determine if peripheral blood contains stem cells that have been mobilized from the bone marrow, it is necessary for the stem cells to demonstrate metabolic viability, functionality and growth. This is performed by culturing the sample cells in a Stem Cell Master Mix containing growth factors that stimulate the stem cells and comparing the results with the same cells cultured in the same Master Mix, but without growth factors. Stem cells cultured in the Control Master Mix (CMM), will either demonstrate no growth or decreased growth over time. Cryopreserved cells usually exhibit a longer lag time to the onset of proliferation than fresh cells. However, if the stem cells are metabolically viability, they will start to exhibit proliferation and growth after only 2 days in culture. Greater sensitivity is obtained for all hematopoietic stem cell sources after 3 days in culture. At this time the difference between the control and stimulated stem cells will be highly significant.

An indication as to whether the stem cell product sample will exhibit proliferation and growth can be obtained by taking the ratio of the ATP concentration exhibited by the stem cells cultured in SCMM and the ATP concentration of the stem cells cultured in the CMM. This ratio should be 1.5 or greater. If a time course is performed from day 1 to day 4 or 5, it will be seen that this ratio will increase with time if the stem cells present in the product are functional. If the cells are not functional and therefore will not grow, the ratio of SCMM : CMM will not increase and will remain at approx. 1.

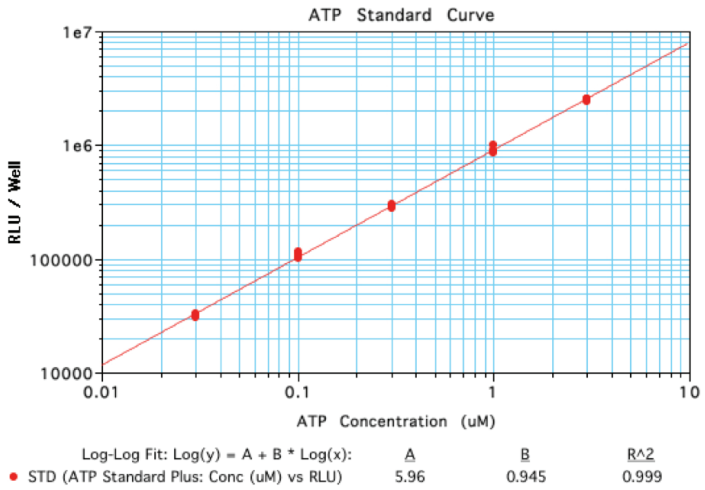
It should be emphasized that a SCMM : CMM ratio of about 1.5 is arbitrary. The user should obtain historical data for specific tissues in order to obtain a value that is considered usable to predict stem cell growth.

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



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

Equipment and Supplies

1. Laminar Flow Biohood
2. Plate luminometer (e.g. Molecular Devices, SpectraMaxL; Berthold, CentroLia)
3. Sterile, capped, plastic tubes (5ml, 10ml, 50ml)
4. Single channel pipettes, preferably electronic (e.g. ViaFlow 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. 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 or other dye exclusion viability assay.

8. The STEMpredict™ PROTOCOL

PLEASE READ THE FOLLOWING PROTOCOL CAREFULLY.
SEE SECTION 9 BEFORE PERFORMING AN ASSAY

Performing STEMpredict™ is a 3-step process.

Step 1 – Cell preparation.
Step 2 – STEMpredict cell culture.
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

STEMpredict™ assay kits have been designed to be used with umbilical cord blood and mobilized peripheral blood. Depletion of erythrocytes is essential since they can interfere with the assay when present at high concentrations (hematocrits > 10%) and cause false positive results.

A. Umbilical cord blood

A mononuclear cell (MNC) suspension is required for best results. It is recommended to deplete the cord blood of red blood cells using a current Hetastarch® protocol, cell lysis or density gradient fractionation 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 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. Human mobilized peripheral blood.

Mobilized peripheral blood samples are usually produced during apheresis of the patient. They normally contain a very low level of red blood cells and are essentially a mononuclear cell (MNC) fraction. However, if the red blood cell content is high, the sample must be further processed.

C. 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 that do not contribute to cell engraftment and result in a dilution of the stem cell content. When the cells are thawed, granulocytes and other cell components will rupture and release DNA. Large amounts of released DNA will clump together encasing valuable stem cells. If the cell preparation originally cryopreserved was a MNC or similar fraction, the chances of

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

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 SpectraMax L 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)

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.

(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 STEMpredict™. This is because red blood cells contain high levels of ATP that will cause false positive results. It is therefore necessary to deplete the sample of red blood cells. 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 umbilical cord blood sample. Density gradient centrifugation is recommended for cryopreserved umbilical cord blood 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, as in some post-thaw umbilical cord blood samples, even density gradient centrifugation may not provide optimal depletion. In most cases, however, density gradient centrifugation will produce a MNC fraction that will be far superior to any total nucleated cell (TNC) fraction for analyzing stem cell quality.

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.

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.

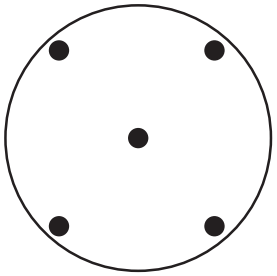
a. First set the integration time to 2 seconds.
b. Next, set the "gain". This must be determined empirically and is best per formed when the ATP standard curve is measured. The gain should be adjusted so that the percent coefficients of variation (%CV) for the mean of the replicates are the lowest value. 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

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



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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 present after separation can be 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.

D. 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 LIVEGlo™ (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 STEMpredict™ CMM or SCMM prepared.

STEMpredict™ Recommended Human Tissues, Cell Preparations, Cell States and Cell Concentrations

Cell Type	Cell Preparation	Cell State	Working Cell Concentration Required (100 x Final Cells/Well)	Final Cell Dose / Well
Umbilical cord blood	MNC	Fresh/Frozen	0.5-0.75 x 10^6	5,000-7,500
Mobilized peripheral blood	MNC	Fresh/Frozen	0.5-0.75 x 10^6	5,000-7,500

STEP 2. STEMpredict™ Cell Culture Protocol

Please refer to Section 9 for recommendations and tips prior to beginning this stage of the procedure.

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

The STEMpredict™ assay kit contains two Master Mixes:

1. Stem Cell Master Mix (SCMM) for detecting hematopoietic stem cells with growth factors included.
2. Control Master Mix (CMM) for the background control. This does not contain growth factors.

The STEMpredict™ Method

1. Thaw the frozen SCMM and CMM bottles by transferring to room temperature or water bath and allow to come to room temperature.
2. When thawed, mix the contents of each bottle thoroughly using gentle inversion or swirling. Do not shake the bottles.
3. Prepare the cell suspension and adjust the cell concentration to the working cell concentration as shown in the table above.
4. STEMpredict™ requires only 0.25ml of the adjusted working cell concentration. *NOTE: the concentration of the working cell concentration in cells/ml is 100 x the final concentration in cells/well. Example—5,000 cells/well requires a working cell concentration of 500,000 cells/ml.*
5. For each sample being tested, label two, 5ml tubes with the same sample number. One of the two tubes will be labeled SCMM, while the other will be labeled CMM.
6. Accurately dispense 0.72ml of the SCMM into each tube labeled SCMM.
7. Accurately dispense 0.72ml of the CMM into each tube labeled CMM.
8. Dispense 0.08ml of the sample cell suspension into the SCMM tube and another 0.08ml from the same cell suspension into the CMM tube. The total volume of each tube will now be 1.0ml.
9. Mix the contents of each tube thoroughly using a vortex mixer. The total

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, mixing the contents of the wells after adding ATP-ER is 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.

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.

STEMpredict™ 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

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volume in each tube will ne 0.8ml.

10. Dispense 0.1ml of the SCMM tube into each of 6 replicate wells into the sterile, 96-well plate provided with the kit. Dispense across the plate in rows. For example Row A, replicate positions 1-6.
11. Dispense 0.1ml of the CMM into each of 6 replicate wells below those of the SCMM. For example, Row B, replicate positions 1-6.
12. Place the sterile lid on the culture plate and transfer to a 37˚C, fully humidified incubator containing an atmosphere of 5% CO_2. If possible, use an incubator gassed with nitrogen to reduce the atmospheric oxygen concentration (21%) to 5% O_2. This reduces oxygen toxicity and increases plating efficiency.
13. Incubate the cells for exactly 3 days.

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 11 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 10 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. High ATP Standard Curve Preparation for Use with Mobilized Peripheral Blood.
Mobilized peripheral blood samples usually exhibit a high iATP concentration. For this reason, it is necessary to modify the ATP standard curve range so that sample ATP values fall within this range. If the ATP concentrations for mobilized peripheral blood samples are found outside of the range from 0.03µM to 3µM, the ATP standard curve will have to be repeated using the dose range from 0.01µM to 1µM (see below). 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.

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
   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 10 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

to maintain. 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.

9. RECOMMENDATIONS AND TIPS PRIOR TO USING THE STEMpredict™ KIT CELL CULTURE MIXES.

(i) Cell Suspension

a. The preferred cell suspension is a mononuclear cell suspension (MNC) of umbilical cord blood or mobilized peripheral blood.
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 page 12 may be required.

(ii) Thawing of STEMpredict™ Master Mix

a. Prior to using the STEMpredict™ Master Mix, remove the bottle from the kit box and thaw either at 37ºC or at room temperature.
b. The STEMpredict™ Master Mixs may be aliquotted into 5ml tubes after mixing and frozen until the expiration date.
c. STEMpredict™ Master Mixes are stable at 2-8°C for 1 month after thawing.

(iii) 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.

(iv) Number of Replicates Performed

STEMpredict™ 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. (Extra samples may not be available to perform an additional experiment. For this reason, performing 6 replicates/sample should be maintained).

(v) Plate Configuration

Samples should be dispensed into the 96-well plates in rows across the plate, not in columns.

(vi) 96-Well Plates Provided

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

(vii) Humidity Chamber

STEMpredict™ requires that the cell are incubated for just 3 days. However, if an incubator is full with other culture vessels, humidity may be difficult

0.90ml of the medium to vial #5

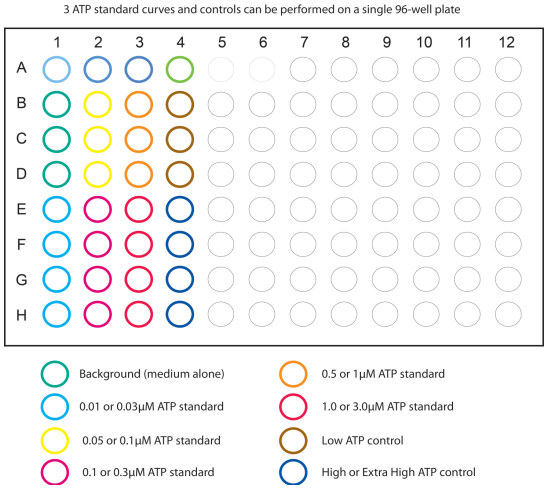
3. Remove 0.1ml of the supplied stock ATP solution (at 10µM) and transfer it to vial #1. Mix by vortexing. This ATP concentration is 1µM.
4. Dispense 0.35ml from vial #1 to vial #2. Mix. This concentration is 0.5µM.
5. Dispense 0.10ml from vial #2 to vial #4. Mix. This concentration is 0.05µM.
6. Dispense 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 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 10 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 10.
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.

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 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 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.

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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

STEMpredict™ can be performed in high throughput mode using 96-well plates. If you intend to perform any part of the STEMpredict™ 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.