

STEMpredict™

A 3-Day Stem Cell Quality Assay Predict
Bankability of Umbilical Cord Blood Units and
Optimization of Mobilization for Peripheral Blood

Technical Manual

(Version 8-19)

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

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Not for clinical diagnostic use.

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1. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. *STEMpredict™ is 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.*

2. Introduction

Present tests used to determine whether an umbilical cord blood unit will be permanently stored for potential transplantation purposes are based whether sufficient total nucleated cells (TNC) and CD34⁺ cells are available. In addition, the viability according to dye exclusion methods should be greater than 85% and results from the 14-day, methylcellulose colony-forming unit (CFU) assay, should indicate growth, by the appearance of colonies, although the range of colony numbers has never been defined.

When stem cells are mobilized from the bone marrow to the peripheral blood by treatment with granulocyte colony-stimulating factor (G-CSF) or other stem cell mobilizing agent, similar tests are usually used for each day of apheresis collection. However, in some cases, patients or donors either do not mobilized or do so, but at a much lower level.

Despite detection of CD34⁺ cells and perhaps other markers by flow cytometry, the ability to rapidly triage high functional quality from low functional quality units for potential stem cell transplantation cannot be performed using present methodology.

Stem cell proliferation ability, also called "stem cell quality", is the amount of proliferation at a specific cell concentration, measured in quantitative units. Proliferation is measured by virtue of the fact that when stem cells are stimulated with a growth factors cocktail the intracellular ATP (iATP) concentration increases. The iATP concentration and, therefore the proliferation status, correlates directly with the number of stem cells plated.

STEMpredict™ is a specialized STEMpredict™ assay that incorporates a fully standardized and validated ATP bioluminescence readout to measure stem cell proliferation ability. STEMpredict™ is an instrument-based, non-subjective measurement of iATP using a luciferin/luciferase reaction. It is the most sensitive and accurate, non-radioactive readout available. STEMpredict™ produces reliable and reproducible results, not only within a single laboratory, but between different laboratories.

To rapidly measure and predict stem cell quality and whether the unit of cells will produce adequate and sufficient proliferation after cryopreservation, the amount of proliferation resulting from a stimulated (proprietary) stem cell population is compared to the same population without stimulation. If the amount of stimulation after 3 days of culture is equal to or greater than 1.5 times that of the control culture, the stem cells present in the unit can be expected to be of high quality and to proliferate sufficiently when the unit is thawed. It should be emphasized, this is not the same as a strength or potency assay, which is determined using different technology.

Like all HALO® assays, STEMpredict™ also incorporates Suspension Expansion Culture™ (SEC) Technology. SEC™ technology is a methylcellulose-free culture system that is fast and easy to use, exhibits far superior sensitivity, accuracy and precision than any CFU assay and requires significantly shorter culture times than the CFU assay.

3. Use and Availability

STEMpredict™ is intended for use by cord blood banks and hematopoietic stem cell processing laboratories that process, cryopreserved and store mobilized peripheral blood. STEMpredict™ is used to rapidly distinguish high from low stem cell quality products, usually in the fresh state.

Although STEMpredict™ can be used to detect stem cells in a total nucleated cell (TNC) fraction, it is NOT recommended since (a) a TNC fraction contains cell impurities that dilute the stem cells, (b) this results in an underestimation of stem cell proliferation ability, and (c) interference by high red blood cell concentrations with the ATP readout results in a false positive result.

The cell preparation used for STEMpredict™ should be a:

- Mononuclear cell (MNC) fraction.
- Purified stem cell fraction, e.g. CD34⁺.

Suspension Expansion Culture™ (SEC™) Technology

STEMpredict™ incorporate Suspension Expansion Culture (SEC) Technology. No methylcellulose is used. This has the following advantages over traditional CFU assay methodology:

- All reagents can be dispensed using normal pipettes.
- Allows cell-cell interaction.
- Produces greater assay sensitivity.

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- Shorter cell incubation times; cell proliferation is measured on the exponential part of the growth curve.
- Coefficients of variation ≤15%.

STEMpredict™ is available with serum-free or low serum Master Mix formulations for a 96-well or 384-well plate format. Both formats, but especially the 384-well plate format, have been designed for high throughput processing of large numbers of samples. For 384-well plates and high throughput processing, it is highly recommended to use a liquid handler.

STEMpredict™ Assay Availability

Catalog No.	Culture Plate Format	Serum Formulation	No. of Plates
K2-SP-1	96-well plate	Low serum	1
K2SF-SP-1	96-well plate	Serum-free	1
K3-SP-1	384-well plate	Low serum	1
K3SF-SP-1	384-well plate	Serum-free	1

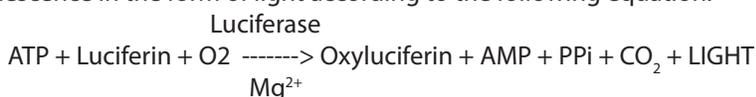
Upon request, assay kits with larger numbers of plates are available.

4. The Concept of ATP Bioluminescence Assays

STEMpredict™ is an ATP bioluminescence assay. The fundamental concept underlying the assay is the measurement of the cell's chemical energy in the form of intracellular adenosine triphosphate (iATP). If a cell is producing iATP, it is demonstrating cellular and mitochondrial integrity and is therefore viable. When hematopoietic cells are stimulated to proliferate, in cultured with growth factors and/or cytokines, the iATP concentration increases several fold. The iATP concentration produced is directly dependent on:

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

Hematopoietic stem cells are incubated in the STEMpredict™ Master Mix provided with this kit for a specific period of time. When the culture period has elapsed, a single-step addition of an 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:



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

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

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

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

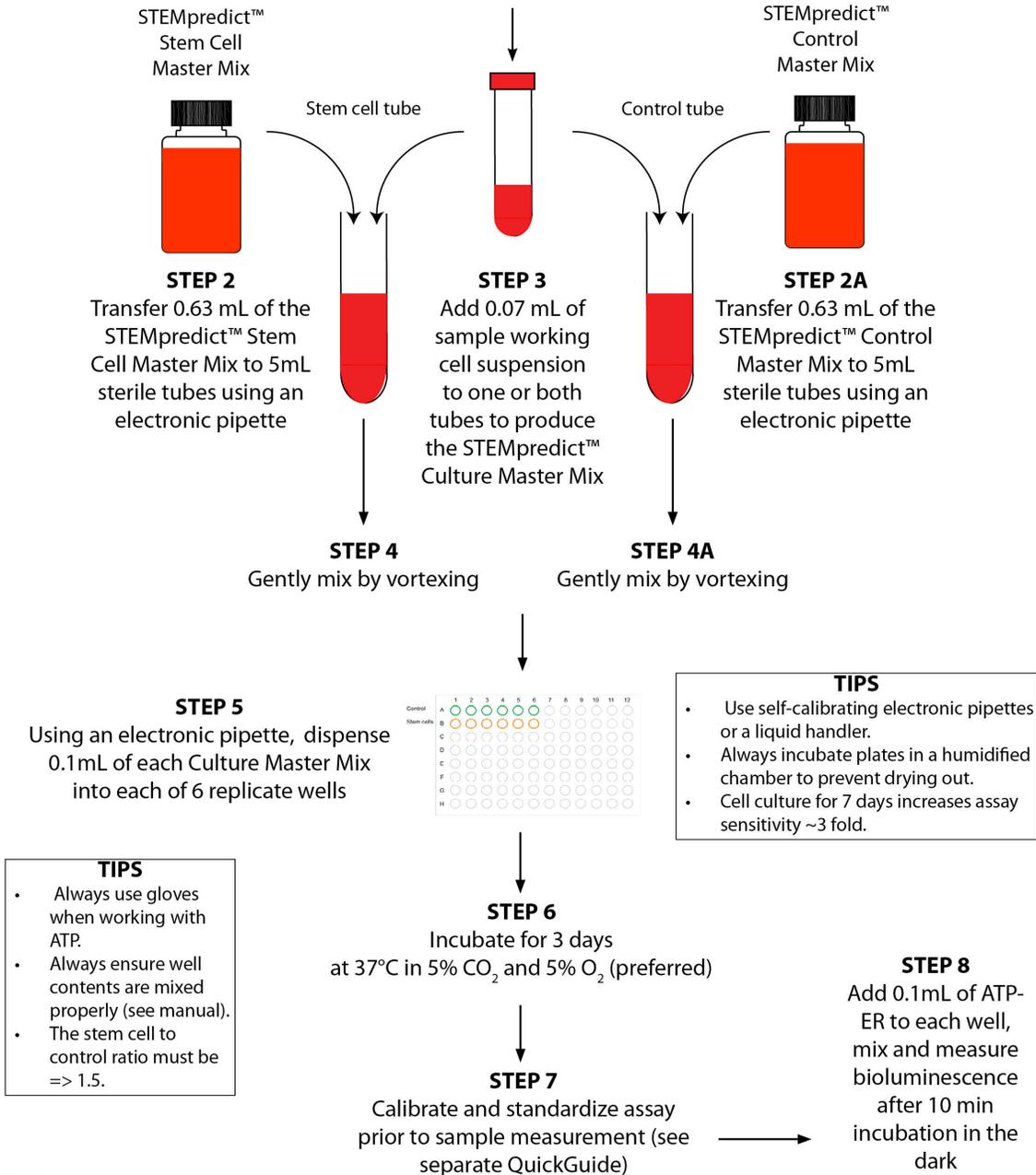
NOTES

5. QuickGuide to STEMpredict™ (Figure 1)

QuickGuide to STEMpredict™

STEP 1

If possible, process cord blood or mobilized PB to a mononuclear cell (MNC) fraction as this will provide the best results. Perform cell count and viability and adjust to working cell concentration. The protocol is for 6 replicates/sample.



QG003.001

6. Kit Contents and Storage Conditions

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™ Master Mix to detect primitive stem cells.	-20°C until used
2	STEMpredict™ Background Master Mix.	-20°C until used
3	Medium (IMDM) for dilution of the ATP standard.	-20°C until used
4	ATP standard.	-20°C until used
5	ATP extra high, high and low controls.	-20°C until used
6	ATP Enumeration Reagent (ATP-ER)*	-20°C in the dark until used
7	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
8	Sterile, solid white 96-well plates for stem cell culture	Can be kept with other kit components
9	Non-sterile, solid white 96-well plates for ATP standard curve determination.	Can be kept with other kit components
	Technical manual can be downloaded from the STEMpredict™ page on the Preferred Cell Systems™ website	

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 Preferred Cell Systems™.

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

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

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

Equipment and Supplies

1. Laminar Flow Biohood
2. Plate luminometer (e.g. Berthold LB962 CentrolIA/pc; Molecular Devices, SpectraMaxL)
3. Sterile plastic tubes (5ml, 10ml, 50ml)
4. Single channel pipettes, preferably electronic (e.g. Rainin EDP pipettes for variable volumes between 1µl and 1000µl).
5. 8 or 12-channel pipette, preferably electronic (e.g. Rainin EDP pipettes for fixed or variable volumes between 10µl and 100µl).
6. Reservoir for 8- or 12 channel pipette
7. Sterile pipette tips.
8. Vortex mixer.
9. Tissue culture incubator, humidified at 37°C with 5% CO₂ (minimum requirement) and 5% O₂ (preferable).
10. 1.5ml plastic vials (5 for each ATP dose response).
11. Hemocytometer or electronic cell counter to determine cell concentration.
12. Flow cytometer or hemocytometer for determining viability.

Reagents

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

8. The STEMpredict™ Protocol

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

Performing STEMpredict™ is a 3-step process.

Step 1 – Cell preparation.

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

Step 3 – Luminescence measurement. An ATP dose response is performed prior to sample luminescence measurements with conversion of RLU to μM ATP.

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

STEP 1 – Cell Preparation

A. Umbilical Cord Blood

1. It is routine for umbilical cord blood to be red blood cell reduced used instrumentation. This fraction is called the Total Nucleated Cell (TNC) fraction. Although a TNC fraction can be used, it is neither suitable nor recommended to use this fraction due to (a) dilution of primitive stem cells being detected, (b) underestimation of primitive stem cells being detected, and (c) interference of high red blood cell concentrations (usually 30% or greater) with the ATP readout resulting in an unsatisfactory high ATP readout.
2. Mononuclear cell (MNC) fraction is the cell preparation of choice. This fraction can be prepared by density gradient centrifugation using, for example, Ficoll-Paque or Lymphoprep from the TNC fraction.
3. Purified stem and progenitor cell populations prepared by cell sorting or magnetic bead separation.

B. Mobilized Peripheral Blood

The collection of mobilized peripheral blood is performed by apheresis. This procedure produces a cell fraction that is similar to the MNC fraction and normally does not need any further fractionation or purification.

Cell Viability, Cell Counting and Cell Culture Suspension Preparation

1. For dye exclusion viability methods, use trypan blue and a hemocytometer or automated method, flow cytometry using 7-AAD or another vital stain.

Note that dye exclusion viability methods detect membrane integrity. They do not detect cellular and mitochondrial integrity and therefore metabolic viability.

A viability of 85% or greater should be obtained when using dye exclusion viability methods only. It is recommended not to use cell suspensions with a viability of less than 85% since these cells will not be able to sustain proliferation ability. Use LIVEGlo™ (Preferred Cell Systems™) as a metabolic viability assay.

2. Determine the cell concentration using either a hemocytometer or electronic cell/particle counter. **NOTE:** Do not base the working concentration on the TNC count or the number of viable cells as this will give erroneous results.
3. Adjust the cell suspension concentration to that recommended in Table 1.
Note the working cell concentration per ml is 100 x the final cell concentration per well. If cells have been treated prior to cell culture, higher cell concentrations may be required.

- Prepare the total volume of cell suspension required using HemoGro™. The volume of the adjusted cell suspension required will be 10% of the total volume of STEMpredict™ Culture Master Mix prepared.

TABLE 1
Recommended Cell Doses for Cell Types, Cell Preparations and Cell States for STEMpredict™

Species	Cell Type	Cell Preparation	Cell State	Working Cell Concentration Required (100 x Final Cells/Well)	Final Cell Dose / Well
Human	Mobilized peripheral blood	MNC	Fresh/Frozen	0.5-0.75 x 10 ⁶	5,000-7,500
	Umbilical cord blood	MNC	Fresh/Frozen	0.5-0.75 x 10 ⁶	5,000-7,500
	Umbilical cord blood	MNC	Frozen	0.5-0.75 x 10 ⁶	5,000-7,500
	Mobilized peripheral blood*	CD34 ⁺	Fresh/Frozen	0.1-5 x 10 ⁵	100-5,000
	Umbilical cord blood	CD34 ⁺	Fresh/Frozen	0.1-5 x 10 ⁵	100-5,000

STEP 2. STEMpredict™ Cell Culture Procedure

- STEMpredict™ Master Mixes are complete and ready-to-use.
- Perform all procedures under a laminar flow, bio-hazard hood.
- Wear protective clothing, including gloves for all operations.

The STEMpredict™ Methodology

- Remove the STEMpredict™ Stem Cell Master Mix and Background Master Mix that came with the assay kit from the freezer and thaw both at room temperature or in a beaker of cold water. Do not thaw in a 37°C water bath or incubator.
- For each sample, label 2 x 5mL tubes, one for stem cell stimulation and one for background.
- Using a calibrated pipette, preferably an electronic pipette with repeat function, dispense the exact volume of each Master Mix, shown in Table 2, into each of the tubes. Notice that the volume of Master Mix is 10% less than the total volume. See Table 2.

TABLE 2

Number of Replicate Wells Required	Volume of STEMpredict™ Master Mix	Volume of Cells (10% of final volume)	Total Volume
6	0.63mL	0.07mL	0.7mL

- Adjust the cell concentration to the working cell concentration shown in Table 1. The working cell concentration should be 100 fold higher than the final cell concentration in the well. Thus, if the final cell concentration is to be 5,000 cells/well, the working cell concentration should be 100 times 5,000 or 500,000 (5 x 10⁵) cells/mL.
- Dispense the volume of cells shown in Table 2 into each of the 2 tubes.
- Mix the contents gently on a vortex mixer. Do not cause bubbles.
- Remove the sterile, individually wrapped, 96-well plate from the assay kit box.
- Using a calibrated pipette, preferably an electronic pipette with repeat function, dispense 0.1mL into each of 6 replicate wells across the plate.
- After replacing the lid, transfer the 96-well plate to a humidified container (see Section 9).
- Transfer the humidified container to the incubator. The incubator should be fully humidified at 37°C and gassed with 5% CO₂ and, if possible, 5% O₂. This reduced oxygen tension improves plating efficiency by reducing oxygen toxicity caused by the producing of free radicals.
- Incubate the cells for 3 days.

STEP 3 – Bioluminescence Measurement

Please note the following important points:

- **FOR ALL OF THE FOLLOWING STEPS, WEAR LABORATORY GLOVES. ATP is present on the skin and can cause erroneous results**
- **PLEASE DOWNLOAD THE DOCUMENT 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.**
- **Please refer to Section 10 for recommendations and tips prior to starting this part of the procedure. In particular, please refer to Section 10 for important information on mixing components.**
- **Remove the ATP Enumeration Reagent (ATP-ER) from the freezer and thaw at room temperature or in cold running water prior to analysis. Do not thaw the ATP-ER in a water bath or 37°C incubator.**
- **If the assay is to be calibrated and standardized, remove the ATP standard, controls and reagents from the freezer and thaw to room temperature or in cold running water prior to analysis.**
- **ATP standard curves performed on previous days or for previous experiments or studies must not be used since the ATP-ER intensity changes with time and lot number.**
- **Use the unwrapped, non-sterile, 96-well plate provided with the kit to perform the ATP standard dose response curve.**

A. Instrument Calibration and Assay Standardization

An Instructional Tutorial on “How to Calibrate and Standardize Any ATP Bioluminescence Assay” is available on the Preferred Cell Systems website under the Resources tab.

It is highly recommended to calibrate the luminescence plate reader and standardize the assay prior to measuring any samples. This will allow a comparison with the expected measurement assurance values (**see Section 13**) that should be obtained prior to measuring samples. Use the non-sterile, 96-well white plate provided with the assay kit for this purpose.

STEMpredict™ Assay Kit includes the following to calibrate and standardize the ATP bioluminescence part of the assay to measure cell proliferation.

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

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

After 3 days of incubation, the ATP concentration will not be high. For this reason, use Protocol 1.

PROTOCOL 1: Use the low and high calibration controls and perform an ATP standard curve from 0.01µM to 1µM. **See Page 19.** ATP concentrations should be within this ATP standard curve range. If they are found to be higher, perform Protocol 2.

It is extremely unlikely that this Protocol will be necessary. However, it is included in case high ATP levels are encountered.

PROTOCOL 2: It is recommended to use the low, high and extra high calibration controls and perform an ATP standard curve from 0.03µM to 3µM. **See Page 20.**

If calibration and assay standardization has been performed, it is extremely important that the ATP standard curve slope, and ATP controls lie within the ranges specified in **Section 13**.

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

C. Sample Measurement

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

1. If possible, place the sample plate(s) in a humidified incubator set at 22-23°C gassed with 5% CO₂ for 30min. Otherwise, allow the plate to come to room temperature for 30 min.
2. If only part of the plate has been used, transfer the plate to a bio-safety hood and remove the lid under sterile conditions. Take a sterile adhesive plate coverfoil from the kit box, remove the backing and layer it over the top of the plate. Using a sharp knife or scalpel, cut away the foil that covers the wells to be processed. The unused, empty wells will now remain sterile for the next samples. (See Section 10, Adhesive Plate Covering Film).
3. Using a multichannel pipette (8- or 12-channel depending on the plate configuration), add 0.1mL of ATP-ER to each well. Mix the contents as described in Section 10.
4. Repeat this procedure for each row or column 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. Preferred Cell Systems™ does not recommend using the automatic dispensers, since the contents of the well are not mixed sufficiently using this method.

E. Using a liquid handler

For cord blood banks that process and triage large numbers of samples, it is recommended to use STEMpredict™ with 384-well plates and a liquid handler. The volume of sample required for this format are significantly lower than for a 96-well plate. However, due to a larger “dead volume”, liquid handlers require slightly larger volumes of reagents. Please contact Preferred Cell Systems if a 384-well plate format is being considered. We can help you set up a high throughput testing system.

9. Recommendations and Tips Prior To Using STEMpredict™.

(i) Cell Suspension

- a. The preferred cell suspension is a mononuclear cell suspension (MNC) or higher purity. **PLEASE NOTE:** Although it is normal for cell processing laboratories to prepare a red blood cell-/plasma-depleted total nucleated cell (TNC) fraction using instrumentation, using this fraction for determining stem cell quality will be severely limited and could result in erroneous conclusions about the quality of the sample and the unit of cells in general.
- b. Extraneous ATP, red blood cells (which have high concentrations of ATP) and hemoglobin interfere with the ATP analysis. The cell suspension must have a hematocrit of 10% or less.
- c. If cells have been treated prior to cell culture, higher cell concentrations than those shown in Table 1 may be required.

(ii) Number of Replicates Performed

STEMpredict™ has been designed for 6 replicates/sample since this will provide superior statistics. Please remember that using fewer replicates may save components in the short term, but may also cause inconclusive results. If outliers are encountered, which may have to be removed from the analysis, the consequence could be that extra experiments would be required resulting in extra time and costs.

(iii) Plate Configuration

When using 6 replicate wells/sample, these should be plated in rows across the plate.

(iv) 96-Well Plates Provided

The reagents have been optimized to work with the 96-well plate(s) provided in the 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 Preferred Cell Systems™ if required.

(v) **Humidity Chamber**

A humidity chamber is recommended due to the small sample volume. Even fully humidified incubators do not keep the humidity level high enough to keep the sample from evaporating. This usually results in so-called “edge effects”. This phenomenon is observed when ATP values in the outside wells are lower than those in the inside wells. A humidity chamber can be assembled using plastic lunch boxes or other plasticware available from a supermarket or discount stores. Holes must be made in the lid to enable adequate gas exchange. Disposable serological pipettes are cut to an appropriate length to fill the bottom of the container. Distilled/deionized water is poured into the container to just below the level of the pipettes. This allows for adequate water to keep the humidity high without the plates sitting in water. Please contact Preferred Cell Systems™ for further information about assembling and using humidity chambers.

(vi) **Incubation Times**

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

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 each use.*
- *Each day bioluminescence is measured, a standard curve MUST be performed. The ATP-ER decays with time. A new ATP standard curve must be performed to ensure accurate conversion of the RLU values to ATP concentrations so that results can be compared.*
- *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 Preferred Cell Systems™.*

Bioluminescence Assay Kit Components

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

Reconstitution of Lyophilized Monitoring Reagent (if included)

- Thaw the ATP Enumeration Reagent Buffer at room temperature, in cold running water, or at 2-8°C overnight.
- Do not use any form of heat to thaw this reagent.
- Allow the lyophilized ATP-ER substrate (brown glass bottle) to come to room temperature.
- Remove the closures from both bottles.
- Carefully pour the entire contents of the buffer bottle into the lyophilized ATP-ER substrate bottle. Swirl gently or invert slowly to mix. Do not shake.
- Allow the ATP-ER mix to reconstitute for 10 minutes at room temperature.
- Reconstituted ATP-ER is stable for 8 hours at room temperature, 48 hours at 2-8°C, or 20 weeks at -20°C.

- ATP-ER can be refrozen up to 11 cycles without significant loss of sensitivity.

Volumes of Luminescence Kit Components Required

- Each vial of ATP standard contains enough volume to perform one or two ATP standard dose responses.
- The amount of ATP-ER added to each well is 0.10mL. Therefore:
Total amount of ATP-ER (μ l) 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.

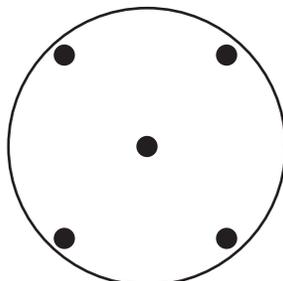
Mixing the Contents of 96-well Plate

Please refer to the Instructional Tutorial on “How to Calibrate and Standardize Any ATP Bioluminescence Assay” is available on the Preferred Cell Systems website under the Resources tab.

Mixing the contents of the wells after adding ATP-ER is one of the most important procedures of the assay. 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 4 for each corner of the well.
5. Try not to cause excessive bubbles in the culture and DO NOT over mix since this can result in drastically reduced luminescence values.
6. This procedure effectively and optimally mixes the contents well.

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



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

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

12. Results

STEMpredict™ is a unique stem cell assay. It provides an instrument-based, non-subjective, quantitative readout of total proliferation ability of stimulated stem cells compared to a background control. There is no comparable CFU assay.

STEMpredict™ is used on fresh umbilical cord blood or mobilized peripheral blood samples to determine if the stem cells will demonstrate the ability to proliferate and grow, if the cells are cryopreserved.

The user should perform the calibration and ATP standardization procedure described in this manual and available as an Instructional Video on the Preferred Cell Systems website under Resources. The ATP controls calibrate the luminescence plate reader. The ATP standard curve allows non-standardized RLU values to be converted into standardized ATP concentrations (μM). The results of the calibration and standardization should be compared with those provided in **Section 13** below. If the results are within the ranges specified in **Section 13**, it is then possible to continue with sample processing and measurement.

These measurement assurance parameters also indicate whether the sample being tested provides acceptable viability and proliferation activity, or whether it should be rejected for use. Please be aware that acceptance criteria for the sample may not necessary apply to the unit of cells from which the sample was obtained. It should also be emphasized that the single values obtained using STEMpredict™, do not represent the potency of the cells. Cell potency is performed using a different methodology to that used for quality control. Please see the related assays at the end of this manual.

After processing and measuring the the background control and the stimulated stem cells, convert the RLU results into standardized ATP concentrations (μM). Divide the Stem Cell ATP Concentration by the Background ATP Concentration. If the ratio obtained is greater than 1.5, the cells in the unit can be transferred to permanent liquid nitrogen storage and the cells would be predicted to proliferate and grow when thawed.

13. STEMpredict™ Assay Measurement Assurance and Validation Parameters

When STEMpredict™ has been calibrated and standardized, ATP bioluminescence technology allows the User's results to be compared to the measurement assurance parameters shown in Table 4. For each control, ATP standard dose and the log-log linear regression curve fit parameters provided, the User's results must lie within the ranges provided. If this is the case, then the following are applicable:

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

IMPORTANT. If the User's results DO NOT comply with those in the table, DO NOT measure the samples. Perform a repeat of the controls and ATP standard curve. If the results still do not comply with those in the Table 4, contact Preferred Cell Systems for help.

Table 4: ATP Controls and Standard Curve Measurement Assurance Parameters

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

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

Samples Values:

- Lowest ATP value indicating unsustainable cell proliferation for hematopoietic cells: \sim 0.04 μ M. The proliferation ability (growth) of the cells in this situation is extremely questionable.
- ATP concentration below which cells are not metabolically viable, i.e. the cells are dead: \sim 0.01 μ M. The cells should not be used in this situation.
- All samples values must lie on the ATP standard curve for accurate RLU to ATP conversion. If ATP values are greater than 3 μ M, the replicate samples should be diluted with IMDM and re-measured. Take the dilution value into account when estimating the true ATP concentration. Alternatively, repeat the culture and ATP measurement using fewer cells.

Assay Validation Parameters

HALO® exhibits the following validation parameters:

- Assay ATP linearity \Rightarrow 4 logs
- Assay ATP sensitivity: \sim 0.001 μ M
- Assay cell sensitivity: 20-25 cells/well (depending on cell type and purity)
- Accuracy (% correct outcomes): \sim 95%
- Sensitivity and specificity detected by Receiver Operator Characteristics (ROC) curve fit and detected as area under the curve (AUC): 0.73 - 0.752 (lowest possible value, 0.5; highest possible value, 1).
- Precision (Reliability and Reproducibility) \leq 15%. At lower limit of quantification (LLOQ): 20%
- Robustness (intra- and inter-laboratory comparison): \sim 95%.
- High throughput capability (Z-Factor): $>$ 0.76 (lowest possible value, 0.5; highest possible value, 1).

14. Troubleshooting

If Calibration and Standardization Results Do Not Conform to Measurement Assurance Parameters (Section 13)

If the investigator has elected to calibrate and standardize the assay using the ATP controls and standard supplied with the kit, the results should be within the ranges provided in Section 13. If the values obtained conform to the measurement assurance parameters, the investigator can continue the assay and process and measure the samples with the assurance that the results can be trusted.

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

High Coefficients of Variation (%CV)

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

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

Low RLU Values

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

- *Reagent decay:* The ATP-ER decays with time, even when frozen. This can lead to low bioluminescence. Once thawed the reagent can be refrozen up to 11 cycles without significant loss of sensitivity. Do not use the reagent after expiry date has elapsed. As a rule of thumb, the RLU value for the lowest ATP standard should be 10 times greater than that of the background value.
- *Inadequate cell growth:* Cells did not exhibit sufficiently high viability. Measure cell viability prior to using cells. A cell viability lower than 85% should not be used. Viabilities lower than 85% can be an indication that the sample was not processed in a time-sensitive manner or that the processing procedures were not standardized and controlled. **PLEASE NOTE:** Dye exclusion viability can produce a false positive results. Even if the cell viability by dye exclusion methods is greater than 85%, this does not mean that the metabolic viability allows the cells to proliferate and grow. It is possible to have high dye exclusion viability values, but the proliferation ability of the cells will be low or non-existent, i.e. the cells might be dead.
- *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.

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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, if the medium contains an indicator, e.g. phenol red. Contaminated cultures will usually be bright yellow in color and probably cloudy in appearance. Cell cultures that demonstrate high proliferation will usually appear orange to light orange, but will not be cloudy. If only “spot” contamination occurs, this is usually due to pipette or repeater tips coming in contact with materials other than the reagents. Contamination will usually lead to outlier RLU values.

Luminescence Reagent Mixing.

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

Culture Plates Drying Out

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

Other Hematopoietic Cellular Therapy Assay Products from Preferred Cell Systems™

HALO® PCA is a 96-well ATP bioluminescence progenitor cell assay to replace the CFU assay for cell processing laboratories.

HemoFLUOR™ PCA is a 96-well, fluorescence readout version of STEMpredict™.

HemoLIGHT™ PCA is a 96-well, absorbance readout version of STEMpredict™.

HALO® SPC-QC is a 5-day, 96-well, standardized, ATP bioluminescence stem cell quality control assay designed to optimized stem cell processing procedures. It is available to measure primitive hematopoietic stem cells as well as primitive lympho-hematopoietic stem cells.

HALO® RS is a 7 day, 96-well, standardized ATP bioluminescence assay to establish cord blood, bone marrow or peripheral blood reference standards for HALO® SC-IPS assays.

HALO® SC-IPS is a 7-day, standardized, 96-well, ATP bioluminescence assay to measure the identity, purity and strength (potency) of 2 primitive stem cell populations in cord blood, bone marrow or peripheral blood samples, prior to use in patients.

HALO® TE, HemoFLUOR™ TE and HemoLIGHT™ TE are time to engraftment assays.

HALO® PMT, HemoFLUOR™ PMT and HemoLIGHT™ PMT are “global” 4-, 5- or 7-population assays to monitor patient reconstitution after transplantation.

Preferred Cell Systems™

Ordering Information

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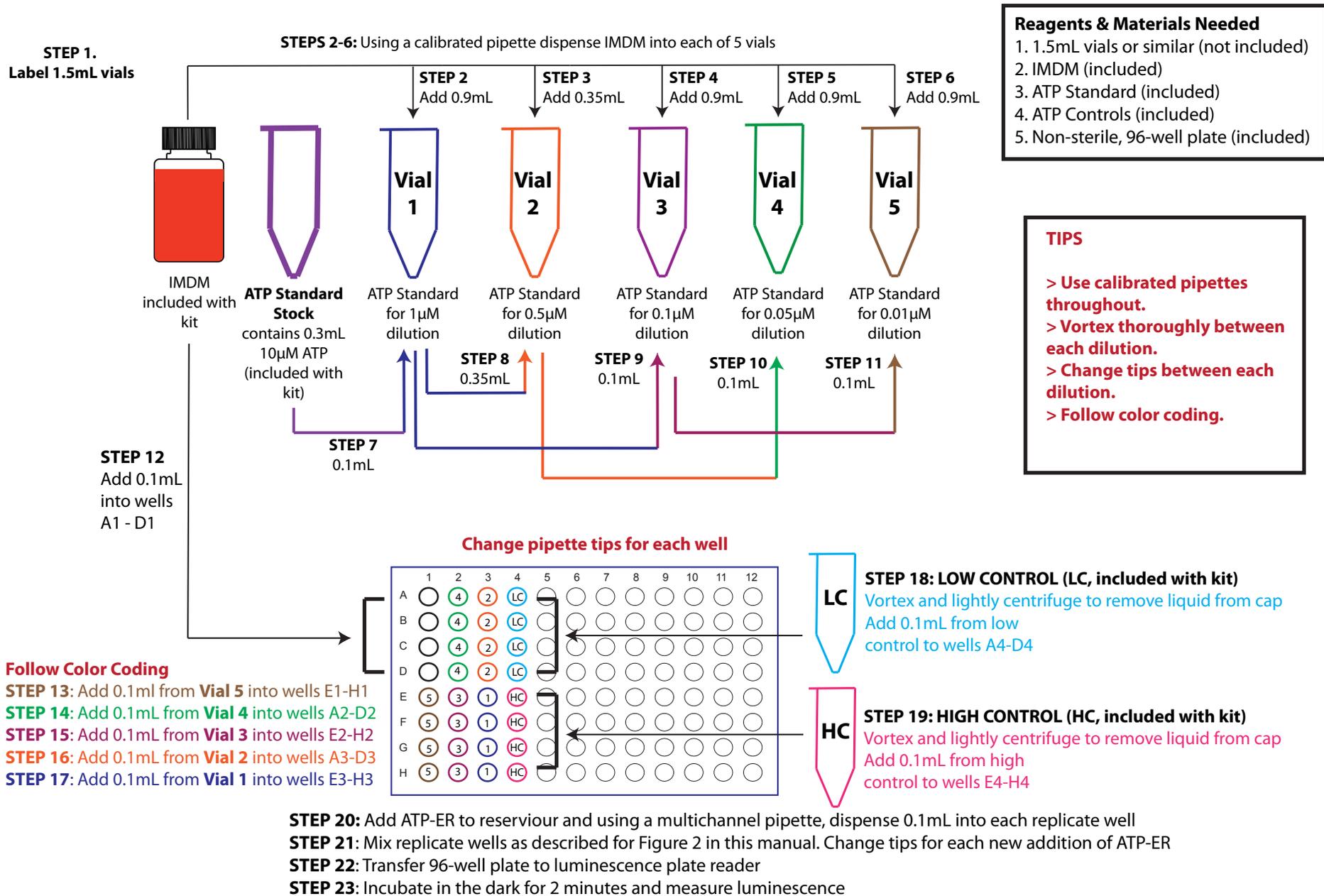
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HALO® was designed and developed by HemoGenix®, Inc.

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Calibration and Standardization Protocol of an ATP Bioluminescence Assay

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



Calibration and Standardization Protocol of an ATP Bioluminescence Assay

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

